Small interfering RNAs targeting peste des petits ruminants virus M mRNA increase virus-mediated fusogenicity and inhibit viral replication in vitro

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Abstract

Peste des petits ruminants (PPR), caused by peste des petits ruminants virus (PPRV), is an acute or subacute, highly contagious and economically important disease of small ruminants. The PPRV matrix (M) protein possesses an intrinsic ability to bind to lipid membranes, and plays a crucial role in viral assembly and further budding. In this study, three different small interfering RNAs (siRNA) were designed on the basis of translated region for PPRV Nigeria 75/1 M mRNA, and were subsequently synthesized for their transfection into Vero-SLAM cells, followed by infection with PPRVs. The results showed that two out of three siRNAs robustly induced cell-to-cell fusion as early as 36 h post-infection with PPRVs, effectively suppressed expression of the M protein by interference for the M mRNA, and eventually inhibited viral replication in vitro. These findings led us to speculate that siRNA-mediated knockdown of the M protein would alter its interaction with viral glycoproteins, thus exacerbating intercellular fusion but hampering virus release.

Keywords:
PPRV, siRNA, M mRNA, Cell-to-cell fusion, Viral replication, Inhibition
In this study, we also demonstrated that siRNAs targeting PPRV M mRNA affected transcription of the M mRNA, suppressed expression of the M protein and finally inhibited viral replication in vitro. More importantly, in comparison with non-siRNA-transfected cells which formed normal syncytia, the siRNA-transfected cell monolayer was largely disrupted as a result of exacerbated cell-to-cell fusion.

In the present study, three siRNAs (M59, M208 and M407) were designed on the basis of translated region for PPRV Nigeria 75/1 (GenBank: HQ197753.1) M mRNA. The siRNA sequences were listed in Table 1 and their targeting sites on the M mRNA were shown in Fig. 1a. All RNA duplexes (Table 1) and a FAM-siRNA (as a fluorescein-labeled negative control) were synthesized by the Shanghai GenePharma Co., Ltd (Shanghai, China), and subsequently were used for transfection of Vero-SLAM cells using the Lipofectamine® 2000 Reagent (Life technologies, Carlsbad, USA) in 24-well plates. A total of eight groups of cells were designed in this study, namely M59-transfected cells (M59), M208-transfected cells (M208), M407-transfected cells (M407), unrelated siRNA-transfected cells (Mock), non-siRNA-transfected cells (U.C.) and treated cells (H.C.), as early as 24 h.p.i. A similar CPE (Fig. 2, red arrowed) appeared only in the group M59 and M407 whereas rapid syncytia formation with multinucleated structure could be visualized in any of the seven groups 0 h.p.i. (Fig. 2), post-infection (h.p.i.), respectively. No cytopathic effect (CPE) was observed on the Leica DM IRB microscope 0, 24, 36, 48 and 72 h in vitro.

In Fig. 1b, demonstrating successful transfection of siRNAs into Vero-SLAM cells. Meanwhile, other cells (except for health cells) were infected with PPRV Nigeria 75/1 at three different concentrations (MOI = 0.2, 0.4 and 0.6) for further incubation.

The group M59, M208, M407, N.C., Mock, U.C. and H.C. were observed on the Leica DM IRB microscope 0, 24, 36, 48 and 72 h post-infection (h.p.i.), respectively. No cytopathic effect (CPE) could be visualized in any of the seven groups 0 h.p.i. (Fig. 2), whereas rapid syncytia formation with multinucleated structure (Fig. 2, red arrowed) appeared only in the group M59 and M407 as early as 24 h.p.i. A similar CPE (e.g., syncytia formation) as shown in both groups 24 h.p.i., however, was observed in the group M208, N.C., Mock and U.C. 36 h.p.i. Interestingly, a hyperfusogenic phenotype without visible multinucleated structure (Fig. 2, yellow wedged) was exhibited still only in the group M59 and M407 as early as 36 h.p.i., and moreover such an atypical intercellular fusion became more extensive over time. For example, a large number of cells in both groups were severely swollen, lost their characteristic morphology and showed an intensive cytoplasmic vacuolization 48 h.p.i., and almost all of the cells eventually detached from the monolayer 72 h.p.i. In contrast, other cell monolayers in the group M208, N.C., Mock and U.C. still maintained typical but further CPE that consisted of rounding, aggregation and slight detachment of cells 72 h.p.i. (Fig. 2).

It has been demonstrated that actin is involved in measles virus budding, and budding itself is possibly the result of a vectorial growth of actin filaments (Bohn et al., 1986). More recently, it has been furthermore proposed that actin filaments associated with the measles virus M protein, alter the interaction between the M and H proteins, thus modulating fusogenic activity and viral assembly (Wakimoto et al., 2013). Specifically, interaction between the M protein and the cytoplasmic tails of viral glycoproteins is required for the downregulation of viral glycoprotein-mediated fusogenicity between infected and adjacent uninfected cells (Muhlebach et al., 2008; Tahara et al., 2007). In this study, M59- and M407-mediated silencing of the PPRV M gene in the cytoplasm possibly resulted in a weakened M protein-glycoprotein interaction at the plasma membrane, thereby improving the lateral mobility of viral glycoproteins, facilitating the formation of active fusogenic complexes of H-F proteins and then increasing the viral fusogenicity in vitro (Moll et al., 2002; Runkler et al., 2008).

