Ovine herpesvirus-2-encoded microRNAs target virus genes involved in virus latency

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Herpesviruses encode microRNAs (miRNAs) that target both virus and host genes; however, their role in herpesvirus biology is understood poorly. We identified previously eight miRNAs encoded by ovine herpesvirus-2 (OvHV-2), the causative agent of malignant catarrhal fever (MCF), and have now investigated the role of these miRNAs in regulating expression of OvHV-2 genes that play important roles in virus biology. ORF20 (cell cycle inhibition), ORF50 (reactivation) and ORF73 (latency maintenance) each contain predicted targets for several OvHV-2 miRNAs. Co-transfection of miRNA mimics with luciferase reporter constructs containing the predicted targets showed the 5′ UTRs of ORF20 and ORF73 contain functional targets for ovhv-miR-2 and ovhv2-miR-8, respectively, and the 3′ UTR of ORF50 contains a functional target for ovhv2-miR-5. Transfection of BJ1035 cells (an OvHV-2-infected bovine T-cell line) with the relevant miRNA mimic resulted in a significant decrease in ORF50 and a smaller but non-significant decrease in ORF20. However, we were unable to demonstrate a decrease in ORF73. MCF is a disease of dysregulated lymphocyte proliferation; miRNA inhibition of ORF20 expression may play a role in this aberrant lymphocyte proliferation. The proteins encoded by ORF50 and ORF73 play opposing roles in latency. It has been hypothesized that miRNA-induced inhibition of virus genes acts to ensure that fluctuations in virus mRNA levels do not result in reactivation under conditions that are unfavourable for viral replication and our data supported this hypothesis.

INTRODUCTION

Malignant catarrhal fever (MCF) is a fatal disease of cattle and other ruminants caused by viruses in the genus Macavirus of the subfamily Gammaherpesvirinae (Russell et al., 2009). The disease occurs as a result of infection of susceptible hosts by contact with an asymptomatic carrier species that acts as a virus reservoir. Ovine herpesvirus-2 (OvHV-2) infects most sheep subclinically and is the major cause of MCF worldwide (Russell et al., 2009). In both sheep and cattle, OvHV-2 infects CD2⁺ T-lymphocytes (Meier-Trummer et al., 2010; Schock et al., 1998), but only in cattle does virus infection cause dysregulation of lymphoid cell function leading to uncontrolled proliferation, cytotoxicity and MCF disease. The proliferation of infected bovine T-cells is dependent on the cytokine IL-2 and immortalized T-cell lines can be cultured from affected cattle. The infected bovine T-cells do not support productive virus replication and have been described as large granular lymphocytes (LGLs) (Reid et al., 1989) in part due to expression of perforin (Nelson et al., 2010); unlike sheep, infected cattle cannot transmit the virus to other susceptible hosts (Russell et al., 2009). The mechanism by which OvHV-2 induces MCF in cattle is unknown; virus-induced cytopathology is thought not to be involved in lesion development and it has been proposed that tissue damage arises from non-antigen-specific, MHC-unrestricted cytotoxicity of the virus-infected LGLs (Cook & Splitter, 1988).

MicroRNAs (miRNAs) are short (21–23 nt) RNAs that act as post-transcriptional inhibitors of gene expression. Cellular miRNAs expressed in the nucleus are derived from primary transcripts (pri-miRNAs) that are processed by the enzyme Drosha to form a shorter precursor miRNA (pre-miRNA). These pre-miRNAs are exported from the nucleus and once in the cytoplasm are further cleaved by the enzyme Dicer to produce a transient double-stranded precursor, where one strand is designated the miRNA* or passenger strand. The miRNA is incorporated stably into the RNA-induced silencing complex and guides it to the target miRNA, which represses translation by a number of mechanisms, including mRNA degradation and inhibition of translation. The interaction of miRNAs
with target mRNAs is mediated by a seed region, nt 2–7 or 2–8 at the 5′ end of the miRNA (Bartel, 2009). Most miRNA targets identified to date are present in the 3′ UTR of mRNAs; however, some miRNAs have been reported that functionally target the 5′ UTR (Grey et al., 2010; Tay et al., 2008).

