RNA Interference with Special Reference to Combating Viruses of Crustacea

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Abstract RNA interference has evolved from being a nuisance biological phenomenon to a valuable research tool to determine gene function and as a therapeutic agent. Since pioneering observations regarding RNA interference were first reported in the 1990s from the nematode worm, plants and *Drosophila*, the RNAi phenomenon has since been reported in all eukaryotic organisms investigated from protozoans, plants, arthropods, fish and mammals. The design of RNAi therapeutics has progressed rapidly to designing dsRNA that can specifically and effectively silence disease related genes. Such technology has demonstrated the effective use of short interfering as therapeutics. In the absence of a B cell lineage in arthropods, and hence no long term vaccination strategy being available, the introduction of using RNA interference in crustacea may serve as an effective control and preventative measure for viral diseases for application in aquaculture.

Keywords RNA interference · Crustacea · Viruses · WSSV · YHV · TSV · PmergDNV · MrNV

Introduction

RNA interference (RNAi) is an evolutionary conserved, natural gene silencing phenomenon in which double stranded RNA (dsRNA) silences gene expression, by either inducing sequence-specific degradation of complementary messenger RNA (mRNA) or by inhibiting translation [56,

126]. This homology-dependent gene silencing was first discovered in transgenic plants [120, 130, 188] where pioneering work by plant virologists established plants responded to viral infections by inducing RNAi, resulting in the specific recognition and destruction of the invading viral RNAs and homologous host RNAs [12, 146]. The phenomenon of RNAi has subsequently been described in a wide range of eukaryotic organisms including arthropods and mammals [40, 45, 48, 77, 100, 121, 147]. To date, RNAi has been used as a strategy to investigate gene function [5, 15, 213] (for example, it has been used to analysis the function of close to 17,000 of the 19,000 (approximate figure) genes in *Caenorhabditis elegans* [76, 186]) and as an antiviral mechanism to combat viral infections in plants [182, 211], invertebrates [29, 185, 209] and vertebrates (in particular, influenza, cancer and human immunodeficiency virus (HIV) [53, 54, 122, 170, 195].

The use of RNAi as an antiviral mechanism is particularly important for invertebrates such as crustaceans that lack an adaptive immune response (however, they have innate immunity) and therefore, cannot be vaccinated as a preventative measure against viral diseases. Viruses contribute to substantial economic losses within penaeid prawn aquaculture so strategies to prevent or combat viral infections are highly desirable.

Discovery and Current Understandings of RNAi

The first indication of the existence of homology-dependent gene silencing emerged when researchers attempting to deepen the colour of petunia flowers by expressing higher levels of the enzyme (chalcone synthase) responsible for the synthesis of pigment, unexpectedly resulted in reduced or lack of floral pigmentation and a reduction in

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gene expression [120, 130, 188]. This occurrence was designated the term co-suppression, indicating that transgenes themselves were inactive and that the DNA sequences were somehow affecting the expression of the endogenous loci [55]. The silencing of homologous genes in the genome for plants may also be referred to post-transcriptional gene silencing (PTGS) or quelling [149].

The term RNAi emerged following the 1998 demonstration of the development of sequence-specific gene silencing by injection of dsRNA into *C. elegans* [45]. Fire and colleagues [45] demonstrated a mixture of antisense and sense RNA (dsRNA) was able to direct the degradation of mRNA and was at least tenfold more potent as a silencing trigger than were sense or antisense RNAs alone. This 'gene silencing' was achieved by simply feeding *C. elegans* the bacterium *Escherichia coli* (the preferred diet of *C. elegans*) containing plasmids with the dsRNA-encoding DNA sequences.

Following the work by Fire and colleagues [45], there was increased interest in the use of RNAi in both plants and animals. To date, RNAi related events have been recognised in almost all eukaryotic animals including algae [202], yeast [151], protozoans [11, 27, 94, 118], plants [43, 196], insects [2, 17, 26, 77, 100], fish [29, 131] and in mammals [38, 48, 121, 198].

RNA Interference Pathway

The mechanism for RNAi remained unexplained for some time. An early crucial observation in determining the mechanism behind RNAi was the production of short RNAs (20–25 nucleotides long) matching the gene being silenced by plants that were silencing genes in a RNAi related process (PTGS) [55]. Similarly, it was revealed by reconstituting the biochemical pathways of RNAi in vitro using fruit fly extracts that long dsRNAs were diced up into short RNAs [39]. Furthermore, the short RNAs had a specific structure involving two 21-nucleotide strands of RNA in a staggered duplex, with 19 nucleotides of dsRNA and two unpaired nucleotides at the ends [39].

Current understandings of the mechanisms underlying RNAi are derived from genetic studies involving the introduction of dsRNA in *C. elegans* [45, 139, 179] and *Drosophila* [39]. To date, the process of RNAi can be divided into two stages: the initiator and the effector stage [59, 96, 133, 167].

Initiator Step

The initiator stage is limited to the cytoplasm of the cell [70, 212] and involves generating short interfering RNAs (siRNA) from long dsRNA, or microRNAs (miRNA) from

endogenous hairpin RNA precursors, that appear in the cell from their primary transcripts by an RNase III-like protein, known as dicer [4, 13, 33, 58]. Dicer is a large protein (approximately 220 kDa) containing a dsRNA binding domain (dsBRD), two catalytic RNase III domains, a helicase domain and a Piwi–Argonaute–Zwille (PAZ) interaction domain which binds small RNAs [16, 81, 116]. Dicer unwinds and cleaves long dsRNA duplexes into siRNAs 21–23 nucleotide DNA fragments with symmetric 2–3 nucleotide 3' overhangs and 5'-phosphate and 3'-hydroxyl groups [37]. Any siRNAs that lack a 5' phosphate are rapidly phosphorylated by an endogenous kinase [134, 165].

The siRNAs are incorporated and bound into a multi-protein RNA-inducing silencing complex (RISC). Functional RISC contains four different subunits including endonuclease, exonuclease, helicase and homology searching domains [96]. The duplex siRNAs are unwound by an RNA helicase [134], leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage [37]. Small interfering RNAs that do not require Dicer processing are able to directly enter the RISC, but induce silencing less efficiently than siRNA precursors induced by Dicer [37, 96, 134].

Effector Step

The effector step is essentially the last process of the RNAi pathway. It is achieved by the multiprotein complex RISC and involves the endonucleolytic destruction of the targeted mRNA. The target mRNA is cleaved at a single site in the centre of the duplex region between the guide siRNA and the target mRNA, 10 nucleotides (nt) from the 5' end of the siRNA [39]. This is achieved through the proteins of the Argonaute family (e.g. *rde-4*, *rde-1* and *drh-1/2* in *C. elegans* [180] or Argonaute2 in *Drosophila melanogaster* [201]) which possess nuclease activity responsible for mRNA target cleavage. Cleavage is endonucleolytic, and occurs only in the region homologous to the siRNA [40, 210]. The cleaved RNA is rapidly degraded and consequently, the protein for which it encodes is not produced.

