Review Article

Antiviral innate immune response of RNA interference

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Abstract

RNA interference (RNAi) is an ancient, natural process conserved among species from different kingdoms. RNAi is a transcriptional and post-transcriptional gene silencing mechanism in which, double-stranded RNA or hairpin RNA is cleaved by an RNase III-type enzyme called Dicer into small interfering RNA duplex. This subsequently directs sequence-specific, homology dependent, Watson-Crick base-pairing post-transcriptional gene silencing by binding to its complementary RNA and initiating its elimination through degradation or by persuading translational inhibition. In plants, worms, and insects, RNAi is the main and strong antiviral defense mechanism. It is clear that RNAi silencing, contributes in restriction of viral infection in vertebrates. In a short period, RNAi has progressed to become a significant experimental tool for the analysis of gene function and target validation in mammalian systems. In addition, RNA silencing has then been found to be involved in translational repression, transcriptional inhibition, and DNA degradation. RNAi machinery required for robust RNAi-mediated antiviral response are conserved throughout evolution in mammals and plays a crucial role in antiviral defense of invertebrates, but despite these important functions RNAi contribution to mammalian antiviral innate immune defense has been underestimated and disputed. In this article, we review the literature concerning the roles of RNAi as components of innate immune system in mammals and how, the RNAi is currently one of the most hopeful new advances toward disease therapy. This review highlights the potential of RNAi as a therapeutic strategy for viral infection and gene regulation to modulate host immune response to viral infection.

Key words: RNA interference, RISC complex, Dicer, viral infection.

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Introduction

RNA silencing is a comprehensive term that has been coined to describe RNA interference (RNAi) in animals, post-transcriptional gene silencing in plants, and quelling in fungi, which are all phenotypically different but mechanistically analogous forms of RNAi [1]. RNA silencing is an evolutionarily ancient RNA surveillance mechanism, conserved among eukaryotes as a natural defense mechanism to protect the genome against invasion by mobile genetic elements, such as viruses, transposons, and possibly other highly repetitive genomic sequence, furthermore to orchestrate the function of developmental programs in eukaryotic organisms [1,2]. Announcement of RNAi as a "breakthrough" by the journal Science encouraged scientists to review their vision of cell biology and cell evolution [3-4]. The discovery of RNAi followed observations in the late 1980s of transcriptional inhibition by antisense RNA expressed in transgenic plants [5], during a search for transgenic petunia flowers that were anticipated to show a more concentrated color of purple. In an effort to alter flower colors in petunias, Jorgensen *et al* [6] sought to upregulate the activity of the chalcone synthase (chsA) enzyme, which is associated with the production of anthocyanin pigments by introducing additional copies of this gene. The overexpressed gene was anticipated to result in darker flowers in transgenic petunia, but instead it generated less pigmented, fully or partially white flowers, indicating that the activity of chsA had been significantly decreased. Actually, both the endogenous genes and the introduced transgenes were downregulated in the white flowers. Unexpectedly, the loss of cytosolic chsA mRNA was not associated with decreased transcription as tested by run-on transcription assays in extracted nuclei. Further examination of the phenomenon in plants indicated that the downregulation was due to post-transcriptional inhibition of gene expression by an augmented rate of mRNA degradation [6]. Other laboratories about the

same time showed that the introduction of the transcribing sense gene could downregulate the expression of homologous endogenous genes [6,7].