To date, >250 virus-encoded miRNAs have been identified, the majority from herpesviruses (Grundhoff & Sullivan, 2011). Herpesvirus-encoded miRNAs have been shown to regulate both cellular and viral gene expression, and to influence cell processes, including proliferation. In Marek’s disease virus, the deletion of a single virus-encoded miRNA abrogates virus-induced cellular transformation (Zhao et al., 2011), and in Epstein–Barr virus (EBV) a cluster of miRNAs has been implicated in controlling virus-induced B cell proliferation and transformation (Feederle et al., 2011a, b; Seto et al., 2010). We have demonstrated previously that OvHV-2 encodes at least eight miRNAs (Levy et al., 2012) expressed within the immortalized bovine LGL line, BJ1035, and hypothesized that these play a critical role in MCF pathogenesis. In order to investigate the role of virus-encoded miRNAs in OvHV-2 pathogenesis it is necessary to identify their viral and cellular targets. In this study, we investigated the role of OvHV-2-encoded miRNAs in regulating selected virus gene expression. There is no in vitro infection system for studying OvHV-2; the only cells in which virus gene expression can be studied are immortalized LGL lines such as BJ1035 (Levy et al., 2012). All cells in these LGL lines are virus genome-positive and in the majority OvHV-2 is latent; however, some lytic cycle gene expression occurs in a small proportion of the cells (Rosbottom et al., 2002; Thonur et al., 2006).

RESULTS

Prediction of OvHV-2-encoded miRNA targets in the OvHV-2 genome

Potential miRNA targets within the OvHV-2 genome were identified initially by scanning the entire OvHV-2 genome using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to align the sequences complementary to the ovhv2-miRs in the OvHV-2 genome (GenBank accession no. AY839756) (Hart et al., 2007). Targets were then mapped to the 5′ UTR or 3′ UTR of OvHV-2 genes. Only a small number of OvHV-2 mRNAs have been mapped previously; therefore for the majority of genes the 5′ UTRs were considered to span the region from the start codon to the predicted TATA box. For 3′ UTRs, the region from the stop codon to the predicted polyA site was used. This analysis identified potential targets in 33 OvHV-2 genes, representing all classes of virus gene, immediate early, early and late, and both structural and non-structural proteins.

For validation of predicted targets we focussed on genes/proteins predicted to play important roles in virus biology and pathogenesis. We chose to analyse targets present in the 5′ UTR or 3′ UTR of three virus genes, ORF20, ORF50 and ORF73. ORF20 has been shown to induce cell cycle arrest in other herpesviruses (Nascimento et al., 2009) and the other two genes encode proteins that play contrary roles in virus latency. ORF50 is crucial for virus reactivation from latency and ORF73 is important for the maintenance of latency (Ackermann, 2006). The positions of the predicted miRNA target sites in ORF20, ORF50 and ORF73 are detailed in Table 1.

miRNAs function by targeting expressed mRNAs. ORF20 and ORF50 are only predicted ORFs from the genomic sequence, and the ORF73 transcript is only partially mapped (Coulter & Reid, 2002). In this study we confirmed the existence of these three virus ORFs as transcripts, including the miRNA target sites, using a reverse transcriptase (RT)-PCR strategy.

The annotated OvHV-2 genome predicts that ORF20 overlaps with both ORF19 and ORF21 (Fig. 1a). To allow detection of ORF20 only, cDNA synthesis was primed using a gene-specific primer located 406 bp upstream (42 530–41 549) of the ORF19 TATA box (41 106). This primer will not prime ORF21 mRNA as it is the same sense as the ORF21 transcript. Fig. 1(b) shows the 118 bp amplicon, the sequence of which was 100% identical to the predicted ORF20 sequence (39 294–41 641) and includes the target sequence (41 617–41 635) for ovhv2-miR-2 (Fig. 1b).