Alternatively, translation may be suppressed without mRNA cleavage, but is more typical for miRNA-mediated gene silencing [56]. Compared with siRNAs that can be both artificial and endogenous, miRNAs are always encoded by the genome itself as hairpin structures [7, 9, 55, 153]. However, both molecules are involved in the same process that leads to mRNA degradation or the inhibition of protein synthesis. As a general rule, siRNAs cause mRNA destruction, whereas miRNAs can cause both mRNA destruction and the inhibition of protein synthesis. If there are variations or mismatches in nucleotide base pairs in the siRNA/miRNA duplex (more common for

miRNAs), the mRNA is not cleaved and gene silencing results from translational inhibition [56].

Triggers of RNA Interference

RNA interference is a conserved eukaryotic gene regulatory system that suppresses gene expression through RNA-mediated sequence-specific interactions. It is initiated by the presence of dsRNA and can be achieved in the laboratory through a number of different strategies. There are six common types of dsRNA that have been identified that trigger RNAi (Table 1). Although all six types of dsRNA have proven to be effective to various degrees [7, 38, 41, 136, 205, 210], siRNAs tend to produce the greatest silencing (often with the least toxicity) [150] and have therefore been the common approach for inducing RNAi-based gene silencing. They are incorporated into the RNAi pathway at a later stage and are therefore less likely to interfere with gene regulation by endogenous miRNA [52, 74]. Furthermore, siRNAs can be manufactured by processes that are amenable to large scale production and can be modified to contain drug-like properties, making them particularly attractive as therapeutics. Hence, they are the class of RNAi therapeutics that is the most advanced in preclinical and clinical studies [30].

Three strategies involving chemical synthesis, in vitro enzymatic synthesis, and DNA plasmid vector exist for generating siRNAs for gene silencing. Each method is associated with specific advantages and disadvantages.

Chemically Synthesized siRNA

The use of siRNAs that are synthesised purified and annealed by chemical processes [39] are becoming increasingly popular. Chemically synthesised siRNAs are directly introduced into the cytoplasm bypassing the ‘dicer’ step. Perfect duplex hairpin RNA is cleaved by Dicer into siRNAs and imperfect duplex hairpin RNA is cleaved by Dicer into miRNAs that are responsible for directing gene

silencing. miRNA are a class of short noncoding RNAs that have an incomplete sequence homology to their targets. miRNA primarily function as siRNAs by preventing the translation of mRNA into protein rather than destroying the mRNA transcript [167]. However, miRNAs cannot be designed by computational methods, unlike siRNAs.

Chemically synthesised siRNAs can be synthesised at higher amounts and have been proven to be the most efficient (despite the higher cost and increased synthesis time) triggers of sequence-specific mRNA degradation when they contain 2 nt 3' overhangs [40]. However, this was originally noted in *D. melanogaster* and what may work best for one species, may not necessarily work optimally for other species. Nevertheless, it should be used as a starting point when chemically synthesising siRNAs. Dicing kinetics has been recorded with the use of siRNAs longer than 27 bp [6]. Even so, chemically generated siRNAs have since been employed as the most efficient means of inducing RNAi. High purity can be obtained using chemically synthesised siRNAs but can be expensive when multiple siRNAs are involved [36].

In Vitro Enzymatic Synthesis of siRNA

Enzymatically generated siRNAs (in vitro siRNA synthesis) involves T7 phage RNA polymerase mediated in vitro transcription and is the quickest and most cost effective method for siRNA synthesis. The polymerase produces individual sense and antisense siRNA strands that form siRNAs when annealed [34, 173, 206]. However, the purity and specificity using enzymatic synthesis is variable [36].

Transcription is generated from short double stranded oligo cassettes containing the promoter sequence upstream of the siRNA template sequence that is to be transcribed [34, 173]. Transcription begins and terminates at specific initiation and termination sequences, determined by the promoter [40]. Small inverted repeats are produced, separated by three to nine nucleotides, termed short hairpin RNAs (shRNAs), that are subsequently converted into siRNAs by Dicer [40, 137, 177]. The siRNA strands are

Table 1 Description of six primary types of double stranded RNA that trigger RNA interference [7, 26, 39, 133, 136, 137, 149, 210]

Short interfering RNAs (siRNAs)	Double stranded RNA approximately 21–22 nucleotides in length, generated from long dsRNA. Gene silencing is achieved through the cleavage of mRNA with exact complementary sequences
MicroRNAs (miRNAs)	Single stranded RNA approximately 19–25 nucleotides in length. Gene silencing occurs at the stage of protein synthesis
Short hairpin RNAs (shRNAs)	Single stranded RNA molecules that contain inverted repeats that allow them to form intramolecular duplexes. shRNAs are processed by Dicer and serve as a template for sequence-specific silencing
Long double stranded RNA (dsRNA)	Double stranded RNA that is introduced into cells and cleaved by Dicer into siRNAs
Short interfering RNA (siRNA)-based hairpin RNA	Produced from the cleavage of duplex hairpin RNA by Dicer
MicroRNA (miRNA)-based hairpin RNA	Imperfect duplex hairpin RNA cleaved by Dicer into miRNAs

synthesised in separate reactions and often contain a GGG leader sequence. Consequently, the siRNA needs to be processed by T7 ribonuclease to remove the single stranded 5' GGG overhang. Furthermore, siRNAs must not be transcribed with a UU 3' overhang, otherwise T7 processing may be incomplete due to the formation of two G:U wobble base pairs, resulting in non-specific inhibition of gene expression [83, 84].

Plasmid Vector

Polymerase III promoter-based DNA plasmids can be used to produce siRNAs for gene silencing [177]. shRNAs are produced which are subsequently processed by Dicer into siRNAs. The use of plasmid generated siRNAs enables stable RNAi expression for up to 2 months post-transfection and is more economical for multiple sequences [22]. However, the construction of a DNA plasmid vector is labour intensive and success can be transfection-dependent [36].

Delivery Strategies

Effective delivery to the appropriate cells or tissue remains a major hurdle for successful RNAi. Numerous *in vivo* delivery strategies exist, ranging from the simple local delivery of 'naked' siRNA duplexes to the more complicated methods involving the systemic delivery of siRNA complexes as conjugates, liposome/lipoplexes, as complexes with peptides, polymers including chitosan nanoparticles or antibodies or vector mediated. Vectors used in DNA based technology can be broadly categorised as those derived from viruses [79] and those that are not (for example, plasmids) [110, 164]. Each approach has its advantages and disadvantages which must be considered when choosing an appropriate delivery strategy. This section provides a review of the various delivery approaches used with siRNA. However, a comprehensive listing of successful *in vivo* efficacy studies can be found in numerous review publications [1, 23, 31].