A homology-dependent gene-silencing phenomenon termed "quelling" was reported in the fungus *Neurospora crassa* [8]. Quelling was recognized during attempts to increase the production of orange pigment expressed by the gene al1 of *N. crassa* [8]. Wild-type *N. crassa* was transformed with a plasmid containing a 1.5 kb fragment of the coding region of the al1 gene. Some transformants were stably quelled and displayed albino phenotypes. In these al1-quelled fungi, the quantity of native mRNA was highly reduced while that of unspliced al1 mRNA was similar to the wild-type fungi. This indicated that quelling, but not the rate of transcription, affected the level of mature mRNA in a homology-dependent manner. Shortly thereafter, plant virologists conducting experiments to improve plant resistance to viral infection made a similar, unanticipated observation. While it was documented that plants produced proteins that mediated virus-specific enhancement of tolerance or resistance to viral infection, a surprising finding was that short, noncoding regions of viral RNA sequences carried by plants provided the same degree of protection. It was concluded that viral RNA produced by transgenes could also inhibit viral accumulation [9].

Homology-dependent RNA elimination was also noticed to occur during an increase in viral genome of infected plants [10]. Ratcliff *et al.* [11] defined a reverse experiment, in which short sequences of plant genes were introduced into viruses and the targeted gene was suppressed in an infected plant. Viruses can be the source, the target, or both for silencing. This phenomenon was named "virus-induced gene silencing" (VIGS), and the whole set of similar phenomena was together named posttranscriptional gene silencing [11]. Not long after these observations in plants, investigators searched for homology-dependent RNA elimination phenomena in other organisms [12,13]. The phenomenon of RNAi first became known after the discovery by Andrew Fire *et al*. in 1998 of a potent gene silencing effect, which occurred after injecting purified dsRNA directly into adult *Caenorhabditis elegans* [2]. The injected dsRNA corresponded to a 742-nucleotide segment of the unc22 gene. This gene encodes nonessential but abundant myofilament muscle protein. The investigators observed that neither mRNA nor antisense RNA injections had an effect on production of this protein, but dsRNA successfully silenced the targeted gene. A decrease in unc22 activity is associated with severe twitching phenotype, and the injected animal as expected showed a very weak twitching phenotype, whereas the progeny nematodes showed strong twitching. The investigators then showed similar loss-of-function knockouts could be generated in a sequence-specific manner, using dsRNA corresponding to four other *C. elegans* genes, and they coined the term RNAi. The Fire *et al*. discovery was particularly important because it was the first recognition of the causative agent of what was until then an unexplained phenomenon.

RNAi can be initiated in *C. elegans* by injecting dsRNA into the nematodes [2], soaking them in a solution of dsRNA [14], feeding the worms bacteria that express dsRNA [15], and using transgenes that express dsRNA in vivo [16]. This method for knocking out genes required only catalytic amounts of dsRNA to silence gene expression. The silencing was not only in gut and other somatic cells, but also spread through the germ line to several subsequent generations [14]. Similar silencing was soon confirmed in plants [17], trypanosomes [18], flies [19] and many other invertebrates and vertebrates. In parallel, it was determined that dsRNA molecules could specifically downregulate gene expression in *C. elegans* [2]. Subsequent genome screening lead to identification of small temporal RNA (stRNA) molecules that were similar to the siRNA in size, but in contrast to the siRNAs, stRNA were single-stranded and paired with genetically defined target mRNA sequences that were only partly complementary to the stRNA [20]. Particularly, stRNAslin-4 and let-7 were determined to bind with the 3' noncoding regions of target lin-14 and lin-41 mRNAs, respectively, leading to reduction in mRNA-encoded protein accumulation. These observations encouraged investigators to look for stRNA-like molecules in different organisms, leading to the identification of hundreds of highly conserved RNA molecules with stRNA-like structural properties [21]. These small RNAs are termed micro RNAs (miRNAs). They are produced from transcript that folds to stem-loop precursor molecules first in the nucleus by the RNA III enzyme Dorsha and then in the cytosol by Dicer, and are present in almost every tissue of every animal investigated [22]. Thus, the RNAi pathway guides three distinct RNA classes, double-stranded siRNA, PIWI-interacting RNAs (piRNAs) and single-stranded miRNA, to the cytosolic RISC complex, which brings them to their target molecules.