The ORF50 and Ov6 transcripts are transcribed in the same direction, with the Ov6 TATA box lying 94 bp downstream of the ORF50 polyA site. ORF50 is spliced and ORF49 lies within the ORF50 intronic sequence transcribed in the opposite direction (Fig. 1a). We were unable to prime cDNA synthesis efficiently using an ORF50 gene-specific primer due to the limited number of nucleotides between the predicted target sequence and the polyA site, we therefore used both oligo (dT) and random primers to prime cDNA synthesis. PCR primers (78 080–78 099 and 79 205–79 181) were designed to generate an 1101 bp amplicon from the

Table 1. Positions of the predicted miRNA target sites in the UTRs of ORF20, ORF50 and ORF73

<table>
<thead>
<tr>
<th>Gene</th>
<th>5′ UTR</th>
<th>3′ UTR</th>
<th>5′ UTR targets</th>
<th>3′ UTR targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF20</td>
<td>41 641–41 538</td>
<td>79 043–79 196</td>
<td>ovhv2-miR-2: 41 611–7</td>
<td>ovhv2-miR-5: 79 161–7</td>
</tr>
<tr>
<td>ORF50</td>
<td>121 049–120 533</td>
<td>120 824–18 : 120 840–34</td>
<td>ovhv2-miR-8</td>
<td></td>
</tr>
</tbody>
</table>

Nucleotide numbers are from GenBank accession no. AY839756 (Hart et al., 2007).
middle of the second exon of ORF50 to the polyA site. The 3' primer (79 025–79 181) is ~1900 bp, and 82 bp upstream of ORF49 and Ov6 TATA boxes, respectively; the 5' primer (78 080–78 099) is 118 bp upstream of the Ov6 TATA box and 2304 bp upstream of the Ov6 polyA site. Fig. 1(c) shows the 1101 bp amplicon, the sequence of which was 100%
identical to the predicted ORF50 and included the target sequence (79,205–79,181) for ovhv2-miR-2.

ORF73 lies downstream of the ORF75 transcript and is transcribed in the same direction (Fig. 1a). The annotated OvHV-2 genome (GenBank accession no. AY839756) (Hart et al., 2007) gives the 5′ terminus of the ORF73 transcript at nt 121,049, 31 bp upstream of the predicted polyA site for ORF75. We used both oligo (dT) and random primers to prime cDNA synthesis. PCR primers (120,514–120,534 and 121,060–121,041) generate a 536 bp amplicon from within the coding sequence of ORF73 to the predicted end of ORF73 mRNA. Fig. 1(d) shows this 536 bp amplicon, the sequence of which was 100% identical to the predicted ORF73 and includes the target sequence (120,817–120,839) for ovhv2-miR-8.

**Inhibition of gene expression**

BJ1035 is a mixed population of cells with respect to virus life cycle; the majority are latently infected, but a small proportion express early and late virus genes (Rosbottom et al., 2002; Thonur et al., 2006). OvHV-2 miRNAs may be also expressed differentially in the proportion of cells in culture in which the virus is latent compared with those cells where the virus is reactivating, making analysis of inhibition of gene expression following introduction of exogenous miRNAs complex. We therefore assessed initially the ability of the ovhv2-miRs to interact with their predicted targets using a luciferase expression assay.

We have shown previously that insertion of a predicted 5′ UTR downstream of an exogenous promoter, e.g. cytomegalovirus immediate early promoter, can result in the expression of transcripts in which the start site does not reflect that of the native transcript (Grey et al., 2010). In keeping with our previous studies, when analysing targets in 5′ UTRs we cloned a region ~1000 bp upstream of the AUG in an attempt to allow correct expression from the natural promoter. For all experiments a luciferase-expressing vector lacking OvHV-2 sequences was used to investigate off-target effects; no significant reduction in luciferase expression was seen using any of the ovhv2-miRs.

The 5′ UTR of ORF20 contained a predicted target site for ovhv2-miR-2 (Table 1, Fig. 3). BHK-21 cells were co-transfected with reporter constructs and miRNA mimics as described, and luciferase expression levels were measured. The combination of the ORF20 5′ UTR reporter and ovhv2-miR-2 resulted in a 57.5 (±10)% reduction (P<0.03) in luciferase expression using 100 nM mimics (Fig. 2a) compared with control miRNA. The same degree of inhibition was observed using 50 nM mimics (data not shown). Ovhv2-miR-2 inhibited luciferase expression by 47.5 (±15)% (P<0.003) even after the mutation of the predicted target site in the 5′ UTR (Fig. 2a), suggesting that inhibition was not due to interaction of ovhv2-miR-2 with the predicted target sequence. The 3′ UTR of ORF50 was predicted to contain a target site for ovhv2-miR-5 (Table 1, Fig. 3). The combination of ORF50 3′ UTR and ovhv2-miR-5 mimic resulted in a 45 (±10)% reduction (P<0.001) in luciferase expression (Fig. 2b) compared with control miRNA. Mutation of the target site from the 3′ UTR abrogated this inhibition.