Virus Vector Mediated

The use of viral vectors or plasmids to express RNAi effector molecules results in the stable expression of RNAi which may otherwise have varied in transfection efficiency [79, 110]. Gene transfer using viral vectors is referred to as transduction. Transduction is defined as the introduction of functional genetic information of the host genome (either plasmid or chromosomal) to a recipient cell through the use of a recombinant viral vector [79, 161, 172]. The life cycle of a virus requires them to efficiently transfer their own genetic material to the cells they infect. The introduction of

the viral genome into the host cell leads to an early phase of gene expression characterised by the appearance of viral regulatory products. This is followed by the expression of structural genes and the assembly of new viral particles. In the case of gene therapy vectors, elements of the viral genome that contribute to replication, virulence and disease are deleted to reduce pathogenicity and immunogenicity and replaced by a therapeutic gene cassette carrying the genes of interest. The residual viral genome and the gene of interest (referred to as a transgene) are integrated into the vector construct which contains the transgene and *cis*-acting sequences that are essential for encapsidation of the vector genome and viral transduction of the target cell. The vector and packaging constructs are subsequently expressed in the packaging cells, which produce the recombinant viral particles [79, 141].

For gene therapy to be successful, an appropriate amount of a gene must be delivered into the target tissue without substantial toxicity. Viral vectors can be divided into three categories: (a) integrating vectors, (b) non-integrating vectors and (c) hybrid vectors. Each viral vector is characterised by an inherent set of properties that affect its suitability for specific gene therapy applications. For example, gene therapies designed to interfere with a viral infectious process may require gene transfer into a large number of cells. For the delivery of therapeutic nucleic acids, viruses provide an efficient means for the delivery of therapeutic nucleic acids (even in cells resistant to transfection with dsRNA and plasmid) due to the inherent ability of viruses to transport genetic material into cells [192]. However, viral delivery systems have a limited loading capacity since it is difficult to produce the genetic material for large scale applications, construction of a viral vector is labour intensive and their use poses several safety risks such as their oncogenic potential and their inflammatory and immunogenic effects which prevent them from repeated administration [99, 111, 178].

This section will briefly describe the properties of viral vectors commonly used for gene therapy and their advantages and limitations in inducing RNAi. There are difficulties associated with determining the vector system most applicable for research applications because each vector has its own specific properties which determine its suitability for research applications. It is therefore up to the discretion of the researcher as to what vector system would be suitable for their specific research application. A description of each vector system and its modification for application to gene transfer is presented here.

Integrating Viral Vectors

Integrating viral vectors are capable of providing long-life expression of the transgene and include adeno-associated

virus (AAV, a parvovirus), lentivirus and retroviral derived vectors. This is in contrast to gene delivery using adenovirus, herpes simplex virus (HSV) and baculovirus viral vectors where the viral genome remains episomal.

Adeno-Associated Virus Adeno-associated virus (AAV) is a member of the *Parvoviridae* and contains a 4.7 kb single stranded DNA (ssDNA) genome. The viral genome consists of two genes (capsid and replicase genes) situated along the genome between two inverted terminal repeats (ITRs) that define the beginning and end of the virus and contain the packaging sequence [79, 192]. Each gene produces multiple polypeptides. The capsid (*cap*) gene encodes the viral capsid protein and the replicase (*rep*) gene product is involved in viral replication and integration. Thus, to generate a recombinant AAV vector, *rep* and *cap* genes can be deleted to leave only the virus's terminal repeats involved in viral replication and integration [158]. A helper virus (commonly adenovirus and HSV) provides AAV with the additional genes it requires for replication. The virus can infect a variety of dividing and non-dividing cell types and remains infectious after heating or freeze-drying [42, 79, 98, 192].

However, in contrast to wild-type AAV, recombinant virus carrying a therapeutic gene in place of the normal viral *rep* and *cap* genes, loses its ability to specifically integrate and in certain cell types, the recombinant AAV is maintained episomally (rather than being integrated into the host genome) where it only expresses the therapeutic gene after second-strand synthesis [44]. It may have limited packaging capacity and can be difficult to produce. Despite this, efficient, long term, stable gene expression has been achieved using recombinant AAV [85].

Retroviruses Retroviruses are lipid-enveloped particles comprising of linear, positive sense, single stranded RNA (ssRNA) genomes of 7–11 kb. Retroviruses are a group of viruses whose genome is retro-transcribed into linear double stranded DNA (dsDNA) and integrated into the cell chromatin following entry into the target cell [79]. The genome consists of three genes (*gag*, *pol* and *env*), which are required in *trans* for viral replication and packaging. These three genes are situated along the genome between two long terminal repeats (LTRs) that define the beginning and end of the viral genome [192]. The LTRs contain a promoter, polyadenylation, and integration sequences, a packaging site termed *psi* (which allows the viral RNA to be distinguished from other RNAs in the cell) and a terminal RNA (tRNA) binding site, as well as several additional sequences involved in reverse transcription and are also involved in controlling the expression of the viral genes. The viral genes encoding the three proteins can be removed and replaced with heterologous genes and

transcriptional regulatory sequences which are subsequently transcribed under the control of the LTRs or enhancer-promotor elements engineered with the transgene [42, 79, 192].

Recombinant retrovirus infects target cells through a specific interaction between the target cells surface receptor and the envelope protein of the virus. The virus is internalised, where it is uncoated and the RNA is reverse-transcribed into proviral dsDNA by means of the virally encoded *pol* gene, and stably integrated into the host genome after being transported to the cell nucleus. The ability of retroviruses to insert their genome into the host DNA allows for stable genetic modification for the life of the host cell [128, 129].

Two members of the Retroviridae are commonly used for gene transfer: lentiviruses (e.g. human immunodeficiency virus) and *Oncoretrovirus* (e.g. Moloney murine leukemia virus) [42]. Lentiviruses are capable of infecting both dividing and non-dividing cells but are unfortunately difficult to produce [101]. Conversely, *Oncoretrovirus* vectors only infect dividing cells but are relatively simple to produce [79, 142].

Non-Integrating Viral Vectors

Non-integrating viral vectors include those based on adenoviruses (Ad), HSVs and baculovirus-derived vectors in which the viral DNA is maintained in an episome in the infected cell.

Adenovirus Adenoviruses are a family of DNA viruses that contain a linear, 30–40 kb dsDNA genome encoding over 50 polypeptides [42, 51, 79]. Adenoviruses can infect both dividing and non-dividing cells efficiently in culture and in vivo and since they do not integrate into the host genome, they are replicated as extrachromosomal elements in the nucleus of the host cell. They are capable of infecting a wide variety of cells through a specific interaction between the viral fiber protein and at least one cell surface receptor and are further enhanced through a specific interaction of the fiber with an integrin 'co-receptor'. Thus, the host range is altered by modifying the fiber protein so it can bind to other components of the cell surface more efficiently [199, 200]. They are extremely useful if expression of the transgene is required for short periods because the incoming adenoviral proteins that package DNA can be transported to the cytoplasm where they are processed and presented on the cell surface, tagging the cell as infected for destruction. Adenoviruses can hold up to 8 kb of foreign DNA [42, 51, 79].

