Virology 377 (2008) 265–272

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

# Inhibition of Marek's disease virus replication by retroviral vector-based RNA interference

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ARTICLE INFO

*Article history:*
Received 10 January 2008
Returned to author for revision
7 February 2008
Accepted 15 March 2008

*Keywords:*
Marek's disease virus
Gallid herpesvirus type 2
Herpesvirus of turkeys
RNA interference
Micro-RNA
Retroviral vector
Avian leukosis virus
Chicken

ABSTRACT

RNA interference (RNAi) is a promising antiviral methodology. We recently demonstrated that retroviral vectors expressing short-hairpin RNAs (shRNA-mirs) in the context of a modified endogenous micro-RNA (miRNA) can be effective in reducing replication of other retroviruses in chicken cells. In this study, similar RNAi vectors are shown to inhibit replication of the avian herpesvirus, Marek's disease virus (MDV, also known as gallid herpesvirus type 2), and its close relative, herpesvirus of turkeys (HVT). Cells expressing shRNA-mirs targeting the MDV or HVT *gB* glycoprotein gene or the *ICP4* transcriptional regulatory gene show significant inhibition of viral replication. Not only are viral titers reduced, but observed plaque sizes are significantly smaller when the virus is grown on cells in which RNAi is effective. We also describe a modified retroviral delivery vector that expresses a shRNA-mir containing up to three RNAi target sequences and employ this vector with multiple targets within the MDV *gB* gene or within both the *gB* and *ICP4* genes. The use of targets within multiple genes potentially can provide a larger antiviral effect and/or make it more difficult for viral escape mutations to evolve.



## Introduction

RNA interference (RNAi) is a promising antiviral strategy (Hu et al., 2002; Berkhout and Haasnoot, 2006; Cullen, 2006; Leonard and Schaffer, 2006). RNAi is mediated by short RNA oligonucleotides that bind to, suppress translation of, and sometimes induce cleavage of complementary mRNAs. RNAi generally arises from two types of intermediary molecules: small interfering RNAs (siRNAs) and micro-RNAs (miRNAs: Tang, 2005; Valencia-Sanchez et al., 2006). siRNAs are ~22 base-pair (bp) double-stranded oligonucleotides that can be chemically synthesized and introduced directly into cells or processed from short-hairpin RNAs (shRNAs) that are transcribed from transfected vectors (Nakahara and Carthew, 2004). More recently, endogenous miRNA genes transcribed as larger "pri-miRNA" precursors have been found to more effectively generate an RNAi effect (Boden et al., 2004; Silva et al., 2005; Dickins et al., 2005). In this case (termed shRNA-mirs; Zeng et al., 2002), the stem region of a miRNA gene is replaced with the target sequence and its guide RNA complement. We previously described retroviral vectors based on the replication-competent avian leukosis virus constructs developed by Hughes and colleagues (Hughes et al., 1987; Federspiel and Hughes, 1997) that express shRNA-mirs either as spliced sub-genomic RNAs transcribed from the retroviral long terminal repeat (LTR) promoter or as short RNAs transcribed from internal promoters (Chen et al., 2007). We further demonstrated the efficacy of such vectors in inhibiting replication of subgroup B avian leukosis virus in cultured chicken cells. Retroviral vectors have been used in large scale RNAi mutagenesis of endogenous genes (reviewed in Chang et al., 2006; Root et al., 2006) but only rarely to inhibit the growth of other viruses.

The replication of several herpesviruses has been inhibited by RNAi, mostly using exogenously administered, synthetic siRNAs. These include murine herpesvirus 68 (Jia and Sun, 2003), Epstein–Barr virus (Chang et al., 2004), Kaposi's sarcoma-associated herpesvirus (e.g., Godfrey et al., 2005), HSV-1 (Bhuyan et al., 2004), herpesvirus-6B (Yoon et al., 2004), human cytomegalovirus (Wiebusch et al., 2004), a duck herpesvirus (Mallanna et al., 2006) and HSV-2 (Palliser et al., 2006). Palliser et al. (2006) demonstrated that siRNAs (including those targeting *gB* glycoprotein mRNA) could block lethal HSV-2 challenges in mice when administered exogenously either before or after the virus. However, retroviral delivery offers the prospect of more stable and cost-effective antiviral RNAi in live animals than do synthetic siRNAs. The goal of this study was to inhibit MDV replication using shRNA-mirs delivered by retroviral infection.