The 5′ UTR of ORF73 was predicted to contain two separate sites for ovhv2-miR-8 (Table 1, Fig. 3). The combination of the 5′ UTR of ORF73 and ovhv2-miR-8 mimic resulted in a 45 (±8)% decrease (P<0.001) in luciferase expression (Fig. 2c). Mutation of either of the predicted ovhv2-miR-8 target sites abolished the inhibitory effect of ovhv2-miR-8.

To further investigate inhibition of the expression of these OvHV-2 genes by the ovhv2-miRs we developed RT-quantitative PCR (qPCR) assays for ORF20, ORF50 and ORF73 transcripts. BJ1035 cells were transfected with the relevant ovhv2-miR mimics or control miRNA for 24 or 48 h, at which point cells were harvested, RNA isolated and qPCR carried out. We were able to demonstrate a 42 (±15)% reduction in levels of ORF50 transcripts...
transfection with ovhv2-miR-2 (Fig. 4b), this was not significant ($P=0.532$). Furthermore, we were unable to demonstrate any reduction in levels of ORF73 transcripts following transfection with ovhv2-miR-8 [+7.3 (±13.9) %] (Fig. 4c, $P=0.532$).

**DISCUSSION**

OVHV-2-induced MCF in susceptible hosts is a disease of dysregulated lymphocyte proliferation and cell function. However, these susceptible hosts cannot transmit the virus. In contrast, infection of carrier hosts is asymptomatic, but these are infectious. A previous study showed that this virus expresses at least eight miRNAs (Levy et al., 2012). In this study, we investigated the control of expression of three virus genes important for control of the cell cycle (ORF20) and virus latency (ORF50 and ORF73) by virus-encoded miRNAs.

The 3' UTR of ORF50 was predicted to be targeted by ovhv2-miR-5. This interaction was confirmed both by the luciferase assay and by inhibition of ORF50 miRNA levels in BJ1035 cells transfected with ovhv2-miR-5. Thus, we demonstrated that expression of ORF50, whose main role is to drive reactivation from latency, can be inhibited by a viral-encoded miRNA.

The 5' UTR of ORF20 was predicted to contain one site recognized by ovhv2-miR-2. Ovhv2-miR-2 did inhibit luciferase expression; however, mutation of the predicted site did not abolish this inhibition. Thus, whilst we have demonstrated that ovhv2-miR-2 inhibits expression of a transcript containing the 5' UTR of ORF20, we cannot definitively show that this inhibition is a consequence of its interaction with the target site predicted in the original analysis. We also investigated the ability of ovhv2-miR-2 to inhibit ORF20 expression in BJ1035 cells. We were able to demonstrate a small reduction in ORF20 mRNA levels in BJ1035 cells transfected with this ovhv2-miR-2; however, this was not statistically significant.

ORF73 was predicted to contain two sites recognized by the ovhv2-miR-8 seed sequence located nine bases apart (Fig. 3). Ovhv2-miR-8 was able to inhibit gene expression in the luciferase assay and mutation of either of these sites resulted in loss of inhibition. Further analysis of the binding of ovhv2-miR-8 to this region using the RNAhybrid tool showed that site 1 was predicted to bind the miRNA with high efficiency, but RNAhybrid did not identify site 2. Site 2 lies in the region that is likely to interact with the 3' end of a miRNA interacting at site 1. It is therefore likely that site 1 is functional and that the loss of inhibition seen when site 2 is mutated is due to loss of non-seed sequence interactions. We were unable to demonstrate any reduction in ORF73 mRNA levels following transfection of ovhv2-miR-8 into BJ1035 cells.

The inability to demonstrate inhibition of ORF20 and ORF73 expression in BJ1035 cells may be due to a number of factors, e.g. the inherent variability within BJ1035 cells.

