Commentary

Antiviral Roles of Artificial microRNAs

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Abstract | Many RNA viruses undergo rapid mutations which compromises the immune protection of conventional vaccines. RNA interference (RNAi) has becoming a feasible strategy against various virus infections. Recently, a significant advance in RNAi technology is the use of artificial microRNAs (amiRNAs) to fight virus infections. However, different strategies are needed to prevent virus variation or mutation escape. This review is intended to present the current situation of antiviral roles of amiRNAs, against variable RNA viruses in particular.

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Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism in eukaryotes (Fire et al., 1998; Ghildiyal and Zamore, 2009). Two types of small RNAs, namely small interfering RNAs (siRNAs) and microRNAs (miRNAs), are the central players in RNAi process, both of which silence gene expression by annealing to the target RNAs (Terasawa et al., 2011). Since its discovery in 1994 as an innate antiviral mechanism, RNAi has become a feasible strategy against a variety of viral infections (Arbuthnot, 2010; Zhou and Rossi, 2011).

MicroRNAs differ from siRNAs by their mechanisms of action and some features of processing. siRNAs are mostly exogenous dsRNA molecules derived from viral RNAs or artificially introduced into the cell (Chu and Rana, 2008), while miRNAs are endogenous non-coding RNAs, typically 18-22bp long, which derive from longer hairpin-shaped precursors called pre-miRNAs (Bartel, 2004). siRNAs are normally designed to be perfectly complementary to their target sequences, while miRNAs are usually fully complementary only to a small (7-8nt) "seed" sequence which determines the specific miRNA targets (Lai, 2002).

Different RNAi strategies have been proposed to inhibit virus replication in cell cultures and animal models. Among them, synthetic siRNAs can imitate endogenous triggers and have great effect for transient virus inhibition, but the toxicity and delivery difficulty prevent their clinical application (Whitehead et al., 2009). Vector-expressed siRNAs and miRNAs can enter the endogenous RNAi pathway and are processed into mature siRNAs (Boudreau et al., 2009; Paddison et al., 2002). Expression of siRNAs from RNA polymerase (pol) III promoters is the most common way to induce RNAi in cells, but the severe toxicity after administration of high doses of siRNA expression vectors has been reported (Grimm et al., 2006). These problems can be circumvented by using weaker pol II promoter to express artificial miRNSs (amiRNAs) which has several benefits, including easier expression of multiple amiRNAs in tissue-specific manners, control of the timing and level of gene silencing, and tracking of amiRNA expressing

cells with correlated markers (Giering et al., 2008). Moreover, the amiRNA strategy has been studied as an antiviral approach for many viruses, including adenoviruses, rabies virus, dengue virus, and porcine reproductive and respiratory virus with minimal or no cytotoxicity (Bauer et al., 2009; Boudreau et al., 2009; Ibrisimovic et al., 2013; Israsena et al., 2009; Xie et al., 2013; Xia et al., 2013).

This brief commentary is intended to present the different amiRNA strategies against virus infections, for prevention of virus variation or mutation escaping in particular.

Prevention of virus variation or mutation escaping by targeting conserved sequences

Many RNA viruses undergo rapid mutations and thus escape mutants after RNAi treatment have been reported due to the sequence-specificity of RNAi mechanism (Boden et al., 2003). Unlike viral proteins, RNA dependent RNA polymerase (RDRP) is central to the replication of many RNA viruses, and thus is highly conserved between different strains. It has been shown that, however, the RDRP-targeted amiRNAs have significant inhibitory effect against the replication of different Venezuelan equine encephalitis virus strains (Bhomia et al., 2013). Foot-and-mouth disease virus (FMDV) is antigenically variable, and consists of seven serotypes and multiple subtypes. It has also been shown that the RDRP encoding 3D gene-targeted amiRNAs can efficiently inhibits FMDV replication (Du et al. 2011). Infectious bursal disease virus (IBDV), the causative agent of a highly contagious disease in chicks, is another example of variable RNA virus. The VP1 protein encoded by the small segment of IBDV RNA genome has both polymerase and capping enzyme activities. It has been shown that the anti-VP1 amiRNA has a more stable anti-IBDV effect than the amiRNA targeting main structural protein VP2 (Wang et al. 2009).

The RNA genomes of plus-strand RNA viruses have highly structured 5'- and 3'-untranslated regions (UTRs) that contain the *cis*-acting RNA elements involved in viral translation, replication and encapsidation (Liu et al., 2009). These conserved elements are also the good target for avoidance of virus variation or mutation escaping from RNAi. For example, Dengue viruses (DENV) have at least four known antigenically distinct DENV serotypes (DENV-1, 2, 3 and 4), and each serotype contains several phylogenetically distinct genotypes (Holmes and Burch, 2000). It has been shown that, however, the lentiviral vector-delivered tandem amiRNAs targeting the 5'- or 3'-UTR of DENV-2 genome can inhibit the viral replication in both stable and dose-dependent manners (Xie et al. 2013). Porcine reproductive and respiratory syndrome virus (PRRSV) is also notorious for strain diversity and rapid mutation (Kimman et al., 2009). Xia et al. (2013) have demonstrated that the plasmid vector-delivered amiRNAs targeting the 5' or 3' UTR of the viral RNA genome have potent effect against replication of three different PRRSV strains.

