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Review

Exploring RNAi as a therapeutic strategy for controlling disease in aquaculture

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ABSTRACT

Aquatic animal diseases are one of the most significant constraints to the development and management of aquaculture worldwide. As a result, measures to combat diseases of fish and shellfish have assumed a high priority in many aquaculture-producing countries. RNA interference (RNAi), a natural mechanism for post-transcriptional silencing of homologous genes by double-stranded RNA (dsRNA), has emerged as a powerful tool not only to investigate the function of specific genes, but also to suppress infection or replication of many pathogens that cause severe economic losses in aquaculture. However, despite the enormous potential as a novel therapeutical approach, many obstacles must still be overcome before RNAi therapy finds practical application in aquaculture, largely due to the potential for off-target effects and the difficulties in providing safe and effective delivery of RNAi molecules *in vivo*. In the present review, we discuss the current knowledge of RNAi as an experimental tool, as well as the concerns and challenges ahead for the application of such technology to combat infectious disease of farmed aquatic animals.

1. Introduction

Aquaculture has expanded rapidly to become a major economic and food-producing sector in the world [1]. In parallel, due to the intensification of rearing methods and systems, the industry has been overwhelmed with a number of trans-boundary aquatic animal diseases caused by viruses, bacteria, fungi and parasites, with newer pathogens being identified every year [2]. Consequently, disease outbreaks have become a significant constraint to the development of the aquaculture industry, affecting the socio-economic development of this sector worldwide [3]. Therefore, in order to meet the increased demands of our expanding population, new technologies and techniques for disease control must be developed and implemented. Of major interest is the application of genetic engineering and other biotechnologies.

One recent technology likely to play a major role in the future of aquaculture is RNA interference (RNAi). RNAi is a recently-discovered mechanism of post-transcriptional gene silencing in which double-stranded RNA (dsRNA) corresponding to a gene, or coding region, of interest is introduced into an organism, resulting in degradation of the corresponding mRNA [4]. Because of this sequence-specific ability to silence target genes, RNAi has been extensively used to investigate the functional role of specific genes by reducing expression, without altering genotypes [5]. The silencing effects could be used not only to study gene function, but also to indentify drug targets and vaccine candidates [6], as well as to control infectious disease by interfering with pathogen transmission, development and proliferation within the host [5].

In this review we summarize the current knowledge regarding the therapeutic applications of RNAi for developing alternative treatment strategies against infectious diseases in aquaculture.

2. RNA interference

RNA interference (RNAi) is a highly evolutionally conserved process of post-transcriptional gene silencing (PTGS) by which double-stranded RNA (dsRNA), when introduced into a cell, causes sequence-specific degradation of homologous mRNA sequences [7]. RNAi as a mechanism of PTGS most likely evolved as a cellular defence strategy to eliminate unwanted nucleic acids (viruses and transposable elements) in plants, fungus and invertebrates [8], but

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is also widely employed in most eukaryotic cells as a mechanism to regulate the expression of endogenous genes [9].

The discovery of RNAi phenomenon was first observed when plant biologists were performing experiments to enhance the hue of purple petunias. The introduction of a pigment-producing gene under the control of a promoter resulted in variegated or completely white flowers, rather than the expected deep purple colour [10]. What was initially thought to be a peculiar effect in flowers was subsequently found to occur in fungi when scientists were attempting to boost the synthesis of orange pigment in *Neurospora crassa* [11]. The phenomenon was first called co-suppression in plants and quelling in fungi. The observation of RNAi in animals came accidently when Guo and Kemphues [12] injected the antisense strand to block expression of the par-1 gene in the nematode *Caenorhabditis elegans*. The expression was disrupted but, upon performing their controls, they found that the sense strand also reduced the expression of that gene. The involvement of dsRNA in gene silencing phenomena, however, was discovered by Fire et al. [13] who found that dsRNA, but not single stranded sense or antisense RNA, mediated gene silencing in microinjected *C. elegans*. Subsequently, RNAi has been recognized as a highly conserved process encountered not just in unicellular protozoans and fungi but also in complex organisms such as plants and animals [14–18].

The basic principle of RNAi involves destruction of mRNA upon interaction with homologous dsRNA, and translational repression through imperfect complementary binding of small RNAs with the 3′ untranslated region of the target mRNA [19]. Genetic and biochemical data indicate a possible two-step mechanism for RNAi: an initiation step and an effector step [4] (Fig. 1).

In the initiation step, long dsRNA, derived from endogenous, transgenic or viral transcripts, are processed into short ~21–22 bp molecules, known as small-interfering RNA (siRNA). Those small molecules have a characteristic 2 nucleotide 3′ overhang that allows them to be recognized by the enzymatic machinery of RNAi that eventually leads to homology-dependent degradation of the target mRNA [20]. The generation of siRNA during the initial steps of RNAi is performed by a Ribonuclease III (RNase III) enzyme, called Dicer, which has the ability to recognize and cleave dsRNA at specific positions or sequences [21]. During the effector step, the short RNA duplexes are incorporated into a multimeric protein complex, known as RNA-induced silencing complex (RISC), which contains an Argonaute (Ago) protein as one of its main components. RISC binds and unwinds the siRNAs into single-stranded molecules. The sense strand is released and the antisense strand remains bound to RISC, serving as guide to select fully complementary mRNA substrates for degradation [22].