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**Fig. 2.** BHK cells ($n=6$) were co-transfected with: (a) PGL-ORF20, which has the 5’ UTR of ORF20 upstream of firefly luciferase (or with a PGL-ORF20 in which the predicted ovhv2-miR-2 target site had been mutated), pRL (which expresses Renilla luciferase) and either an ovhv2-miR-2 mimic or a scrambled miRNA control; (b) psi-ORF50, which has the 3’ UTR of ORF50 downstream of Renilla luciferase (or with a psi-ORF50 in which the predicted ovhv2-miR-5 target site had been mutated) and either an ovhv2-miR-5 mimic or a scrambled miRNA control; or (c) PGL-ORF73, which has the 5’ UTR of ORF73 upstream of firefly luciferase (or with a PGL-ORF73 in which the predicted ovhv2-miR-8 target sites had been individually mutated), pRL (which expresses Renilla luciferase) and either an ovhv2-miR-8 mimic or a scrambled miRNA control. At 24 h post-transfection, firefly luciferase levels were measured, normalized to the Renilla luciferase levels, and expression in the control and test miRNA samples compared.
could result in variation in baseline levels of virus gene expression or the ovhv2-miRs may inhibit translation (the luciferase assay measured protein levels) of ORF20 and ORF73 without affecting significantly mRNA levels. ORF73 is the main latency-associated protein and so will be expressed in the majority of BJ1035 cells. Our transfection efficiency for BJ1035 cells is ~40–45% (data not shown) and it is possible that the level of inhibition

![Fig. 3](image)

**Fig. 3.** (a) RNAhybrid analysis of the predicted target sites with the relevant miRNA. MFE, mean free energy. (b) Sequence of the ORF73 5' UTR surrounding predicted sites 1 and 2 (underlined); the shaded box represents the sequence shown to interact with ovhv2-miR-8 in (a); 1 kcal = 4.18 kJ.

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![Fig. 4](image)

**Fig. 4.** BJ1035 cells (n=3) were transfected with control miRNA mimic, or (a) ovhv2-miR-5 mimic, (b) ovhv2-miR-2 mimic or (c) ovhv2-miR-8 mimic. At 24 h post-infection RNA was extracted and transcript levels of (a) ORF50, (b) ORF20 and (c) ORF73 analysed by RT-qPCR (three technical replicates per biological replicate). Levels were compared with levels in control transfected cells; error bars represent the SEM as produced by the linear mixed-effect (LME) statistical models, taking into account the technical and biological replicates.
obtained is not sufficient to be seen under these experimental conditions.

All three classes of herpesvirus, homologues of ORF20 (the UL24 family) have been shown to induce cell cycle arrest and inactivate the cyclin B/cdc2 complex (Nascimento et al., 2009). The conservation of the structure and function of these proteins during herpesvirus evolution suggests strongly that they are important in herpesvirus biology (Nascimento et al., 2009). Using ORF20 deletion mutants of MHV, Nascimento et al. (2011) also demonstrated that ORF20 is not essential for virus replication; there was a delay in virus clearance from the lungs of animals infected with the mutant virus and the mutants established latency normally. The role, if any, of ORF20 in MHV pathogenesis is therefore not clear. A recent study showed that viral cyclins play a key role in the control of latency and reactivation of the gammaherpesvirus MHV-68 (Lee et al., 2012). Different mammalian cyclins could substitute for some of the functions of the MHV-68 cyclins, with different cyclins mediating persistence or reactivation (Lee et al., 2012). It is possible that changes in expression of ORF20, mediated in part by ovhv2-miRs, could result in a change in the cyclin expression profile in infected cells, influencing the balance between productive and latent life cycles. A major part of MCF pathogenesis is uncontrolled proliferation of the infected LGLs, whose aberrant cytotoxic function leads to pathology. Inhibition of ORF20 expression by ovhv2-miR-2 may downregulate an ORF20-mediated block to the cell cycle, contributing to the dysregulated lymphocyte proliferation in susceptible hosts. OvHV-2 miRNAs were identified as being expressed in a bovine cell line, but their expression pattern in infected ovine cells is unknown. However, it is possible that a difference in the regulation of these miRNAs in ovine and bovine cells may result in differences in expression of ORF20, leading to the observed differences in the outcome of OvHV-2 infection in cattle and sheep.