A recombinant adenovirus for gene transfer is generated by removing the E1 gene important for viral gene expression and replaced with the therapeutic gene under the

regulation of a heterologous promoter. However, E1-deleted viruses can be propagated only in a cell line that provides the E1 gene products *in trans*, such as 293 cells and express low levels of viral antigens following infection, resulting in a low level of DNA replication (particularly at a high multiplicity of infection) and often, the loss of therapeutic gene expression after seven to fourteen days [51, 203, 204]. Alternate recombinant adenoviruses and their complementary cells lines for production have been constructed by deleting the E2 and/or E4 genes. However, these viruses show reduced antigenicity *in vivo* and a reduction in the duration of gene expression, possible due to a loss of viral DNA replication or transregulation of gene expression [87, 194].

Herpes Simplex Virus Herpes simplex virus (HSV) is an enveloped virus with a large genome composed of 152 kb of linear dsDNA containing 84 viral genes, approximately half of which are nonessential for replication in cell culture. These features provide for multiple sites of foreign DNA insertion, capable of harbouring up to 75 kb or more of foreign DNA. HSVs can be used for efficient gene transduction in a variety of cell types. However, there are difficulties associated with long term transgene expression in certain tissues. This is thought to be since the mechanism of HSV attachment is complex, involving multiple viral envelope glycoproteins [79]. Using at least two of the viral envelope glycoproteins (gB and gD), the virus binds to cells through an interaction with heparin sulfate moieties on the cell surface. The virus enters the host cell by fusion, and there the linear DNA circularizes. Gene expression is initiated by the viral protein VP16, which is carried into cells as part of the viral tegument, stimulating expression of a set of proteins that activate a series of genes required for DNA synthesis and viral packaging. The newly synthesised viral particles are released from the infected cells by lysis. Since many of the HSV proteins are nonessential for viral replication, they can be removed and replaced with the target therapeutic genes [68, 105].

Baculovirus Baculoviruses provide a powerful tool for foreign gene expression at high levels in insect cells. Viruses within the Family *Baculoviridae* possess a closed dsDNA genome ranging from 80–220 kb in length, encoding for over 100 genes [117, 125]. Very little is known about the function of many baculovirus genes. However, two genes (p10 and polyhedrin) have been mapped and sequenced [67, 91, 100]. The polyhedrin gene has an important role in the transmission of the virus in nature, but is nonessential for viral replication. Hence, the polyhedrin gene can be exchanged with other genes to create a productive recombinant virus [160]. Furthermore, it is a gene with a strong promoter, and it can be utilised as

a marker, allowing easy detection by light microscopy [117]. Recombinant viruses for transfection are obtained by the recombination in host cells transfected with the wild-type DNA and a recombinant transfer plasmid vector containing the foreign gene and the 3' and 5' flanking region of the polyhedrin gene, including the promoter [117, 125]. During the final phase of gene expression (20–72 h post infection), polyhedrin becomes the predominant protein of the cell, expressing the gene of interest [125].

Baculoviral vectors contain multiple sites for foreign DNA insertion and are therefore able to encapsidate large amounts of foreign DNA [171] whilst maintaining stable and prolonged silencing of targeted genes [108]. However, cell lysis-associated proteolysis (if the protein of interest is a secreted protein, proteinases from lysed cells could severely compromise the yield of protein production the restricted host range [97]. Furthermore, the restricted host range (invertebrates) obviously makes baculovirus expression inconvenient for some applications.

Non-Viral Vectors

In light of the limitations of viral vectors such as the size of genes, random integration in the host genome, insertional mutagenesis and immunogenicity of the viral vector, there have been substantial considerations for the utilisation of non-viral vectors as a promising alternative for gene therapy since they avoid some of the potential hazards associated with the use of viral vectors. Gene transfer through the use of non-viral vectors is referred to as transfection. Transfection is the uptake by a cell of a fragment of naked DNA and the incorporation of this DNA into the host cell chromosome in an inheritable form [164].

Non-viral carriers have several advantages over the use of viral vectors: (1) they are generally safer *in vivo* and are cheaper to produce, (2) they do not elicit a specific immune response (less immunogenic than viral vectors) and can therefore be administered repeatedly, (this is not a problem in invertebrates as they have no antibodies) (3) they are easy to prepare and (4) they are more flexible with regard to the size of the DNA fragment being transferred [164].

Several DNA-vector-mediated mechanisms have been developed to use for RNAi to avoid some of the potential hazards associated with the use of viral vectors. There are three main non-viral vector systems that involve the treatment of cells by chemical (cationic liposomes and polymer complexes) or physical means (direct injection of naked plasmid DNA) [64, 89, 108, 110, 164]. The use of chemical and physical methods of gene transfer is relatively simple and does not provoke specific immune responses of the host. However, they are generally less efficient in delivering DNA and in initiating gene expression compared to the use of viral vectors [110, 164].

Liposomes and Lipoplexes

There are two types of liposomes, anionic and cationic, with the cationic liposomes more frequently used for gene therapy. Liposomes are artificial carrier molecules composed of lipids that are used to encapsulate DNA and permit foreign DNA to be introduced into cells. Liposomes form large complexes (either positive (cationic) or negatively (anionic) charged) that facilitate DNA fusion with the host cell. The majority of cationic liposomes used for transfection consist of a positively charged lipid mixed with a neutral helper lipid, facilitating the formation of stable lipid bilayers and interaction with the targeted cell membrane. The positively charged DNA complex is taken up from the extracellular compartment by endocytosis and transferred into the nucleus of the target cell [95]. Conversely, lipoplexes are spontaneously formed from the interaction of cationic lipids and negatively charged nucleic acids. However, they are structurally more heterogeneous and unstable compared to liposomes and there are major disadvantages for reproducibility, manufacturing, and drug administration [30].

In contrast to viral vectors, liposomes and lipoplexes are non-pathogenic, can be used for multiple treatments, and are relatively cheap and easy to produce. Furthermore, there is no limitation on the length of DNA that can be incorporated into cationic liposomes for gene delivery. However, the efficiency of transfection using liposomes is often less than that achieved using viral vectors. Hence, liposomes can be conjugated to defective viral particles, viral protein, or virally derived peptides that are able to disrupt the lysosome and/or increase DNA transport to the nucleus [47, 75, 98, 156].

Polymer Complexes

Polymer complexes offer an alternative to liposomes and are believed to protect DNA from degradation by condensing DNA molecules [98, 183]. Polymer complexes can be naturally or synthetically derived. Natural polymer complexes that have been employed as vectors for gene delivery include proteins such as histones [46] and aminopolysaccharides such as chitosan [18]. Synthetic polymer complexes include numerous peptides and polyamines [144].

