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Artificial microRNAs as antiviral strategy to FMDV: structural implications of target selection

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RNA interference (RNAi) appears as a promising strategy to control virus replication. While the antiviral power of short-hairpin RNAs or small-interfering RNAs against FMDV has been demonstrated widely, safer RNAi effectors such as artificial microRNAs (amiRs) have not been evaluated extensively. In this work, transgenic monoclonal cell lines constitutively expressing different amiRs targeting FMDV 3D-coding region or 3′UTR were established. Certain cell lines showed an effective, sequence-specific amiR-mediated silencing activity that was accomplished by degradation of the target mRNA, as demonstrated in co-transfection experiments of reporter genes fused to FMDV target sequences. However, FMDV replication in these amiR-expressing cells was affected barely. Experiments aimed at elucidating the cause of RNAi failure demonstrated limited accessibility of the targeted region in the molecular environment of the viral RNA. Since RNAi is mediated by large-dimension silencing complexes containing the siRNA and not simply by a linear oligonucleotide, we propose that target selection should consider not only the local RNA structure but also the global conformation of target RNA.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of major socioeconomic importance affecting domestic and wild cloven-hoofed animals (Grubman and Baxt, 2004). The etiological agent, FMD virus (FMDV, member of the genus *Aphthovirus* in the *Picornaviridae* family), consists of a single-stranded positive-sense RNA molecule surrounded by an icosahedral capsid composed of four viral proteins (Rueckert, 1996). The viral genome is approximately 8500 nucleotides long and encodes a polyprotein precursor that is cleaved post-translationally to originate the mature viral proteins including the RNA-dependent RNA polymerase (3D), a highly conserved enzyme that catalyzes viral RNA replication (Carrillo et al., 2005). At both its 5′ and 3′ ends, the FMDV RNA contains highly structured untranslated regions (UTRs) relevant for the viral replication cycle. Additionally, the genomic RNA is covalently linked to a viral polypeptide (VPg) at its 5′ end and contains a genetically coded poly(A) tail at its 3′ terminus (Grubman and Baxt, 2004).

The virus is antigenically highly variable, and there are seven serotypes (A, C, O, Asia, SAT1, SAT2 and SAT3) and multiple subtypes (Domingo et al., 2003). Current vaccines based on inactivated whole virus have been effective in controlling FMD from susceptible animal populations, albeit without efficient cross-protection across serotypes or subtypes. Furthermore, inactivated vaccines need ~7 days to induce immune protection, and so their use is limited in case of outbreaks particularly in disease-free countries. Consequently, there is a need to develop new antiviral tools that provide early protection or ideally block viral replication thereby restraining FMD spread.

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing phenomenon triggered by double-stranded RNA and mediated by small RNAs (small interfering RNAs [siRNAs] or microRNAs [miRs], among others) that leads ultimately to degradation or translational repression of the targeted transcript. RNAi has been proposed as an alternative means to control virus replication including FMDV (Grubman and de los Santos, 2005; van Rij and Andino, 2006; Arbuthnot, 2011). In fact, the antiviral activity of RNAi against FMDV has been demonstrated widely both *in vitro* and *in vivo* using synthetic siRNAs as well as plasmid- or virus-encoded short hairpin RNAs (shRNAs) targeting different regions of the viral genome (Chen et al., 2004; Kahana et al., 2004; Liu et al., 2005; Chen et al.,

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2006; Kim et al., 2008, 2010). However, shRNAs may disrupt the endogenous miR pathway in transfected cells, so the use of plasmid-encoded artificial miRs has been favored (Boudreau et al., 2009). Indeed, the antiviral power of amiRs has been demonstrated (Son et al., 2008; Israsena et al., 2009; Du et al., 2011).

A major obstacle faced by RNAi technology for therapeutic use is the need of effective, safe and reliable delivery systems (Castanotto and Rossi, 2009). Given the rapid spread of FMD, RNAi-based antivirals should block ideally the first cycle of viral replication and thus limit disease dissemination. In this sense, recent studies aimed at the development of transgenic animals naturally resistant to FMDV are challenging (Pengyan et al., 2010; Wang et al., 2012).

In this work, we have investigated the silencing activity of transgenic cell lines constitutively expressing amiRs targeting essential regions of FMDV genome. Our results point out the critical role played by RNA accessibility that is often overlooked during the design of RNAi-based antiviral approaches.

2. Materials and methods

2.1. Cells, viruses and virus titration

Baby hamster kidney (BHK-21 clone 13) cells were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. Transgenic $amiR_{FMDV}^{+}$ cell lines were grown in DMEM supplemented with 10% FBS and 7 μg/ml blasticidin S (Invitrogen, Carlsbad, USA). FMDV A/Arg/01 (prototype strain MC267 from Trenque Lauquen, Genbank accession number AY593786) was obtained from the National Institute for Animal Health (SENASA, Argentina) (García Núñez et al., 2010).

Virus titers were determined in BHK-21 cells by plaque assay (pfu/ml) or alternatively the 50% tissue culture infective dose ($TCID_{50}$) was calculated using the formula of Reed and Muench (Reed and Muench, 1938).