ORF50 is a homologue of the replication and transcription activator (RTA) of EBV and Kaposi’s sarcoma herpesvirus (KSHV). In KSHV, the virus-encoded miR-K5 and miR-K9 have antagonistic effects on latency: miR-K5 attenuates expression of RTA, leading to a reduction in reactivation, whilst miR-K9 targets BCLAF-1, a host transcription factor that is associated with reduced viral replication (Ziegelbauer et al., 2009). Using a PARclip approach, Gottwein & Cullen (2010) reported that ORF73 of KSHV is targeted by KSHV miR-K10/miR-142-3p. Deletion of three EBV-encoded miRNAs has been shown to result in a significant increase in expression of virus latent genes (Feederle et al., 2011a, b; Seto et al., 2010), and Riley et al. (2012) showed that EBV-encoded miRNAs target virus-encoded, latency-associated genes and suggested that these miRNAs play a role in the control of EBV latency. The OvHV-2-immortalized LGL cell line, BJ1035, is a mixed population of cells with respect to virus life cycle; the majority are latently infected, but a small proportion express early and late virus genes (Rosbottom et al., 2002; Thonur et al., 2006). Thus, OvHV-2 miRNAs may be expressed differentially in the proportion of cells in culture in which the virus is latent compared with those cells where the virus is reactivating.

The proteins encoded by ORF50 and ORF73 play important but contrary roles in relation to latency: ORF50 is critical for virus reactivation and ORF73 is important in the maintenance of viral latency (Ackermann, 2006). Bellare & Ganem (2009) proposed that miRNA-induced inhibition of virus genes is not the sole regulator of reactivation/latency, but rather that miRNAs ensure that fluctuations in virus miRNA levels do not result in reactivation under conditions that are unfavourable for viral replication, i.e. the miRNAs act as a type of rheostat to control the levels of virus gene expression. The apparent contradiction of having miRNAs that act to both inhibit and encourage reactivation expressed in the same cell line can be explained by considering the mixed nature of the BJ1035 line. The differential expression of individual virus miRNAs in individual cells could respond to changes in the cellular environment acting to regulate virus gene expression. The factors that determine the maintenance of, or reactivation from, latency are complex; by demonstrating miRNA-mediated control of both ‘pro- and anti-reactivation genes’ our data add support to the hypothesis that miRNAs represent an additional layer of control exerted by the virus to control the latent state.

**METHODS**

**Target identification.** Potential miRNA targets within the OvHV-2 genome were identified using Blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to align the sequences complementary to the ovhv2-miRs in the OvHV-2 genome (GenBank accession no. AY839756) (Hart et al., 2007). Targets were mapped to the 5' UTR or 3' UTR of OvHV-2 genes. Only a small number of OvHV-2 miRNAs have been mapped previously; therefore, for the majority of genes, the 5' UTRs were considered to span the region from the start codon to the predicted TATA box. For 3' UTRs, the region from the stop codon to the predicted polyA site was used. Sites chosen for validation were also analysed by RNAhybrid (Rehmsmeier et al., 2004) allowing no G:U pairing in the seed sequence and a helix constraint of nt 2–8.

**Cell culture.** Baby hamster kidney cells (BHK-21) were cultured in Glasgow’s minimum essential medium (Gibco) supplemented with 10% new born calf serum, 1% (v/v) penicillin/streptomycin, 1% (v/v) l-glutamine (Sigma) and 10% (v/v) tryptose phosphate broth. BJ1035 cells were grown in suspension in Iscove’s modified Dulbecco’s medium (Invitrogen) (Hart et al., 2007) supplemented with 10% (v/v) FCS, 1% (v/v) penicillin/streptomycin and 350 U proleukin (IL-2) ml⁻¹ (Novartis Pharmaceutical), and cultured at 37 °C with 5% CO₂.