Mechanism of action of RNA interference

Prior 1980s, RNA was not considered more than a passive messenger of transferring information from DNA to protein. RNAs are now recognised as backbone of cellular biology. RNAi was observed first by a plant scientist in the late 1980s, but the molecular basis of its mechanism remained unknown until the late 1990s, when research using the *C. elegans* nematode showed that RNAi is an evolutionarily conserved gene-silencing mechanism [2]. Sequence-specific posttranscriptional RNAi gene silencing by double-stranded RNA is conserved in a wide range of organisms: plants (*Neurospora*), insects (*Drosophila*), nematodes (*C. elegans*), and mammals. This process is part of the normal defense mechanism against viruses and the mobilization of transposable genetic elements [2,3]. Although RNAi first discovered as a response to experimentally introduced RNA sequence, it is now known that RNAi and related pathways regulate gene expression at both transcriptional and posttranscriptional levels. The key steps in RNAi underlie several gene regulatory mechanisms that include downregulation of the expression of endogenous genes, direct transcriptional gene silencing and alteration of chromatin structure to promote kinetochore function, and chromosome segregation and direct elimination of DNA from somatic nuclei in tetrahymena [23].

The dsRNAs, generated by replicating viruses, integrated transposons, or one of the recently discovered classes of regulatory noncoding miRNAs, are processed into short dsRNAs [20]. These short RNAs generate a flow of molecular and biochemical events involving a cytoplasmic ribonuclease III (RNase III)-like enzyme, known as Dicer, and a multi-subunit ribonucleoprotein complex called RNA-induced silencing complex (RISC). The antisense (guide) strand of the dsRNA directs the endonuclease activity of RISC to the homologous [target] site on the mRNAs, leading to its degradation and posttranscriptional gene silencing. The naturally occurring miRNAs are synthesized in large precursor forms in the nucleus. An RNA III enzyme called Drosha mediates the processing of the primary miRNA transcripts into pre-miRNA (70-80 mers), which are then exported via the exportin-5 receptor to the cytoplasm [24]. In the cytoplasm, Dicer cleaves dsRNA, whether derived from endogenous miRNA or from replicating viruses, into small RNA duplexes of 19-25 base pairs (bp). These have characteristic 3'-dinucleotide overhangs that allow them to be recognized by RNAi enzymatic machinery, leading to degradation of target mRNA [25]. Dicer works with a small dsRNA-binding protein, R2D2, to pass off the siRNA to the RISC, which has the splicing protein Argonaute 2 (Ago2). Argonaute cleaves the target RNA between bases 10 and 11 in relation to the 5'-end of the antisense siRNA strand [26]. The siRNA duplex is loaded into the RISC, whereupon an ATPdependent helicase (Ago2) unwinds the duplex, allowing the release of "passenger" strand and leading to an activated form of RISC with a single-stranded "guide" RNA molecule [27, 28]. The extent of complementarities between the guide RNA strand and the target mRNA decides whether mRNA silencing is achieved by site-specific cleavage of the mRNA in the region of the siRNA-mRNA duplex [29] or through an miRNA-like mechanism of translational repression [30]. For siRNA-mediated silencing, the cleavage products are released and degraded, leaving the disengaged RISC complex to further survey the mRNA pool. siRNA, piRNAs and miRNA were considered the three major types and central to RNAi. siRNAs were believed to be exclusively processed from the exogenous RNA of microbial pathogens that infect the cell. However, that opinion changed with the discovery of plentifully expressed endogenous siRNAs (endo-siRNAs) in mammalian cells. miRNAs and piRNAs are endogenous, small non-coding RNA sequences transcribed from cellular loci and subsequently processed to produce small fragments that interact with the downstream silencing machinery. Right now, hundreds of thousands of different piRNAs, produced from gene clusters of repetitive elements, more than 1,000 different miRNAs and number of endo-siRNAs are reported in mammalian cells [31]. Several reported evidences propose that piRNAs act via different cellular pathways from siRNAs and miRNAs and thus could provide another targeting approach for therapeutic targets [32].