An internal ribosome entry site (IRES) allows for translation initiation in the middle of an mRNA sequence as part of the greater process of protein synthesis. These elements are often used by viruses when the host translation is inhibited. Chang et al. (2014) have showed that the co-expression of two IRES-targeted amiRNAs can effectively inhibit the replication of different FMDV strains. Furthermore, injection of the co-cistronic expression vector can partially or completely protect suckling mice against the challenge with prevalent PanAsia-1 and Mya98 strains of FMDV serotype O (Du et al., 2014).

Prevention of virus variation or mutation escaping by combinatorial RNAi strategy

Vector-based constructs have been developed for combinatorial RNAi approaches to silence multiple oncogenes or to enhance gene silencing efficiency. Such combinatorial RNAi approaches are particularly important for durable inhibition of escape-prone viruses. Several groups have shown that enhancement of RNAi activity can be obtained by concatenating multiple miRNA hairpins in a single construct (Sun et al., 2006; Chung et al., 2006). This approach has also been employed to inhibit pathogenic viruses that require a combinatorial RNAi attack to prevent viral escape, including HIV-1 (Liu and Berkhout, 2008) and HBV (Ely et al., 2009). In addition, the biosafety evaluation has shown that the three polycistronic amiRNAs expressed from a single construct have no apparent impact on cell proliferation, interferon response and interruption of native miRNA processing (Zhang et al. 2012). Although the siRNAs have a powerful inhibitory effect against Japanese encephalitis virus (JEV) replication, the high rate of genetic variation between JEV strains hampers their broad-spectrum

application. Wu et al. (2011) have shown that tandem amiRNAs targeting the highly conserved regions of JEV genes are not only effective against the wild strains of genotypes I and genotype III, but also predicted to effective against other JEV genotypes (*e.g.*, genotypes II and IV).

Prevention of virus variation or mutation escaping by targeting viral receptors

Most viruses enter the target cells via receptor-mediated endocytosis. Unlike the viral proteins which undergo rapid mutations, the cellular receptors for virus binding and/or entry are highly conserved proteins and thus can avoid the escape of gene-silencing effects by different viral strains (Fechner et al., 2007). Chicken heat-shock protein 90α (Hsp90α) has been identified as a functional component of the cellular receptor complex essential for IBDV infection (Lin et al., 2007). Yuan et al. (2012) have shown that the plasmid vector-delivered anti-Hsp90α amiRNAs have a potent inhibitory effect against IBDV infection. Furthermore, our recent studies showed that the avian adeno-associated viral vector-delivered anti-Hsp90α amiRNA was effective against three different IBDV strains (unpublished data). Various viruses, such as picornavirus, adenovirus, herpesvirus, Hantavirus, and reovirus, use integrins to initiate infection through a variety of mechanisms (Stewart and Nemerow, 2007). It has been shown that FMDV uses αvβ1, αvβ3, αvβ6 and αvβ integrins as the cellular receptors to initiate infection (Jackson et al., 2004). Recently, Du et al. (2014) have demonstrated that the plasmid vector-delivered amiRNAs targeting the αv subunit have significant inhibitory effect against FMDV replication in cells. Moreover, the amiRNA transgenic mice can be partially protected against the challenge with a large dose of FMDV. To date, at least three PRRSV receptors have been identified on porcine alveolar macrophages (PAMs), including heparan sulphate as the general attachment factor, sialoadhesin (CD169) for the viral binding and internalization, and CD163 for the viral genome release (Van Gorp et al., 2008). More recently, our group has shown that the Ad vector-delivered CD169- and CD163-targeted amiRNAs have an additive anti-PRRSV effect against different viral strains. The partial silencing effect is possibly due to alternative cellular factor(s) involved in PRRSV infection, rather than the low anti-vial activity of amiRNAs.

Cross transfer of anti-viral amiRNAs between different cells via exosomes

More recently, the exosomes derived from human and mouse cells have been shown as an efficient small RNA transfer vehicle (Valadi et al., 2007). This is very important for in vivo amiRNA delivery using viral vectors since most viruses have restricted tissue tropisms. For example, PRRSV targets the cells of monocyte-macrophage lineage, which are resistant to Ad vector transduction due to the lack of high affinity Ad receptor (Kaner et al., 1999). Our preliminary study has shown that, however, the CD163- or CD169-targeted amiRNA can be secreted from and taken up by pig cells via exosomes (Zhu et al., 2014). However, the exosome-mediated amiRNA transfer efficiencies in livestock such as pigs have not been investigated. More recently, our group showed that, after rAd transduction, the PRRSV 3'-UTR-targeted amiRNA was efficiently secreted from and taken up by different pig cell types. Moreover, the exosome-transferred amiRNA had potent anti-PRRSV effect against different viral strains (our manuscript). This indicates that pig cell-derived exosomes could also serve as an efficient small RNA transfer vehicle, including anti-viral amiRNAs.

In summary, a number of amiRNA approaches are presently available which have been proved to be the effective strategy against various virus infections, including prevention of virus variation or mutation escaping. Delivery remains the single greatest hurdle to in vivo use of the RNAi method. However, exciting advances have been made and new delivery systems under development may help to overcome these barriers.

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