Dynamic polyconjugates and cyclodextrin-based nanoparticles have yielded robust results involving siRNA in vivo. Dynamic polyconjugates is a multicomponent polymer system involving a membrane-active polymer to which siRNAs are covalently coupled via a disulfide bond where a polyethylene glycol (PEG) lipid and *N*-acetylgalactosamine groups are linked via PH-sensitive bonds [159]. The polymer complex disassembles in the low-pH environment

of the endosome, exposing its positive charge, resulting in endosomal escape and cytoplasmic release of the siRNA from the polymer [159]. Cyclodextrin-based nanoparticles contain polycation nanoparticles and like dynamic polyconjugates, involve targeted delivery and endosomal escape mechanisms [63]. Atelocollagen and chitosan are two additional polymer-type approaches that have also been reported to effectively deliver siRNA in vivo [69, 142, 148, 181].

Peptide and Protein Complexes

Short interfering RNAs may also be integrated with positively charged peptides or proteins. Briefly, cationic peptides and proteins are used to form complexes with the negatively charged phosphate backbone of the siRNA duplex. These complexes may incorporate a targeting element, such as a receptor-specific peptide or antibody [90, 174]. Peptide or protein complexes can also be non-targeted such as polyethylenimine (PEI) polymers and cell-penetrating peptides [1, 19].

Polyethylenimine polymers are synthetic linear or branched structures with high cationic charge densities and protonable amino groups that interact with the targeted cell surface through electrostatic interaction after incorporation with the siRNA. They are taken up by cells through endocytosis whereby endosomal escape is hypothesised to occur, resulting in the osmotic release of the polyplexes into the cytoplasm [1, 19]. Despite successful in vitro delivery of siRNA, there has yet to be successful published reports of in vivo silencing utilising non-targeting peptide or protein complexes.

'Naked' siRNA

The direct injection of naked plasmid DNA is the simplest non-viral gene transfer system and refers to the delivery of siRNA (unmodified or modified) in an excipient such as saline. Hence, the ease of formulation and administration of the delivery of naked siRNA to tissues makes this an attractive therapeutic approach. However, the expression level after the direct injection of naked plasmid DNA is severely limited due to rapid degradation of the DNA by nucleases [64, 89, 123]. Furthermore, plasmid based strategies require transcription and in the case of hairpin DNA, Dicer processing (compared with siRNA transfection that do not require Dicer processing) so the initiation of siRNA-transfected silencing is not immediate. The utility of plasmids is also limited in cell lines that are unable to be propagated for long periods (such as primary cells) and are difficult to transfect. The advantage with the use of plasmid DNA compared with the use of siRNAs is that plasmid DNA can be readily generated [37].

Hybrid Vectors

Hybrid vectors are designed to combine the advantages of more than one vector into a single reagent to achieve greater infection efficiency. For example, adenovirus/retroviral hybrid vectors combine the integrating potential of retroviral vectors (leading to persistent, long term gene expression) with the ability to infect dividing and non-dividing cells) and the high output of adenoviral vectors [127]. Similarly, prolonged transgene expression has been achieved through the use of an Ad/AAV hybrid vector compared with the single use of Ad vectors [49]. Adenovirus and AAV hybrids [49, 50], Ad and lentivirus hybrids [88], Ad and retrovirus hybrids [127, 208] and AAV and baculovirus hybrids [138] have successfully been established and have greater infection efficiencies compared to the use of either of the single virus vectors.

An alternative approach to the use of hybrid vectors involves the use of vectors that combine the merits of both viral and non-viral systems. For example, virus-cationic-liposome DNA complexes are capable of achieving a level of gene transfer greater than 2,000-fold above the achieved baseline using a cationic liposome (transferrin-polylysine) alone [28]. Furthermore, the inclusion of the adenovirus in a hybrid vector with transferrin-polylysine permits gene transfer in a number of cell lines that would otherwise have been refractory to gene transfer using transferrin-polylysine conjugates [28].

While these hybrid vectors have shown promise, at least in vitro systems, their efficiency for in vivo applications should be a focus for further investigation because there may be additional factors operating in vivo that reduce the efficiency of the hybrid vector. Furthermore, hybrid vectors have primarily been investigated in mammalian systems so the use of hybrid vectors in plant and alternative animal systems, warrants investigation before it can be assumed they work just as efficiently as they do in mammalian systems. However, the evidence from research to date suggests there is potential for the use of hybrid vectors to be used in preclinical and clinical applications for gene therapy.

Summary of Vectors for Gene Delivery

The delivery of siRNAs to target cells or tissues remains the primary challenge regarding RNAi therapeutics. A delivery vector, of either viral or non-viral origin, must be used to carry the foreign gene into a cell. No single gene delivery vector currently contains all the desirable characteristics, and each has its advantages and limitations. Viral vectors take advantage of the facile integration of the gene of interest into the host cell and high probability of its long term expression but are plagued by safety concerns

including toxicity and mutagenesis. Non-viral vectors, although less efficient in delivering DNA and in initiating and maintaining foreign gene expression and lacking specificity, are non-pathogenic and amenable to large scale production. Hence, non-viral gene transfer will probably become more important as better delivery methods become available. Whatever the method, all approaches have been demonstrated to provide selective and potent target gene suppression and offer great promise for the development of RNAi therapeutics.

Ideally, a gene transfer vector would have the properties from viral and non-viral vectors including high infection efficiency, convenience and reproducibility of production, ability to target the desired cell type and non-pathogenic to the host. All the desirable properties do exist individually in disparate vectors so perhaps the use of a combination of several vector systems will achieve the desired result of the vector being non-pathogenic, efficient and a specific gene delivery method for RNAi. However, the selection of the delivery approach will also be influenced by the nature of the application, the route of administration to be used, and the cell types and tissues to be targeted.

Limitations on the Use of RNA Interference

Although RNAi is an extremely powerful tool as a gene silencing and antiviral mechanism, RNAi has several limitations.

‘Off-Target’ Effects

Although the effects of RNAi are generally thought to be sequence-specific, there is still debate within the literature as to whether or not some of the effects are ‘off-target’. ‘Off-target’ effects include silencing of the wrong genes or the degradation of closely related but non-identical mRNAs [10, 35, 59]. Conversely, the use of some siRNAs can enhance expression instead of having a silencing effect. There may also be cross reactivity of the siRNAs with targets of limited similarity [72, 78, 162]. Hence, ‘off-target’ gene silencing is clearly unwarranted since the cellular consequence of altered gene activity is unknown and largely unpredictable.

Genome-wide monitoring gene activity by microarray technology has demonstrated that ‘off-target’ silencing of genes can result from siRNA treated cells. Initial analyses demonstrated that a match of as few as eleven nucleotides can result in ‘off-target’ knockdown [72]. More recently, a six to seven nucleotide match to the siRNA has been identified in experimentally verified ‘off-targets’ [24, 73, 106]. Thus, an understanding of the specificity of siRNA-mediated gene silencing is essential for the appropriate

design and interpretation of RNAi experiments and RNAi-based therapeutic strategies. However, as the intensive research into the specificity of siRNAs and miRNAs continue, it is likely that sequences can be targeted with minimal side effects.