MDV (also known as gallid herpesvirus type 2, GaHV-2) is a herpesvirus that causes Marek's disease, a lymphoproliferative disorder in which aggressive T-cell lymphomas result from infection of susceptible chickens (Calnek and Witter, 1997). MDV is a major chronic infectious disease concern for the world's poultry industry

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doi:10.1016/j.virol.2008.03.019

and is of increasing interest as a model for herpesvirus oncology (Osterrieder et al., 2006). Since 1970, Marek's disease has been controlled primarily by vaccination (Calnek and Witter, 1997) with HVT or with one or more attenuated MDV isolates. However, since vaccination blocks tumor formation rather than viral replication, MDV continues to multiply and evolve in vaccinated flocks. In addition to escaping vaccine control, MDV also appears to be evolving greater virulence and the ability to generate more acute disease symptoms (Witter, 1997; Nair, 2005; Osterrieder et al., 2006). MDV genomes range from about 174 to 180 kb in size, and each encodes approximately 100 proteins. Genetic analysis has demonstrated that the *gB, gE, gI, gM, UL49.5* and *UL49–VP22* genes are required for MDV growth (Schumacher et al., 2000, 2001; Tischer et al., 2002; Dorange et al., 2002). Several other MDV genes are necessary either for full infectivity and/or for pathogenesis *in vivo* (reviewed in Osterrieder et al., 2006). We initially chose to test RNAi using the *gB* gene (Yoshida et al., 1994) as our target, as it encodes a major surface glycoprotein that is easy to detect with a monoclonal antibody. The *gB* glycoprotein also has a major role in protective immunity (Ross et al., 1996). Subsequently, we also used the key transcriptional regulatory gene, *ICP4*, as a target. The HSV1 *ICP4* gene has been demonstrated to be essential for viral replication (Preston, 1979; Dixon and Schaffer, 1980; DeLuca et al., 1985; Compel and DeLuca, 2003). Moreover, Trang et al. (2000, 2001) showed that expression of RNaseP-derived ribozymes against HSV1 *ICP4* mRNA reduced viral growth. Jia and Sun (2003) demonstrated RNAi against murine herpesvirus 68 using the *Rta* gene as a target, and *Rta* functions as an early regulator of viral transcription in gamma-herpesviruses in a somewhat analogous fashion to the role of *ICP4* in MDV (Pavlova et al., 2003; Feederle et al., 2000). These experiments suggest that reductions in *ICP4* mRNA levels can generate an antiviral effect. In addition to its role in lytic infection, *ICP4* is one of a few genes expressed in cells that harbor latent MDV genomes. Several labs have demonstrated that latently infected cells naturally express a family of antisense transcripts to *ICP4* (LATs: Cantello et al., 1994, 1997; Li et al., 1994, 1998; McKie et al., 1995; Ohashi et al., 1994). The function of LATs and the mechanism(s) by which they might modulate *ICP4* activity remain unclear, but Xie et al. (1996) showed that antisense oligonucleotides or expression of antisense RNA to *ICP4* inhibited the proliferation of MDV-transformed MSB1 cells. In addition, Burnside et al. (2006) recently identified two clusters of miRNAs in the MDV genome, one of which was at the 5′ end of the LAT region, just downstream of *ICP4*.