**Cloning of target sites.** Sequences spanning the 3’ UTR and 5’ UTR of the selected genes were amplified by PCR. For 3’ UTRs, the region from the stop codon to the predicted polyA site was amplified; for 5’ UTRs, ~1000 bp upstream of the ATG was amplified. 3’ UTRs were cloned into the psiCHECK-2 Vector (Promega) downstream of a Renilla luciferase reporter gene (RLuc) and 5’ UTRs were cloned into the pGL4.10 vector (Promega) upstream of a firefly luciferase reporter gene (Fluc).
Transfection. BHK-21 cells \( (n=6) \) were co-transfected with reporter vectors \( (1.5 \mu g) \) with or without the 5’ UTR or 3’ UTR of interest and with 50 or 100 nM mimic miRNAs \((\text{mScript} \text{ miRNA Mimics; Qiagen}) \) or control miRNA \((\text{AllStars} \text{ Negative Control siRNA; Qiagen}) \) using Lipofectamine 2000 (Invitrogen) \((\text{mimic sequences: ovhv2-miR-2, 5’-AUCUGGACAGCAUCUGCUAGUAG-3’; ovhv2-miR-5, 5’-UGAAGUACAGCCGACGGAU-3’; ovhv2-miR-8, 5’-UGGGUCAGCGGACUGGCUUC-3’})\). After 24 h, samples were harvested and luciferase levels assessed using the Dual-Luciferase Reporter Assay System \((\text{Promega}) \). For psiCheck \((3’\text{ UTR})\)-based vectors, Rluc \((\text{target})\) expression was normalized to Fluc expression from the same plasmid. For pGL4.10 \((5’\text{ UTR})\)-based vectors, Rluc \((\text{target})\) expression was normalized to Fluc expression from the same plasmid. For pGL4.10 \((5’\text{ UTR})\)-based vectors, Rluc \((\text{target})\) expression was normalized to Fluc expression from the same plasmid. For pGL4.10 \((5’\text{ UTR})\)-based vectors, Rluc \((\text{target})\) expression was normalized to Fluc expression from the same plasmid. For pGL4.10 \((5’\text{ UTR})\)-based vectors, Rluc \((\text{target})\) expression was normalized to Fluc expression from the same plasmid.

Site-directed mutagenesis. Site-directed mutagenesis was performed using the Quick Change Site-Directed Mutagenesis \((\text{Stratagene}) \) protocol. Primers for mutagenesis \((\text{HPLC purified; Eurofins MWG Operon})\) were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\).

RT-PCR analysis of transcripts. ORF20 cDNA was amplified with the primer pair TCTATAGCAGTGGTGCC and CTGAAACA-TGGCCTCCTCAACG (nt 41 617–41 635 and 41 530–41 549). ORF50 cDNA was amplified with the primer pair CCCCCAAACCGTCAAGCATTIT and CACATTCTTTACTCATACCACAC (nt 78 080–78 099 and 79 205–79 181). ORF73 cDNA was amplified with the primer pair CACATTCTGAAAGACCCGATT and GTATCCCTG-CCACACGTGTA (nt 120 514–120 534 and 121 060–121 041). PCRs contained 8 pM primers, 120 \( \mu M \) dNTPs and 1 U HotStarTaq Plus \((\text{Qiagen})\) in a final reaction volume of 25 \( \mu l \) for 30 cycles \((30 s \text{ at } 95 °C, 30 s \text{ at } 58 °C \text{ and } 1 \) min 5 s \text{ at } 72 °C). Samples were run on a 2 % agarose gel, visualized, captured from the image and sequenced.

RT-qPCR. RNA was isolated using an RNeasy Kit \((\text{Qiagen})\). RNA \((1 \mu g)\) was digested with RQ1 DNase \((\text{Promega})\) for 30 min at 37 °C. For analysis of ORF20, cDNA was primed with 250 or 66 ng specific primer \((\text{oligo (dT)}\) and random primers \((\text{Promega})\); equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \)). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\). Primers for ORF20 were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\). Primers for ORF50 were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\). Primers for ORF73 were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\). Primers for ORF73 were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\). Primers for ORF73 were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\). Primers for ORF73 were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\). Primers for ORF73 were designed such that each primer was equivalent to 0.5 \( \mu g \) RNA \( \mu g^{-1} \). cDNA was synthesized using avian myeloblastosis virus \((\text{AMV})\) RT for 1 h at 42 °C \((\text{oligo (dT)}\) and specific primer) or 37 °C \((\text{random primer})\).

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OvhV-2 miRNA targets

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