Rapid control of foot-and-mouth disease outbreaks: is RNAi a possible solution?

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RNA interference (RNAi) is a mechanism of gene silencing that is being examined as an antiviral strategy in mammalian cells. Chen et al. have used this approach to examine its potential against foot-and-mouth disease (FMD), an extremely contagious viral disease of cloven-hoofed animals. This Research Focus discusses these experiments and comments on the possible use of RNAi, as well as other antiviral strategies, as a rapid method of FMD control.

Foot-and-mouth disease (FMD) is a highly contagious viral disease of domestic and wild cloven-hoofed animals [1]. The etiological agent, FMD virus (FMDV), is a member of the family Picornaviridae and contains a positive-sense, single-stranded RNA (ssRNA) genome of ~8500 nucleotides, surrounded by an icosahedral capsid consisting of sixty copies each of four structural proteins (VP1–4) [2]. The virus is antigenically highly variable and consists of seven serotypes and multiple subtypes [1]. The disease has been designated by the Office International des Epizooties (www.oie.int), World Organization of Animal Health, as a transmissible disease that has the potential for serious and rapid spread and is of major socioeconomic importance. As an example of its economic significance, outbreaks in Taiwan in 1997 and the UK in 2001 resulted in the slaughter of millions of animals and multibillion-dollar economic losses [1].

Currently, disease control measures include culling of infected and in-contact susceptible animals, restriction of susceptible animal movement, decontamination of infected premises and possibly vaccination with an inactivated whole-virus antigen. The vaccine has been successful in eliminating FMD from the susceptible animal population in Western Europe and parts of South America. However, there are limitations in its effective use in the event of an outbreak in disease-free countries. In particular, this vaccine, as well as a newly developed live-virus vector-delivered subunit vaccine [3], requires ~7 days to induce complete protection from experimental challenge. FMDV replicates and spreads extremely rapidly resulting in the appearance of clinical disease in infected animals as early as 2–3 days post-exposure. Thus, there is a need to develop new tools that provide early protection in susceptible, uninfected animals surrounding disease foci soon after an outbreak has been detected, to block, or at least limit, virus shedding, thereby containing disease spread and potentially reducing the numbers of animals slaughtered.

RNA interference (RNAi) as a potential antiviral approach

To address the limitation of FMD vaccines to induce rapid protection, Chen et al. [4] have examined the use of RNAi to inhibit virus replication. RNAi is a sequence-specific gene silencing phenomenon mediated by 21–23 nucleotide double stranded RNA (dsRNA) that results in degradation, repression of transcription or inhibition of translation of targeted mRNA [5] (Figure 1). This process, first described in Caenorhabditis elegans [6], operates in a wide range of eukaryotic organisms and probably has multiple functions [7,8]. RNAi appears to be a major antiviral mechanism in plants ([9,10] and references therein) and is induced in mosquito cells by virus replication [11]. However, it is unclear whether RNAi is a natural host defense mechanism against viral infection in mammals. Nevertheless, this technology has been exploited as a potential antiviral strategy to inhibit replication of several RNA and DNA animal viruses [12].

Gitlin et al. [13] have demonstrated that the replication of poliovirus, which like FMDV is a member of the Picornaviridae family, can be inhibited by addition of synthetic 21–23 small RNAs (siRNAs) targeted to different regions of the viral genome. These workers showed that pretreatment of cells with siRNAs directed to the viral capsid or the 3D polymerase coding regions reduced virus titers in single-step growth experiments by 97–99% and significantly reduced the synthesis of both poliovirus RNA and proteins. However, not all siRNAs selected blocked virus replication. An siRNA that targeted the 5’ non-coding region was ineffective [14], suggesting that RNA secondary or tertiary structure, as well as RNA–protein interactions, might affect the ability of siRNAs to function. Furthermore, these authors demonstrated that viruses that survive siRNA treatment have a one-nucleotide substitution in the middle of the targeted sequence and are barely sensitive to the siRNA.

Can RNAi inhibit FMDV replication?