RNA Interference Suppressor Proteins

There is the additional concern of PTGS/RNAi suppressor proteins that have been found in several plant and animal viruses. Viruses encoding proteins that are suppressors of RNA silencing was first reported from plant viruses in 1998 [8, 14, 21]. A number of plant [146, 169, 193] and insect [103] viruses have since been reported to express different RNAi suppressor proteins. For example, the tomato bushy stunt virus p19 protein suppresses PTGS in plants by binding siRNAs generated after virus infection [169] and the Cucumber mosaic virus 2b protein inhibits the spread of PTGS throughout the host plant [152]. The insect virus, Flock house virus B2 protein suppresses RNAi activity in both plants and *Drosophila* S2 cells [104]. A similar B2 protein in *Macrobrachium rosenbergii* nodavirus (MrNV) [61] has been found suggesting an evolutionary conserved RNAi pathway (or at least part of) having a natural antiviral role. Hence, viral suppressor proteins may be useful tools to investigate the mechanism of RNAi.

The molecular mechanism of RNAi inhibition is still relatively unknown. Research concerning RNAi suppressor proteins has primarily concerned plants and to date, three types of viral suppressors have been identified using a variety of silencing suppression assays. Two types of viral suppressors are capable of a complete or partial reversal of pre-existing RNA silencing, whereas the third does not reverse RNA silencing but prevents RNAi signalling [102]. Although the suppression of RNAi could be essential for efficient viral infection, many of the viral suppressors identified have previously been shown to be required for virulence determination [102]. Hence, the suppression of RNA silencing by plant and animal viruses probably represents a viral adaptation to a novel host antiviral defence mechanism. Since the RNA silencing pathway seems to be conserved in all eukaryotic organisms and as viruses of plant and animal hosts encode homologous proteins, the same assays for silencing suppression established in plants should be applied to look for animal viral suppressors (preferably in animal viruses that have proteins which share an evolutionary origin with known plant viral suppressors for initial investigations).

The Use of Synthesized siRNA

The siRNA sequence is not, surprisingly, a crucial aspect in determining the efficiency of the siRNA-directed mRNA

cleavage which results in gene silencing. However, there are a number of other factors including the binding of the RNA-binding proteins and the secondary structure of the mRNA target that are likely to affect the efficiency of gene silencing involving siRNAs [37]. Not every siRNA sequence is efficient in mediating gene silencing and it has been suggested that most researchers achieve a 33 % success rate [35]. Furthermore, siRNAs that are not perfectly homologous to their target sequence can repress their translation (recorded in plant and mammalian systems [33, 162] and in invertebrates [93]). However, further research is required to determine if there is any interplay between the characteristics of the mRNA target sequence and the effectiveness of siRNA-directed gene silencing.

Another drawback with the use of siRNAs is cost, particularly since different siRNAs targeting the same gene can have a range of efficacies. Consequently, several siRNA sequences may need to be assessed for knockdown efficiency before finding the one that works well enough to use reliably. However, it has been suggested that whichever strand of the RNA duplex is most easily unwound from 5' to 3' will be preferentially assembled into the RISC complex [166]. This has led to the conclusion that siRNAs designed to be unstable at the 5' end of the antisense strand are the most effective, and have the least likelihood of sense strand-directed silencing [82].

Multiple sequences are often required to be screened because different siRNA sequences have different efficacies. Hence, it has been suggested that by pooling enzymatically generated siRNAs, there is a greater chance of achieving the required result with increased efficiency, eliminating the need to identify a single effective siRNA. Although this may be a quicker and more cost effective process, there are increased concerns of non-specific translational inhibition due to any residual unprocessed long dsRNAs and increased off-target effects [6, 66, 71, 72, 162, 163]. If several siRNAs were pooled, one would have to assume there would be some competition between the highly efficient and less efficient siRNAs resulting in fewer efficacies compared to the use of one optimal siRNA sequence. By utilising only one optimal siRNA, any observed phenotypic changes can be observed and linked to that siRNA and can be verified through the application of a second siRNA targeting the same gene. This certainly has its advantage over pooling siRNAs where the specific sequence for the phenotypic changes is unknown, requiring additional confirmation through the use of target-specific and mismatched siRNAs to verify the sequence specificity of the observed phenotype. This bound to be an energy/metabolic cost to cells and organisms of the processing of all these siRNA which will lead to slower growth rates.

The development of siRNA gene based therapies can also be difficult, particularly for RNA viruses which can

have a high degree of sequence diversity between difference genotypes. In addition, rapid evolution through mutations and recombination can also make the development of siRNA gene based therapies difficult [86]. Furthermore, large sections of highly unstructured translated regions of viral RNA genomes are resistant to PTGS [86]. Hence, gene targeting must avoid these areas of the RNA genome for effective silencing.

The use of siRNAs has two other potential problems. The first is that the downregulation of gene expression mediated by siRNA frequently only lasts for 3–5 days in cell culture or 3–5 cell divisions post-transfection, due to dilution rather than degradation of siRNAs [36, 66], which may not provide a sufficient amount of time to determine if functional depletion has occurred. This may not be such a problem in eutelic animals with a constant cell number like nematodes, or pseudoeutelic animals like insects and crustacea. Secondly, there is variability in transfection efficiency, particularly when working with difficult-to-transfect cell lines. Despite the potential problems associated with inducing RNAi with siRNAs, they have been applied and continue to be successfully applied in plant, invertebrate and vertebrate systems [25, 38, 124, 175, 197].

Concerns with the Use of Viral Vectors

In general, viruses are very efficient vectors for gene transfer but there are significant limitations and concerns associated with their use. There are obvious safety issues that arise when working with viral vectors such as carcinogenesis and viral integration into the host genome which has the potential for insertional mutagenesis, resulting in inappropriate activation or inactivation of endogenous genes. Furthermore, repeated administration of a viral vector into vertebrates can induce an immune response which abolishes the transgene expression [110]. However, the question still remains as to the long term effect of the integrated transgene and of the virus in the host individual and should be a focus for further research, particularly if viral vectors are to be used for therapeutic purposes.

Toxic immunological reactions are another major drawback of viral vectors. RNAi is an endogenous pathway with its own cellular machinery so there are concerns that utilising this machinery for therapeutic purposes could disrupt its intrinsic functions [149]. Furthermore, viral vectors have been implicated in the death of at least one patient. In 1999, a teenager died following the administration of recombinant adenoviral vector received during gene therapy treatment. The recombinant adenoviral vector invaded not just the intended target organ (the liver), but also other organs triggering an activation of innate immunity that led to respiratory system distress and fatal multiple organ failure [119, 191].