## Results

### *Inhibition of HVT by retroviral delivery of shRNA-mirs targeted against the gB gene*

We previously described RCASBP(A)miRNA (Supplemental Fig. 1), a retroviral vector containing 417 bp of the chicken *miR-30a* gene modified to contain MluI and NcoI restriction sites for insertion of 99 bp synthetic duplex target sequences using a modified pENTR3C entry plasmid and Gateway® (Invitrogen Corp.) recombination technology (Chen et al., 2007). Subsequently, an alternative *miR-30a*-based entry vector that accepts shorter synthetic duplexes (78 bp) between SphI and NgoMIV sites has been created (pENTR3C-miR30a-sphngo, Supplemental Figs. 1 and 2). In both cases, the modified *miR-30a* transcript is expressed from the retroviral LTR via a sub-genomic spliced mRNA. The viral vectors were used to transfect DF-1 avian fibroblasts (Schaefer-Klein et al., 1998) in which they are replication-competent. After the vectors spread throughout the culture, cells were challenged with HVT followed by a plaque assay (Materials and methods). Five different target sequences (Supplemental Table 1) were chosen within the HVT *gB* sequence using methods described previously (Chen et al., 2007). Three out of the five shRNA-mirs (FgB2, FgBcshl2 and FgBcshl3) significantly reduced HVT plaque numbers in comparison either to mock-transfected DF-1 cells or to DF-1 cells treated with RCASBP(A)miRNA containing a scrambled target sequence (Table 1). The most effective target (FgB2) reduced plaque numbers to about 30% of that of the controls. As previously described for other targets (Chen et al., 2007), no reduction in HVT titer was observed when the same *gB* sequences were inserted into a *miR-30a* gene downstream of a chicken U6 polymerase III promoter in the RCANBP(A) delivery vector (data not shown). Two of the effective HVT *gB* targets, FgB2 and FgB-cshl2, were subsequently inserted as shorter duplexes into the pENTR3C-miR30a-sphngo entry vector followed by

**Table 1**
Effect of retroviral RNAi vector treatment using *gB* gene targets on FC126 HVT infection

| FC126 HVT *gB* target | shRNA-mir30a[a] | | shRNA-mir30a-sphngo[a] | |
|---|---|---|---|---|
| | Mean[b] | Std. error | Mean[b] | Std. error |
| FgB1 | 1.1 | 0.13 | ND[c] | ND[c] |
| FgB2 | 0.3[d] | 0.04 | 0.32[d] | 0.03 |
| FgB3 | 1.1 | 0.13 | ND[c] | ND[c] |
| FgBcshl2 | 0.4[d] | 0.03 | 0.45[d] | 0.02 |
| FgBcshl3 | 0.5[d] | 0.02 | ND[c] | ND[c] |
| Scrambled | 1.1 | 0.03 | 0.93 | 0.05 |

[a] Type of pENTR3C entry vector employed to insert target.
[b] Average titer of each retroviral treatment divided by the average titer after mock infection.
[c] ND, not done.
[d] Statistical difference of $P<0.01$ from mock treatment within a column.

**Table 2**
Reduction in MDV and HVT plaque sizes due to RNAi against *gB*

| Retroviral vector and RNAi target | | | | | |
|---|---|---|---|---|---|
| RCASBP(A)miRNA Md11 MDV *gB* | | | RCASBP(A)miRNA-sphngo FC126 HVT *gB* | | |
| Construct | Mean* | SD* | Construct | Mean* | SD* |
| Mock | $135^{AB}$ | 47 | Mock | $129^{A}$ | 50 |
| Scrambled | $140^{A}$ | 48 | Scrambled | $83^{AB}$ | 34 |
| 2541 | $153^{A}$ | 69 | gB2 | $27^{C}$ | 13 |
| 1874 | $28^{C}$ | 11 | gBcshl2 | $41^{BC}$ | 29 |
| 1518 | $72^{BC}$ | 31 | | | |
| 596 | $57^{C}$ | 42 | | | |

*All units are in $\mu m^2 \times 10^3$. Mean values within a column not sharing a common letter superscript are statistically different at $P<0.005$. SD = standard deviation.