Besides siRNA, another class of small RNAs, known as microRNAs (miRNAs), have been characterized, sharing a common RNase-III processing enzyme (Dicer) and closely related effector complex (RISC), for post-transcriptional repression. However, while siRNAs originate from exogenous DNA or dsRNA, aberrant transcripts from repetitive sequences in the genome or long hairpin forming transcripts, miRNAs are derived from precursor transcripts called primary miRNAs (pri-miRNAs), which are typically transcribed by RNA polymerase II [23]. Pri-miRNAs are cleaved in the nucleus by a Dicer-like enzyme called Drosha to produce a characteristic stem-loop structure of about 70 bp long, known as pre-miRNA [24]. Subsequently, pre-miRNA is transported to the cytoplasm, where the loop end is cleaved off by Dicer activity, resulting in a mature miRNA, a dsRNA approximately 22 bp in length [25]. The mature miRNA then enters RISC and exert its regulatory effect via either degradation of complementary mRNA or binding to imperfect complementary sites within the 3′ untranslated region (UTRs) of their mRNA targets, leading to translational repression [26]. The mature miRNA then enters RISC, which recognizes target mRNAs based on sequence complementarity between the guide miRNA and the mRNA transcript resulting in either mRNA degradation or translational repression [26] (Fig. 1).

Apart from PTGS, another homology-dependent gene silencing mechanism called transcriptional gene silencing (TGS) is recognized in plants and animals [27]. TGS is induced by the same molecules that trigger PTGS, but results in inactivation of the gene for transcription rather than by RNA destruction [28]. TGS inhibits transcription by DNA methylation and histone post-translational modifications, and corresponding remodelling of chromatin around the target gene into a heterochromatic state [29]. As TGS is not the focus of the present, more detailed information can be found in other reviews [27,30,31].

The multi-step processes of RNAi can be experimentally activated at different stages by specific forms of regulatory RNAs. These include *in vitro* transcribed [32] and bacterially expressed dsRNA [33], *in vitro* [34] and chemically synthesized siRNA [35], Dicer-generated siRNA pool (d-siRNA) [36], viral/plasmid-based hairpin RNAs (shRNAs) [37,38], miRNA [39,40] and pre-miRNA [41]. Among the delivery methods that have been developed for efficient administration of these classes of nucleic acid, both *in vivo* and *in vitro*, are ingestion [42], injection [43], immersion [44], transfection [45] and electroporation [40].

3. RNAi as a natural antiviral defence mechanism in vertebrates and invertebrates

To protect themselves from viral infections, most organisms have evolved several defence mechanisms to sense and fight those pathogens. Among those systems are the interferon (IFN)-mediated [46] and the ancient recently described RNAi-based antiviral mechanism [47]. In both systems, dsRNAs present in the viral genome or commonly generated during viral replication are recognized by the host as molecular pattern associated with viral infection, inducing a range of immune responses [8].

The post-transcriptional activity of the RNAi machinery to degrade cytoplasmic RNA in a sequence-specific manner is the key to its antiviral function in invertebrates [48]. In this process, virus-derived siRNAs originated by Dicer cleavage are incorporated into the RISC, leading to the degradation of the corresponding viral RNAs and, consequently, inhibition of viral replication [49].

In vertebrate cells, on the other hand, the introduction of long dsRNA typically induces innate immune responses, constituting the first line of defence to limit viral replication [50]. The intracellular presence of viral dsRNA activates the Toll-like receptor (TLR) 3 pathway, as well as dsRNA recognition proteins (dsRNA-dependent protein kinase PKR and 2′–5′-oligoadenylates/RNase L), leading to the nonspecific degradation of RNA transcripts, the production of IFN response and the overall shutdown of host cell protein syntheses [51]. The nonspecific inhibitory effects caused by activating the IFN pathway initially hampered the application of RNAi in vertebrate cells. This problem was later circumvented by Elbashir et al. [52], who showed that siRNAs (roughly 20–25 bp), rather than long dsRNA (>30 bp), effectively knockdown the amount of transcript of a given gene without activating the IFN system. However, the minimum effective siRNA dose is often recommended to be employed, as high siRNA concentrations have also proven to activate components of the IFN system [53–56]. Such discovery ignited an explosion of work on RNAi in vertebrate cells. Nevertheless, it is still unclear whether RNAi naturally acts as a system of defence against viral infection in vertebrates.

Given the potential of the innate immune response to promote an effective antiviral response based on protein recognition, it is expected that in vertebrates the RNAi machinery has been conserved for other purposes, rather than an ancient nucleic-based