Biological Applications for RNAi

Pathogen Resistance and Maintenance of Normal Gene Expression

The primary focus for the use of RNAi has been for pathogen resistance and there is no doubt that RNAi has a potential role in pathogen (viral) resistance in plants, invertebrates and vertebrates. The appearance of dsRNA within a cell (for example, as a result of viral infection) triggers an RNAi response. The cellular enzyme Dicer cleaves the dsRNA into siRNAs. The siRNAs bind to the cellular enzyme RISC which degrades the mRNA, silencing the expression of the viral gene.

Following the 1998 demonstration of dsRNA-mediated RNAi in *C. elegans* [45], the use of RNA silencing as a nucleic acid-based antiviral immunity in invertebrates has been established in a number of arthropod species including the fruit fly [80], honeybee [17] and mosquitoes [2]. Furthermore, RNAi inhibition of viral replication has been demonstrated in vitro for a variety of viruses in plants and mammals [12, 38, 48, 55, 121]. Hence, RNAi has the potential to be harnessed as a first response against viruses where protective vaccines are unavailable or useless (in particular arthropods since they have an innate immune system and therefore cannot be treated or protected against viral diseases using vaccines).

In mammalian systems, RNAi has been suggested to be involved in maintaining order in the genome (by suppressing the movement of mobile genetic elements such as transposons and repetitive sequences) and maintaining normal cellular gene expression [38]. However, since it has only been suggested for mammalian systems and the mechanism of RNAi seems to be evolutionary, it may be likely that it has the same role in plants and invertebrates. Additional research is required to determine the possible role RNAi has in maintaining order in the genome and normal gene expression in plant and animal systems.

Transgenic RNAi

The application of RNAi technology has progressed towards the development of transgenic animals (the passing and amplification of the RNAi signal from cell to cell), often done by the infection of embryonic stem cells [3, 37]. The first indication that RNAi silencing could be amplified came from the observation that RNAi had the ability to spread throughout *C. elegans* after the ingestion of bacterially expressed dsRNAs [45, 168, 184]. However, transitive RNAi can result in the silencing of a number of unintended genes within the genome. In this process, siRNAs bind to complementary transcripts after being denatured. New dsRNAs (secondary siRNAs) are created via a

5' to 3' extension reaction performed by an RNA-dependent RNA polymerase (RdRP) [32]. Consequently, there may be non-specific gene silencing if the secondary siRNAs are complementary to other gene sequences.

Much of the work concerning transitive RNAi has concerned plants [140, 189, 190]. However, the phenomenon of transitive RNAi has also been reported in *C. elegans* (where the transitive RNAi phenomenon was first observed) [3] and in mice and rats [60, 115]. For example, mice injected with a polymerase III expression vector as embryos were crossed to produce F1 progeny that showed virtually complete silencing of enhanced green fluorescent protein (EGFP) in the brain, liver, kidney and spleen suggesting the silencing effect was widespread throughout the body [60]. Similarly, transgenic mice and rats have been produced after infecting embryos with a lentivirus vector containing the gene of interest [115]. These findings suggest transgenic RNAi could function as an alternative method for gene silencing even in species where cell lines are not established.

The work concerning transitive RNAi in plants suggests the targets of RNA silencing are involved in the expansion of the pool of functional siRNAs. There is also some indication that RNA silencing can expand across target RNAs and to regions downstream of the primary genome target [20, 57, 187]. Since transitive RNAi has been reported in plant, invertebrate and vertebrate systems, it is likely that this phenomenon is more widespread than what has been reported but the lack of studies in this area has hampered our knowledge on how widespread the phenomenon of transitive RNAi is. Furthermore, experiments concerning transitive RNAi have involved a limited number of siRNAs and cell types. It is therefore necessary to extend these findings to other cell types and test different siRNAs (since different siRNAs have different efficiencies), particularly if the siRNAs are to be used as therapeutic agents. Nonetheless, the results to date indicate RNAi-mediated gene silencing is heritable and stable and that RNAi functions in all cell and tissue types tested from embryos to adult animals.

RNAi with Special Reference to Controlling Viruses in Crustacea

Effect on Crustacean Mortality

Quite a lot of research has been undertaken investigating RNAi in crustacea, but as yet there is no compilation of its effectiveness. Most of the major viruses of crustacea have been investigated. In almost all cases reported, survival increased following the administration of dsRNA, triggering of the RNAi pathway. RNAi has down regulated White

Spot Syndrome Virus and allowed a 85 % reduction in mortality when targeting the VP28 envelope gene, suggested to play a role in WSSV infection [114, 148]; 78 % reduction in mortality when targeting RR2 ribonucleotide reductase; 70 % reduction in mortality against VP19 envelope gene [154]; 47 % reduction in mortality against viral ubiquitin ligase [62]; 44 % reduction in mortality when antagonistic to the DNA polymerase. Similarly, when yellow head virus was down regulated by attacking its protease gene and a host host Rab7 GTPase, mortality was reduced by 90 % [143]. Almost the same reduction in mortality (88 %) was achieved when the polymerase gene of Taura Virus Syndrome was knocked down.

Even in experimental animal models for crustacean viruses, RNAi has been effective. *Cherax quadricarinatus* had 50 % less mortality when RNAi against the B2 gene of MrNV was administered [61]. This is particularly interesting as the B2 gene produces a protein that specifically stops the activity of RNAi. So as long as the RNAi is administered before viral exposure, the RNAi machinery can out-compete the viral encoded anti-RNAi system which stops the enzyme Dicer from working. In a cricket model, mortality was reduced by 21 % when RNAi was designed against the capsid protein of *Penaeus merguensis* densovirus (PmergDNV) (previously named hepatopancreatic parvovirus). Whilst most studies do not report any negative, off-target or detrimental effects of RNAi, dsRNA targeting the NS2 gene of PmergDV resulted in faster time to mortality and higher mortalities (70 % mortality) than the virus alone (46 % mortality) [92] even though the viral titres were reduced by one log. Whilst the role of NS2 is unknown it can be speculated that NS2 might be homologous to important host housekeeping genes to have this effect. However, this does demonstrate that RNAi can have an unpredicted deleterious effect and the effect of all dsRNA designed to trigger the RNAi pathway should be tested before any theoretical “*in silico*” application.

Effects on Viral Titre

Very few studies have looked at the change in viral titre in animals administered RNAi. Early studies showed down regulation of genes based on the presence/absence or lighter amplicons on gels [112, 155, 207]. When the titre was measured by quantitative PCR, the viral titre was reduced twofold to 1 log reduction. RNAi targeting the viral ubiquitin ligase WSSV222, an anti-apoptosis protein of WSSV dropped the titre **20 fold** from 2.6×10^7 to 1.3×10^6 [62]. RNAi against the capsid gene of PmergDNV reduced the titre from 3.4×10^3 to 3.6×10^2 [93]. Similarly, against NS2, titre dropped from 9.9×10^4 to 8.1×10^3 [92]. Interestingly, the non-specific controls also reduced titre to 4.0×10^2 and 2.2×10^3 respectively

for the experiments described above demonstrating non-specific down regulation (see below). In a study investigating RNAi against the *B2* gene of MrNV (known to have protective effects), titres only reduced from 3.5×10^1 to 1.3×10^1 but mortality reduced from 60 to 10 % [61].