Antiviral RNA Silencing in Mammals

Antiviral systems are indispensable in all living organisms to protect themselves from viral infections cells have evolved several mechanisms. In plants, worms, and insects, RNAi is a strong antiviral defense mechanism. The interferon [IFN] response of innate immunity is a well-known and defined antiviral mechanism in mammals. In mammalian cells, virus-specific dsRNA induces the IFN pathway via the pattern recognition receptors that include Toll-like receptors or via a replication-dependent pathway involving the cytoplasmic dsRNA sensors retinoic-acid-inducible protein-1/melanoma-differentiation-

associated gene 5 (RIG-1/MD5) [33,34]. Antiviral proteins that are induced by dsRNA also include the 2'-5'oligoadenylate synthases (2'-5'OAS)/RNaseL/ PKR [35, 36]. As RNAi, IFN responses, and 2'-5'OAS/RNaseL/PKR are initiated by dsRNA, it is most likely these pathways work together in the antiviral innate immune response. The helicase RIG-1/MDA5 pathway can be stimulated by siRNA, therefore it is likely that these proteins could link antiviral RNAi and IFN responses [36, 37]. Stimulation of RNAi in mammalian cells by endogenous expression of short hairpin RNAs (shRNAs) is a potent, novel antiviral mechanism [38].

In most cases of viral infection of mammalian cells, however, virus-specific siRNA could not be easily detected [39]. Pfeffer *et al.* have analyzed siRNA expressed in the cells infected by a variety of viruses including DNA viruses, such as human cytomegalovirus (CMV), Kaposi sarcoma-associated herpes virus (KSHV), murine herpes virus and Epstein-Bar virus (EBV), as well as the human retrovirus HIV-1 and the RNA viruses, such as yellow fever virus and hepatitis C virus (HCV)]. Although they failed to identify antiviral siRNA, they were able to identify several virally encoded miRNA, particularly in DNA virus-infected cells, which clearly suggested that viruses use host cellular RNAi machinery as defense mechanisms [39].

Several studies have implied a direct role of RNAi in controlling viral infections in mammalian cells. In addition, it was reported that mutations in RNAi machinery components improved the replication of human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV), and influenza virus, in mammalian cells. To date, virus-specific siRNA has been detected in human cells for HIV-1 and the LINE-1 retrotransposon [32,40-41]. Virus-specific siRNA accumulation in mammalian cells is low in comparison with plants, insects, and nematodes. The explanations for this remain unclear. One reason could be the lack of RNA-dependent RNA polymerase enzyme (RdRp) function in mammals. In insects and plants, RdRp is responsible for amplification of RNAi signals. The absence of RdRp enzyme activity, in combination with viral RNA silencing suppressors (RSS) activity, could also explain the low siRNA in mammalian cells. An additional explanation is that antiviral RNAi in mammalian cells is stimulated by cellular miRNA rather than production of completely new siRNA [42]. This was suggested for the retrovirus primate foamy type 1 (PFV-1) in which the endogenous cellular miR-32 was found to target sequences of PFV-1. PFV-1 overcomes this micro-RNA-mediated defense mechanism by expressing and producing RSS Tas protein [42]. Recently, it has been reported that virus replication was enhanced in cells with defective RNAi machinery. HIV-1 replication is increased in human cells in which Dicer and Dorsha expression is knocked out [43]. This is another indication that RNAi plays an important role in the anti-HIV-1 defense mechanism in human cells. The antiviral activity of RNAi was confirmed in a report showing enhanced accumulation of the mammalian vesicular stomatitis virus in *C. elegans* with defective RNAi machinery [44]. A good indication for the role of RNAi-mediated antiviral activity in mammalian cells came from evidence that many mammalian viruses express strong RSS activity [41]. Activation of RNAi in vivo in mammals was initially reported in animals infected with hepatitis B virus, siRNAs targeting Fas mRNA (Fas cell surface death receptor CD959) in a mouse model of autoimmune hepatitis, resulting in defence of the treated animals against liver fibrosis [32]. Endogenous cellular miRNA is significant for regulation of cellular genes, but recent evidence shows that miRNA can also provide antiviral defense. miRNA interrupts the viral life cycle, viral tropism, and pathogenesis of viral diseases. Human miR-32 contributes to the suppression of replication of retrovirus PFV-1 in human cells by partial complementary binding to the 3'UTR regions of five different mRNAs generated by PFV-1. The downregulation of these five genes by miR-32 repressed the replication of PFV-1 [42]. This study highlighted the antiviral activity of miRNA and suggested a possibly broad effect of these molecules on viral infection. In support of this, other scientists recently reported that the IFN pathway, which has a central role in defense against viral infection in mammalian cells, works in coordination with miRNA to control viral infection [45]. In this study, Pedersen *et al*. reported that IFN-β can initiate the expression of several cellular miRNAs, including miR-1, miR-30, miR-128, miR-196, miR-296, miR-351, miR-431, and miR-448, that form almost perfect nucleotide base pair matches with the HCV genome, and some of these have specific targets in the virus [45]. In support of their antiviral role, when these miRNA are experimentally introduced they reproduce the antiviral effects of IFN-β on HCV, and the IFN defense is lost when they are experimentally removed. These host-encoded miRNA might contribute to the antiviral defense mechanism of IFN-β against HCV [44]. Surprisingly, IFN-β was also reported to downregulate