In order to determine whether all three siRNAs have an ability to inhibit M mRNA transcription or not, all cells in the group M59, M208, M407, N.C., Mock and U.C. were harvested 72 h.p.i. for total RNA isolation using a nucleic acid extractor (TIANLONG, Xi’an, China). The total RNAs were subsequently subjected to real-time qRT-PCR analysis using a One Step SYBR® PrimeScript™ Plus RT-PCR Kit (Takara, Dalian, China) and the following specific primers: forward 5'-tgcataagacaacccacct-3' and reverse 5'-cgaagtctacttcca-3'.

In the present study, three siRNAs (M59, M208 and M407) were designed on the basis of translated region for PPRV Nigeria 75/1 (GenBank: HQ197753.1) M mRNA. Fluorescence (left) and bright-field (right) photomicrographs of FAM-siRNA-transfected Vero-SLAM cells 6 h post-transfection (b). Bar = 50 μm.

Table 1

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Position</th>
<th>Sequence (5’ → 3’)</th>
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<tbody>
<tr>
<td>M59</td>
<td>59–77</td>
<td>GCAUAGAACCCACCACCUAdTdT</td>
</tr>
<tr>
<td>M208</td>
<td>208–226</td>
<td>GGUGAACCUUCGGCGUUCUAdTdT</td>
</tr>
<tr>
<td>M407</td>
<td>407–425</td>
<td>GCCGCAACAGGGCUUCAGGAdTdT</td>
</tr>
<tr>
<td>N.C.</td>
<td>Not available</td>
<td></td>
</tr>
</tbody>
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* Nucleotide positions in the translated region of viral M mRNA.
that by real-time qRT-PCR (Fig. 3). Nevertheless, the reason why both the efficient siRNAs caused partial inhibition of the M mRNA transcription by qRT-PCR but “complete” suppression of the M protein expression by western blotting might be that the probe-based qRT-PCR was more sensitive than the western blotting using the anti-M protein polyclonal antibodies.

Finally, supernatants were collected from the group M59, M208, M407, N.C., Mock and U.C. 72 h.p.i., and were subsequently subjected to viral titration by 50% tissue culture infective dose (TCID50) assay, respectively. The viral titer for each sample was calculated by the Spearman-Kärber equation. In comparison with that in the group U.C., the viral titer in the group M59 and M407 significantly declined by nearly 2.8 and 2.0 logTCID50/mL, respectively, whereas in the group M208 slightly declined by only 0.4 logTCID50/mL (Fig. 4). In other words, a maximum reduction of virus yield was approximately 630- and 100-fold in the group M59 and M407, respectively, but was only 2.5-fold in the group M208. Therefore, in vitro replication of PPRVs was considerably inhibited by the M407 and especially by the M59, rather than by the M208.

It is widely believed that M proteins are adapters which link together the structural components of virions, driving their assembly and budding (Harrison et al., 2010). In addition, it was demonstrated in our previous studies that the PPRV M protein is the necessary viral component as a driving force promoting the formation of virus-like particles (Liu et al., 2014a,c). Therefore, we speculate that the PPRV M protein, if inhibited in some way in vitro, would hamper the release of progeny viruses to some extent. Fortunately, this speculation was validated by the present study, showing that siRNA-induced silencing of the PPRV M gene resulted in at least hundred-fold lower viral titer than those determined in different controls (N.C., Mock and U.C.) (Fig. 4).
In conclusion, we demonstrated that two out of three siRNAs targeting PPRV M mRNA have a potent ability to inhibit the M mRNA transcription, the M protein expression, and the viral replication in vitro. Unlike siRNAs targeting the PPRV N mRNA (Holz et al., 2012; Keita et al., 2008; Servan de Almeida et al., 2007), although they could result in a more efficient inhibition (about 10,000-fold) of PPRV progeny (Keita et al., 2008) than that caused by either the M59 or the M407 in this study, the phenomenon of intercellular hyperfusogenicity was only related to the M mRNA-targeted siRNA interference. Indeed, we also independently demonstrated that siRNAs targeting the PPRV N, P and L mRNAs were incapable of inducing such a hyperfusogenic phenotype in vitro (data not shown). Therefore, it can be suggested that the PPRV M protein plays a key role both in inducing cell-to-cell fusion and in promoting viral replication.

It has been reported that matrix-less viruses lose acute pathogenicity but penetrate more deeply into the brain parenchyma than standard viruses (Cathomen et al., 1998). Accordingly, it is exciting to speculate whether there are PPR-unspecific pathological changes caused by the M gene-targeted siRNAs in goats or sheep infected with PPRVs. If so, the M gene-targeted RNA interference as therapeutics may be impractical for the control of PPR, unless no unwanted side-effects conferred by siRNAs were observed in small ruminants infected with PPRVs. Unfortunately, due to a lack of appropriate animal models for PPRVs, siRNA-related experiments were not performed in vivo in this study. In addition, it was demonstrated that PPRVs were able to systematically escape interference conferred by PPRV N gene-targeted siRNAs after 3–20 consecutive passages (Holz et al., 2012). The M gene-targeted siRNAs, if proven to be ineffective for the inhibition of PPRVs due to an innate mechanism of PPRVs for escaping RNA interference, would be further reconsidered for their infeasible application in the control of PPR.

Acknowledgments

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References