Dose and Timing of Prophylactic Delivery of RNAi

Within crustacea, the dose of the dsRNA required to trigger the RNAi pathway and subsequently knock down viruses does not seem to be that critical, probably because the iRNA template is recycled within the RISC to be reused. With crustacea, doses have ranged from 2 doses of 7.5 µg/g of crustacean to 0.5 µg/g with a mean of 2.6 µg/g (SD = 2.27 µg/g, $n = 10$).

However, the timing of the delivery of the prophylactic dose is much more critical with many authors reporting loss of antiviral activity with time. Most authors have delivered the iRNA 24 h before challenge with the virus with satisfactory results. Even up to 4 days but not 5 days administration prior to exposure was protective against YHV [207]. Only one study has shown that delivery of dsRNA 24 h after exposure to YHV was acceptable but 48 h after exposure was too long [143]. As usual, prophylaxis is better than treatment, but interestingly, treatment is not impossible immediately after a potential exposure to a virus.

A seminal piece of research by Rajeshkumar et al. [148], showed that after 1 week of dietary uptake of chitosan-bound plasmid against WSSV VP28, there was relatively long term protection. Challenge with WSSV 1, 2, and 3 week after cessation of administration of the dsRNA lead to survival of 85, 65 and 50 % respectively. Even this result at 3 weeks after delivery could be practical at the farm level where survival of 50 % is still profitable. The other beauty of this research was that it showed that delivery by diet rather than inoculation was feasible for juvenile prawns.

Non-Specific Protective Effects of dsRNAs

One observation that has occurred a few times in the literature is the fact that non-target, non-specific dsRNA used as negative controls in viral knockdown experiments has had a protective effect against some viruses including WSSV [154], YHV [207] and even totally unrelated, non-specific dsRNA (green fluorescent protein) down regulated YHV genes in *Penaeus monodon* [207]. Similarly, La Fauce and Owens [93] found non-specific dsRNA knocked down against PmergDNV (see above).

In other animals this would suggest that an interferon pathway (INF) via toll-like receptors was operating in

crustacea. However, other published work suggests that the decapod INF pathway has regressed to be non-functioning [132] despite the presence of an *Interferon Regulatory Factor (IRF)* gene present in Chinese mitten crab, *Eriocheir sinensis* [132]. Indeed experimental exposure of crayfish to doses of α -INF from 100 to 210 IU/g BW showed increasing mortality in a dose-dependent manner (unpublished data). Perhaps the therapeutic dose of INF is below 100 IU/g BW as the available literature supports the presence of the INF effect. At this time, it appears many of the components (genes) of a historic interferon pathway are present in crustaceans [113] but there is no evidence of a functioning pathway. The interferon components may have been subverted for other functions, perhaps related to viral accommodation which may be how the nonspecific dsRNA can result in a protective effect.

Use of RNAi to Test Host Gene Function

In biomedicine, RNAi has been used frequently as an experimental tool to investigate gene function. This technique has been some what underexploited regarding crustaceans, perhaps due to the lack of a readily available in vitro cell culture system. dsRNA targeting crayfish anti-lipopolysaccharide factor (ALF) resulted in a tenfold increase in WSSV demonstrating a critical role of ALF in combating WSSV [112]. Target RNAi assays against *P. monodon* Rab 7 GTPase involved with viral endocytosis in endosome plus an anti-YHV resulted in 20 % less mortality than the anti-YHV alone [143]. When the same host gene was targeted in *P. vannamei*, TSV mRNA was also dramatically reduced [135]. Su and colleagues [176] actually targeted Dicer itself and by knocking down the key enzyme of the RNAi pathway allowed GAV to express more quickly and lethally. However, supplemental targeting host GTPase is unlikely to be a sustainable treatment as GTPases have such widespread functions in host cell maintenance so there is bound to have some detrimental side effects.

Conclusions on RNAi in Crustacea

Double stranded RNA triggering the RNAi pathway has been successful against all the major viruses that affect crustacean aquaculture except infectious myonecrosis virus and so the concept is well and truly tested. Furthermore, dsRNA can be delivered via a pelleted diet using chitosan nanoparticles, thus clearing the way for commercial delivery. It remains to be seen if these can be manufactured in a cost effective manner, but it should be possible. The long term future might involve transgenic crustacea where the dsRNA is injected into the 4-cell stage of the embryo [145]. Coupled with the SPF breeding programs, this would

deliver animals not only free of specific pathogens but truly resistant as well.

Future Research Concerning RNAi Based Technologies

Although the effects of RNAi seem to be beneficial, the available literature lacks the answers to some potential problems that may be associated with the artificial delivery of dsRNA. For example, it is still unknown whether cells undergoing RNAi-mediated gene silencing are as healthy as cells not undergoing RNAi-mediated gene silencing. There have been few studies concerning the overall health of the cells undergoing RNAi-mediated gene silencing. However, available literature suggests cells not undergoing RNAi are healthier than their affected counterparts. Certainly, there must be a metabolic cost to processing RNAi which may end up being expressed as lower biomass production of commercial animals. There is evidence that siRNAs and members of the RNAi machinery have a role in chromosome architecture in several organisms including the fission yeast *Schizosaccharomyces pombe* (the enzyme Dicer is involved in chromatin silencing and heterochromatin assembly). Secondly, there is evidence that the RNAi machinery can be saturated [76]. Consequently, the question remains whether a cell devoting much of its RNA machinery to the process of mRNA degradation could become more susceptible to virus infection or even defective in chromosome function. Therefore, siRNA-directed against a particular gene may silence that gene but may also affect normal cell division.

The injection of dsRNA into the nematode *C. elegans* triggers its spread to several tissues but it is unknown how the dsRNAs exit the cell in which they are produced, how they are systemically disseminated, or how they are taken up by distant target cells. It has been proposed for plants and lower eukaryotes such as *C. elegans* and embryos of *Drosophila*, that siRNAs possess the capability to function as primers that are extended on the targeted RNA by an RdRp to amplify the dsRNA trigger [109, 168]. Although a similar phenomenon has been detected in *Anopheles gambiae* and in *Drosophila* (work following the initial observations in *Drosophila* embryos) [65, 157], siRNAs that function as primers to amplify the dsRNA trigger have not proven to play a significant role in the RNAi pathway. It is also unknown if there is a threshold level of dsRNA needed to trigger an RNAi response. Additional research is therefore required into the spread of RNAi-mediated silencing.

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