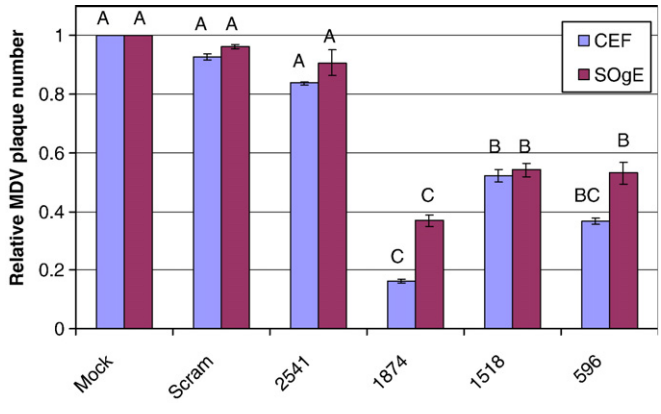


**Fig. 1.** RCASBP(A)shRNA-mir directed against Md11 MDV *gB* reduces plaque numbers in both CEF (blue bars) and SOgE cells (purple bars). Four different shRNA-mirs against MDV *gB* (Supplemental Table 1), along with a scrambled control sequence, were delivered to CEF and SOgE cells via the RCASBP(A)miRNA vector (Supplemental Fig. 1). Targets are identified by the number of their first nucleotide within the Md11 *gB* coding sequence (Supplemental Table 1). Cells were infected with 100 to 400 pfu of Md11 MDV. Plaques were counted at 5 dpi and normalized to the mock-infected control as 1.0. Histogram bars labeled with different letters are significantly different within either the CEF or SOgE cell groups, respectively ($P<0.05$).

transfer to RCASBP(A), resulting in a similar antiviral effect (Table 1). The use of shorter synthetic target inserts should provide higher sequence reliability at lower cost.

Since *gB* functions as a membrane fusion protein, any reduction in *gB* expression could impact viral spread between cells and thus produce a distinct plaque phenotype. Table 2 shows that the mean plaque sizes for the FgB2 and FgB-cshl2 groups were significantly smaller than those observed on mock-treated cells ($P<0.005$). Indirect immunofluorescence (IIF) staining using a monoclonal antibody specific for the HVT gB protein confirmed the small plaque phenotype and also demonstrated a reduction in gB fluorescence within the stained plaques treated with effective RNAi vectors (data not shown).

*Antiviral RNAi against MDV (GaHV-2) using gB gene targets*

We next investigated whether RNAi against the viral *gB* gene could inhibit pathogenic serotype 1 strains of MDV (GaHV-2) in cell culture. MDV and HVT gB proteins are only 81% identical and their respective genes are about 75% identical (Tulman et al., 2000; Afonso et al., 2001; Kingham et al., 2001) in nucleotide sequence, so four specific RNAi target sites were designed for the MDV *gB* gene (strain Md11; Niikura et al., 2006). These assays were performed in chicken embryo fibroblasts (CEF) since they are more permissive to serotype 1 MDV infection. When delivered using RCASBP(A)miRNA, three of the four targets gave rise to a significant ($P<0.05$) reduction in plaque number, with the most effective target (1874) reducing titers to about 16% of the control (Fig. 1). Again, MDV plaque sizes were reduced and showed reduced *gB* expression by IIF (Fig. 2 and Table 2) in the three cultures treated with effective RNAi target sequences (1874, 1518, and 596, where the number indicates the first nucleotide of the target site in the *gB* coding sequence, Supplemental Table 1).

Recently, a permanent cell line, SOgE, that constitutively expresses the MDV gE glycoprotein, has been established for propagation of virulent strains of MDV (Schumacher et al., 2002). We delivered RCASBP(A)miRNA vectors into SOgE cells via nucleofection (Materials and methods) and monitored their spread through the culture by fluorescence microscopy and flow cytometry. In a RCASBP(A)-GFP transfected culture, more than 90% of the cells were positive for GFP expression after four passages (data not shown). We then employed these transfected SOgE cells in plaque assays of Md11 MDV. The three constructs that reduced plaque numbers in CEF also significantly inhibited plaque formation in SOgE cells ($P<0.05$), although the most effective target sequence (1874) showed less inhibition in SOgE cells than in CEF (Fig. 1). As expected, the other target (2514) and a control scrambled sequence target did not significantly reduce plaque numbers. The reduced inhibition in SOgE cells may relate to the use of transfection to deliver the RNAi vector to these cells or to the fact that, in our hands, Md11 generates smaller plaques on these cells even in the absence of RNAi.