2.2. Plasmids

2.2.1. Pre-amiR encoding plasmids

Target sequences were predicted on the FMDV genome (serotype A, strain A/Arg/01) using the *siRNA target Finder* (Ambion). To take into account target site accessibility during the amiR selection procedure, the candidate target sites were mapped within the secondary structure of the full-length FMDV RNA predicted as indicated in Section 2.9. Target sites located at positions 6853–6873, 7945–7965 and 8154–8174 of the viral genome (designated 3D1, 3D2 and 3UTR, respectively) were predicted to be less structured as other candidates within the same portion of the genome and accordingly they were selected to design the corresponding amiRs. Targets showing polymorphic positions mainly outside the seed region among FMDV serotypes circulating in South America (Fig. 1A) were selected intentionally to study the impact of such differences on the silencing ability of transgenic cells. Mature amiR sequences were: $amiR_{3D1}$ 5′-UUUCGUGUCUCCUUUGUGUUU-3′, $amiR_{3D2}$ 5′-UCCUGCCACAGAGAUCAACUU-3′ and $amiR_{3UTR}$ 5′-AGGAAGCGGAGAAAGCUCUUU-3′. Target sequences showed no similarity with mammalian genes.

Complementary single-stranded DNA oligonucleotides encoding each pre-amiR (Table 1) were synthesized, annealed and cloned into pcDNA®6.2-GW/miR vector (BLOCK-iT Pol II miR RNAi expression vector kit, Invitrogen, Carlsbad, USA) following the manufacturer's instructions. The resulting plasmids were named pcDNA6.2/$amiR_{3D1}$, pcDNA6.2/$amiR_{3D2}$ and pcDNA6.2/$amiR_{3UTR}$.

2.2.2. Luciferase reporter plasmids

Reporter plasmids encoding the *Renilla* luciferase gene (RLuc) fused to FMDV fragments enclosing amiR target sequences from serotypes A (A/Arg/01) or O (O1 Campos, Genbank Accession number AJ320488.1) were constructed. The RLuc coding sequence was amplified by PCR using oligonucleotides RLfor and RLrev (Table 1) and plasmid pRNull (Promega, Madison, USA) DNA as template. The amplified product was digested with *Eco*RV and cloned at the *Dra*I site of pcDNA6.2®-GW/miR-neg, originating plasmid pcDNA6.2/RLuc/miR-neg. DNA fragments encompassing amiR target sequences were obtained by fill-in (3D1 and 3UTR) or PCR (3D2) reactions using the primers listed in Table 1. For fill-in reactions, oligonucleotides were annealed and double-stranded DNA was elongated with Klenow fragment of DNA polymerase (Promega, Madison, USA). Products were purified from a 20% polyacrilamide gel and cloned into pGemT-Easy vector (Promega, Madison, USA). The 3D2-encompassing fragment was amplified from cDNAs derived from strains A/Arg/01 and O1 Campos and cloned into pGemT-Easy vector (Promega, Madison, USA). Fragments containing each target sequence were subcloned between the *Bam*HI and *Xho*I sites of vector pcDNA6.2/RLuc/miR-neg. Control plasmid pcDNA6.2/RLuc lacking the miR expression cassette was obtained by enzymatic digestion of plasmid pcDNA6.2/RLuc/miR-neg with *Bam*HI and *Bgl*II, followed by re-ligation of compatible ends.

To assess *in vitro* accessibility of target sequences, the T7 RNA polymerase promoter was excised from plasmid pGemLuc (Promega, Madison, USA) and subcloned between *Nde*I and *Sac*I restriction sites located upstream of the RLuc coding sequence in selected pcDNA6.2/RLuc-derived plasmids.

To construct plasmid pcDNA6.2/FLuc, the firefly luciferase (FLuc) coding sequence was excised from plasmid pGemLuc (Promega, Madison, USA) with *Bam*HI and *Xho*I enzymes and subcloned into plasmid pcDNA6.2®-GW/miR-neg.

All recombinant vectors were confirmed by automated sequencing.

2.3. Establishment of transgenic cell lines

Pre-amiR encoding plasmids were transfected into 95% confluent BHK-21 cells using Lipofectamine (Invitrogen, Carlsbad, USA), according to manufacturer's instructions. Transfected cells were subcultured after 24 h in DMEM medium containing 10% FBS and 7 μg/ml blasticidin S (Invitrogen, Carlsbad, USA). Polyclonal cell lines were further cloned by limiting dilution. Briefly, cells were seeded into 96-well culture plates at a density of 0.5 cells/well in DMEM supplemented with 10% FBS and 20% conditioned medium obtained from supernatants of BHK-21 cell cultures. The development of foci corresponding to unique clones of cells was monitored daily and monoclonal cell lines were subcultured in DMEM supplemented with 10% FBS and 7 μg/ml blasticidin S. Transgenesis was confirmed in established cell lines by PCR.

2.4. RNA isolation

Total RNA was isolated from monolayers of transfected or infected cells using Trizol® reagent (Invitrogen, Carlsbad, USA) according to manufacturer's instructions. Similarly, total RNA was isolated from pooled supernatants of infected BHK-21 cells using Trizol® LS reagent (Invitrogen, Carlsbad, USA).

2.5. Real-time PCR

2.5.1. Reverse transcription (RT)

Except for experiments involving viral RNA, total RNA was treated with DNAse I (Invitrogen, Carlsbad, USA) prior to RT. First-strand cDNA was synthesized either using Superscript III