Chen et al. [4] selected an siRNA directed to the VP1 structural protein coding region of FMDV serotype O/HKN/2002 and delivered this molecule to baby hamster kidney-21 (BHK-21) cells by transfection with a plasmid expressing either 21 or 63 nucleotide short hairpin RNAs...
(shRNA). Transfected cells infected with FMDV O/HKN/2002 were examined 24 h post-infection (hpi) and had a delay in the development of the characteristic cytopathic effects (CPEs) that occur after FMDV infection. The effect was specific because transfected cells infected with pseudorabies virus or FMDV O/CHA/1999, an isolate that differs in the targeted region of O/HKN/2002 VP1 by at least 2 nucleotides, developed typical CPEs. Virus yield, in O/HKN/2002 infected cells examined at 12 and 24 hpi, decreased by 1000-fold in cells pretreated with specific siRNAs but there was no significant decrease at 48 hpi. Control siRNAs had no effect on O/HKN/2002 yield and the O/HKN/2002-specific siRNAs had no effect on virus yield in FMDV O/CHA/1999 or pseudorabies virus infected cells. The authors extended this work to examine the potential antiviral effects in an animal model. Suckling mice, which succumb to FMDV infection, were inoculated with the various plasmids expressing shRNAs and challenged with FMDV O/HKN/2002 6 h later. All control mice died by 1–2 days after infection, whereas ~75% of the mice administered with the VP1-specific shRNAs survived after a low-dose challenge (20 LD$_{50}$) and 20% survived after a high-dose challenge (100 LD$_{50}$). An additional intriguing observation was that mice treated with a plasmid expressing VP1 before the administration of the VP1-specific shRNA had increased survival as compared to a group challenged with FMDV after only being given the specific shRNA. The authors attributed this additional protection to the presence of a rudimentary form of immune memory but additional experiments are clearly needed to explain this observation.

Although these results suggest that an siRNA antiviral strategy has potential use in FMD therapy, significant additional information is required before this approach can be of practical use.

**What hurdles need to be addressed?**

Several concerns need to be addressed to improve RNAi effectiveness as an antiviral strategy against RNA viruses, particularly in animal model systems and naturally susceptible animals. These are listed below:

(i) **Improving delivery systems.** Current methods of delivery of siRNAs to tissue culture cells as well as limited studies in animals [15,16] mainly use either synthetic RNAs or plasmids containing shRNAs under the control of RNA polymerase III promoters.
Characterizing viral suppressors induced rapid and long-lasting protection and the dual-inoculated influenza and vaccinia viruses are also able to block induction of genes whose protein products can inhibit various stages in a virus life cycle. To overcome the host response, viruses, including FMDV, have developed various mechanisms that interfere with IFN induction, synthesis of IFN-induced genes or the function of these gene products. Although FMDV can circumvent the innate immune response, virus replication is highly sensitive to the direct action of IFN-α/β protein [1]. Pretreatment of cells with IFN-α/β inhibits replication of several FMDV serotypes. Recently, Chinsangaram et al. [24] and Moraes et al. [25] demonstrated that adenovirus-delivered IFN-α can prevent clinical disease and viremia in swine challenged one day post-inoculation and protection can last for 3–5 days. Furthermore, addition of IFN-α one day post-infection can reduce the level of viremia and virus shedding and decrease disease severity. The combination of an adenovirus-delivered IFN-α and an adenovirus-delivered FMD subunit vaccine induced rapid and long-lasting protection and the dual-inoculated swine developed a significant boost in neutralizing antibody response after challenge, even though there was no evidence of virus replication. In efficacy studies in cattle, Wu et al. [26] demonstrated that adenovirus-delivered IFN-α can delay viremia and delay and reduce clinical disease when the animals are challenged 1 or 2 days post-inoculation, compared to control animals. The reduced level of protection of cattle compared to swine correlated with the reduced levels of IFN-α detected in their plasma. These proof-of-concept studies have demonstrated the feasibility of rapidly inducing complete protection in naturally susceptible animals.

Rosas et al. [27] used antisense technology as an antiviral strategy against FMDV. They demonstrated that virus yield was reduced in infected BHK-21 cells first transfected with antisense RNAs of ~150 or 400 bases that are homologous to the 5’ and 3’ non-coding regions of FMDV serotype C. Subsequently, they produced BHK-21 cell lines that stably expressed these antisense transcripts and demonstrated inhibition of virus replication of all seven FMDV serotypes. These results suggest that a nucleic acid-based antiviral approach could be used to control FMD. Furthermore, as the authors proposed, transgenic animals expressing these antisense molecules might display reduced susceptibility to FMDV.

These methods require relatively large amounts of material to be successful. The use of adenoviral or lentiviral vectors, which enable a more effective delivery of siRNA based on the highly efficient entry of viruses into cells, provides a promising alternative. This approach has been examined both in cell culture and in mice [17,18]. Moreover, viral vectors could be targeted to specific cell types.