*Antiviral RNAi against MDV using a three target vector with ICP4 and/or gB target sequences*

We modified our vector system such that multiple shRNA-mirs can be transcribed within a single pri-miRNA-like precursor. At least two putative endogenous chicken pri-miRNA-encoding genes generate transcripts predicted to contain 6 miRNA hairpins, one on chromosome 1 (employed by Das et al., 2006) and another on chromosome 4. We chose to use the second locus for ease of construction. The sequence in question was modified to generate a cassette that contains three miRNA sites (miR-20b, miR-92-2 and miR-19b), each of which is flanked by a pair of unique restriction sites allowing for the insertion of

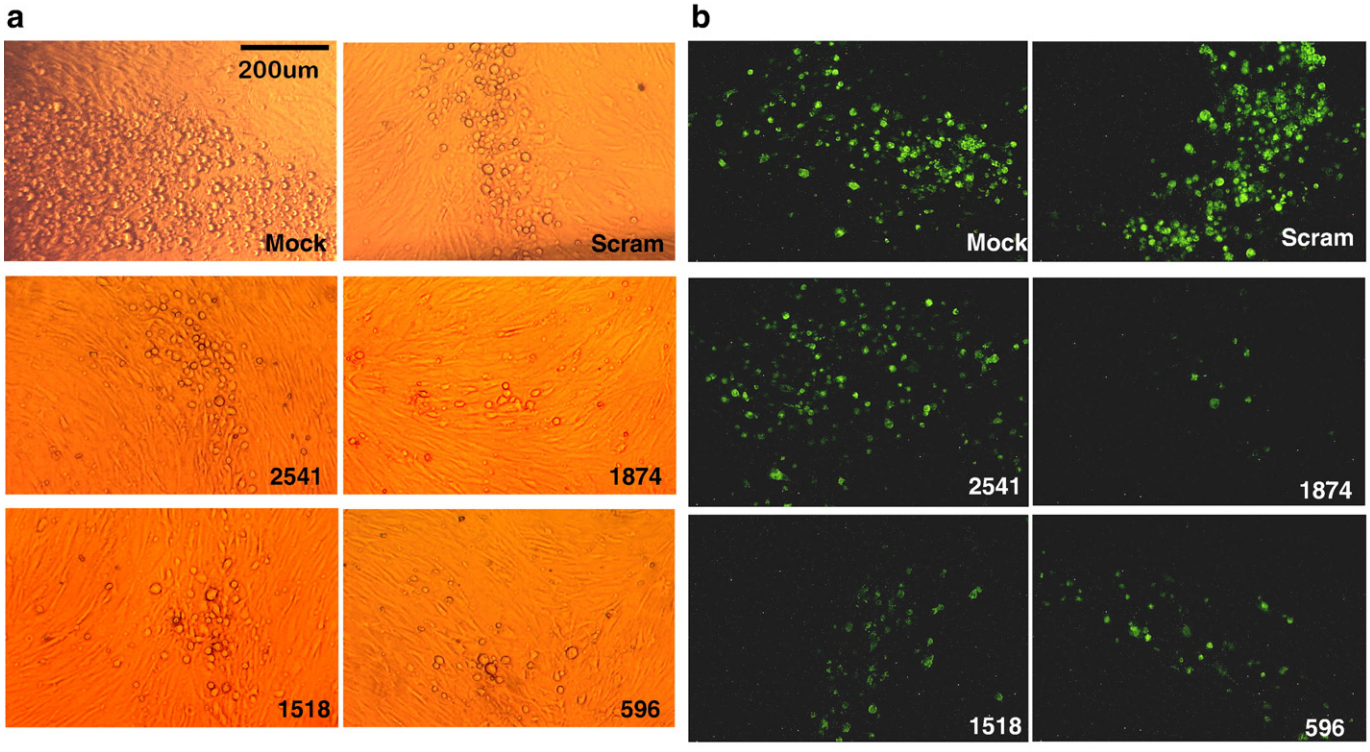


**Fig. 2.** a. Morphology of Md11 MDV plaques on CEF at 5 dpi. Four different MDV *gB* target sequences (Supplemental Table 1), along with a scrambled control sequence, were delivered to CEF via the RCASBP(A)miRNA vector. CEF cells were infected with 100 to 400 pfu of Md11 MDV. Two *gB*-specific RNAi treatments (1874 and 596) of CEF cells show a reduced plaque size. A magnification scale is inserted in the Mock panel. b. IIF analysis of representative Md11 plaques in CEF. CEF were infected with RCASBP(A)miRNA vectors with *gB* target sequences as described in Fig. 2.a. At 5 dpi, cells were fixed with acetone: alcohol (60:40) and incubated with MDV *gB* MAb IAN86. The secondary antibody was anti-mouse immunoglobulin G conjugated to FITC (Molecular Probes). Three *gB*-specific RNAi treatments (1874, 596 and 1518) demonstrate reduced *gB* expression and plaque size. Magnification, ×250.