the expression of miR-122, a miRNA that has been reported to be essential for HCV replication in hepatic cells [46]. Furthermore, over half dozen of human miRNAs, containing miR-199a-3p, miR-210, and miR-125a-5p are reported to suppress the replication of hepatitis B virus (HBV) in hepatic cells [31]. It was reported that EBV and KSHV are targeted by numerous cellular miRNAs, comprising the miR-17/92 and miR-106b/25 [31]. It becomes clear that host miRNA can also modulate cellular genes involved in the IFN response, as reported for mir-146. The expression of mir-146 is induced by the EBV-encoded latent membrane protein (LMP1) [47], which suggests a complicated role of miRNA in viral-host interactions. Other examples of antiviral miRNAs include miR-323, miR-491, and miR-654 against influenza virus. Furthermore, miR-27 and miR-93 against VSV, and miR-28, miR29a, miR-125b, miR-150, miR-223, and miR-382 against HIV-1 [31]. These results provided proof that cellular miRNA is part of the innate immune system, and they revealed a component of innate defense based on direct reaction between host-produced miRNA and viral-encoded nucleic acid.

The list of miRNAs in human cells that associated with regulation of viral infections promises to extend and grow. Indeed, the conclusion that human microRNAs effectively contributes to the innate host defense by targeting essential viral genes, thereby reducing the replication efficiency of the virus, came after analysis of more than 25,000 individual HCV, HIV-1, HPV and HBV sequences and reported that there is strong conservation of cellular miRNA-targeted sites within the sequence of those viruses [48]. Therefore, the provided findings support the notion that excessive miRNAs expressed in host cells represent one layer of host immune response against invading viruses that form a part of the cell's overall antiviral immunity.