(ii) Systemic spread. To be an effective antiviral mechanism in animals, it is essential that the RNAi silencing signal is spread systemically. In C. elegans, a dsRNA transporter (SID1) has been identified and is required for systemic RNAi [19]. The presence of human and murine SID1 homologs suggests that RNAi could have a systemic component in mammals; however, this hypothesis remains to be proven.

(iii) Circumventing the emergence of resistant mutants. RNA viruses exist as a quasispecies because of the high error rate of RNA replication. Thus, it is not surprising that a 21 nucleotide siRNA that effectively inhibits FMDV O/HKN/2002 replication fails to act on FMDV O/CHA/1999, presumably owing to the two nucleotide difference in the target sequence [4]. Similarly, Gitlin et al. [13] found that polioviruses that survived siRNA treatment had point mutations in the targeted region. One approach to address this problem could be to use siRNAs directed to multiple sites on the viral genome or perhaps identify siRNAs that target portions of the genome coding for conserved regions of non-structural proteins essential for virus replication. Because FMDV is an antigenically variable virus, the use of multiple conserved siRNAs might also broaden the antiviral effects of this approach across serotypes.

(iv) Characterizing viral suppressors. Plant and insect viruses have developed ways to counteract the effects of RNAi [9,11,20]. In addition, recent studies have shown that suppressors of the interferon (IFN)-induced innate immune response encoded by influenza and vaccinia viruses are also able to block RNAi in virus-infected insect cells [21]. These results anticipate a potential resistance mechanism to RNAi in animal virus-infected cells. Identification and specific inhibition of suppressors might overcome this problem.

(v) Avoiding side-effects. Several studies have indicated that siRNAs can have off-target effects. Pairing of as few as 11 consecutive nucleotides can affect target RNA expression [22]. Despite the short length, in some cases siRNAs might trigger a non-specific response by upregulating IFN responsive genes [23]. Clearly it will be necessary to determine if particular siRNAs have these effects before their use in antiviral therapy. Furthermore, a more complete understanding of all parameters involved in identification of effective and specific siRNAs is necessary.

What can we expect?
To be effective in controlling outbreaks occurring as a result of the accidental or deliberate introduction of FMDV into disease-free countries, both rapid and relatively long-lasting disease control approaches are required. RNAi is an evolving field and its use as an antiviral treatment in humans and animals is of considerable interest. Although the antiviral effect of siRNA in animals appears to be rapid, the duration of protection and its ability to spread systemically are unclear. To control FMD, administration of a rapidly acting antiviral, such as siRNA or type I IFN (Box 1), in combination with a vaccine that induces long-lasting protection, can potentially be effective. However, the challenge of converting exciting new laboratory approaches to effective and accepted procedures in the field has just begun.

Acknowledgements
We thank E. Rieder, L. Rodriguez, C. de los Santos and S. Botton for critical reading of the manuscript.
Gfi-1: another piece in the HSC puzzle

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Recent work addresses the complex issue of stem cell regulation mechanisms. Both groups involved have studied the role of Gfi-1 in preserving the functional integrity of the adult hematopoietic stem cell (HSC) pool through a series of experiments, including phenotypic analysis of the primitive hematopoietic compartment, transplantation assays and cell cycle analyses. The results suggest that Gfi-1 deficiency leads to an exhaustion of HSCs in the adult mouse bone marrow, presumably through unrestrained cycling of these cells.

Introduction
Recent work on Gfi-1 (growth factor-independent-1), performed by Zeng et al. [1] and by Hock et al. [2], might shed some light into the utterly complex enigma of hematopoietic stem cell (HSC) regulation. Gfi-1, a transcriptional repressor harboring a Snail/Gfi-1 (SNAG) domain and six zinc-finger motifs, is expressed in several compartments of the hematopoietic system and is a member of the same oncogenic complementation group as the polycomb group gene Bmi-1 [3–9]. Evidence suggests that a dominant negative mutation of Gfi-1 causes the human congenital neutropenia syndrome and leads to an increase in the elastase (ELA2) gene expression, linking these two genes in a common myeloid differentiation pathway [10].

The hematopoietic system relies on tissue-specific stem cells to consistently replenish its short-lived cellular components. Attributes of ‘stemness’ confer to this rare multipotent cell the capacity to proliferate and give rise to a more differentiated progeny, responsible for sustaining....

References

1471-4906/$ - see front matter. Published by Elsevier Ltd.
Corresponding author: Sauvageau, G. (guy.sauvageau@umontreal.ca). Available online 7 January 2005