Many viruses encode miRNA to exploit this gene regulatory mechanism and to facilitate infection. Viral-encoded miRNA regulates both viral and host genes [49]. The best-studied mammalian viral suppressors of RNA silencing (VSRs) are Ebola virus VP35, VP30, and VP40 proteins; severe acute respiratory syndrome (SARS) virus 7a accessory protein; NS1 of influenza A virus; HIV-1 TAT; capsid protein of HCV; and Tasprotein of PFV. The list of viruses that encode these miRNA also includes EBV, KSHV, and CMV [39,50-52]. Different miRNA are expressed at different stages in cells latently infected with EBV, indicating that viral miRNA is involved in the regulation and maintenance of viral latency [39,50]. Herpes simplex virus-1 (HSV-1) encodes miR-LAT to maintain the host cells latency and to inhibit apoptosis of the cells by decreasing expression of transforming growth factor-β(TGF-β) and mothers against decapentaplegic homologue 3 (SMAD-3) in host cells, which interferes with TGF-β-dependent signaling pathways and prevents host cell apoptosis [53]. Human CMV-encoded miR-UL112 represses the expression of MHC-class-1-polypeptiderelated sequence B (MICB). MICB is a natural killer cell [NK]-activating receptor group-2, member D (NKG2D) stress-induced ligand. NKG2D is required for NK cell-mediated killing of infected cells [54]. These findings indicate that CMV escapes host immune surveillance by encoding viral miRNA, which attacks cellular mRNA. This suggests that viruses use miRNA not only to regulate their own life cycles, but also to evade the host immune system and facilitate infection. Specifically, hepatic-cell-produced miR-122 has been reported to mediate the replication of HCV by interacting with 5'UTR of HCV RNA [46]. Although animal miRNA are reported to work at the posttranscriptional level to downregulate gene expression, this finding shows that HCV evolved to develop miRNA-mediated gene regulation to escape host immune surveillance and to facilitate viral replication by yet to be determined mechanisms.

Surprisingly, most of the viral miRNAs discovered so far lack extensive homology to each other or to animal miRNA. It is also interesting that miRNA is mostly detected in DNA viruses and not in RNA or retroviruses [50]. It is also noteworthy that no virus-encoded siRNA has been detected in virus-infected cells [39,50]. In addition to virus-encoded miRNA that allows viruses to regulate their genes and host genes, some viruses were found to produce silencing suppressor proteins that counteract miRNA or siRNA-mediated immune defense response. A good example of such a mechanism was found in PFV-1, which encodes the silencing suppressor Tas that can interfere with the miR-32-mediated downregulation of its mRNA and allows the PFV-1 to infect and replicate in infected cells [43]. In the same way, HIV-1 uses one of its own transcriptional activators, Tat as a miRNA-silencing suppressor that interferes with RNAi machinery enzyme, Dicer functions to prevent processing of dsRNA into siRNA [41, 44]. In agreement with these results, a HIV-1 strain that is deficient in Tat does not spread effectively in human cells, perhaps due to its inability to suppress RNAi in host cells [41, 43].

Conclusions

The identification and characterization of RNAi has exposed a formerly underappreciated mechanisms of posttranscriptional gene regulation in mammalian cells. RNAi is a part of the innate immune defense of mammalian cells against viral infections. Since RNAi has been confirmed to play crucial roles in antiviral defense of invertebrates, and all components of the RNAi machinery required for robust RNAi-mediated antiviral response are conserved through evolution in mammals, its contribution to mammalian antiviral innate immune defense has been a matter of facts.

The dynamic attack and counter attack relationship between cells in which RNAi serves to fight viruses and viruses evade RNAi to replicate effectively in cells. The efficiently replicating viruses must encode RNAi suppressors to evade RNAi. An RNAi suppressor function is only one of numerous functions such as shielding of the virus genome from RNAi, sequence changes in the viral genome to evade RNAi, virus regulation of cellular miRNA expression, and virus adaptation to cellular RNAi, for the virus to escape cellular RNAi restriction. Certainly, shielding of the viral RNA genome from RNAi, changes in viral sequences to evade RNAi, and virus-regulation of cellular miRNA-transcription have all been reported for HIV-1. Viruses such as HIV-1 that are extremely mutable may escape RNAi efficiently through target sequence changes; these viruses do not want a strong RNAi suppressor. However it is also known that less mutable viruses may need strong RNAi suppressors to lessen RNAi restriction in order to replicate efficiently.

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