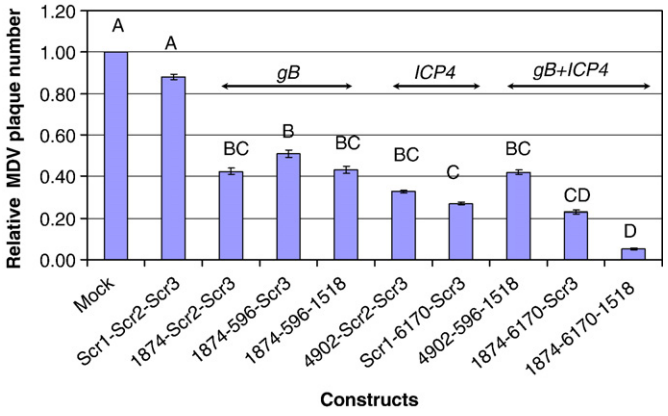


**Fig. 3.** RCASBP(A)3mirs directed against subgroup MDV *gB* and/or *ICP4* reduces titers in CEF. miRNAs against MDV *gB* and/or *ICP4* (Supplemental Table 1), along with scrambled control sequences, were delivered to CEF via the RCASBP(A)3mirs vector (Supplemental Fig. 1). Targets are identified by the number of their first nucleotide within the MDV *gB* or *ICP4* coding sequence, respectively (Supplemental Table 1). In each case, the first sequence in a series is in the miR-20b site, the second in the miR-19b site and the third in the miR-92-2 site. Scr1, 2 and 3 indicate different scrambled control sequences with similar base compositions to the targets. *gB* targets are 1874, 596 and 1518, whereas *ICP4* targets are 4902 or 6170. Cultures were infected with 100 pfu of Md5 MDV. Plaques were counted at 5 dpi and normalized to the mock-infected control as 1.0. Histogram bars labeled with different letters are significantly different ($P<0.01$).

up to three targets (Supplemental Fig. 3). Initial tests of this vector (RCASBP(A)3mirs, Supplemental Fig. 1) using single MDV *gB* target sequences demonstrated that each of the three sites individually can generate an effective RNAi response (data not shown). RCASBP(A)3mirs was then used to deliver one, two or three of the *gB* gene targets previously shown to be effective. In this case, including two additional *gB* targets (596 and 1518) did not significantly add to the antiviral effect observed using the best single *gB* target (1874) alone (Fig. 3).

The multi-target RNAi vector then was used to explore the effectiveness of the MDV *ICP4* gene as a target. We first tested individual target sequences within *ICP4*. Two of four *ICP4* targets chosen were able to significantly reduce plaque numbers to about 30% of the control (Fig. 3, only the two effective targets are shown). The effective *gB* and *ICP4* targets were then combined within single multi-target vectors. Fig. 3 shows that the addition of the best *ICP4* target sequence (6170) to one or two *gB* targets enhances the antiviral effect observed, with the best construct (1874-gB, 6170-ICP4, 1518-gB) reducing the MDV titer to nearly 5% of the mock-infected control (Fig. 3). Given that RNAi reduced MDV viral replication as a whole, we expected at least a concomitant reduction in viral target mRNA levels. Indeed, reverse transcriptase PCR (RT-PCR) measurements demonstrated that reduction of *gB* mRNA levels correlated well with the reduction in MDV plaque number (Fig. 4). For one of the four experiments whose results are summarized in Fig. 3, we also measured replication of MDV viral DNA via quantitative PCR comparing the level of MDV *gB* gene DNA to that of the host cell *GAPDH* gene (Supplemental Fig. 4). These results correlated well (correlation coefficient = 0.70, similar to, or higher than, those observed by Bumstead et al., 1997 and Baigent et al., 2005) with the titers shown in Fig. 3, although the inhibition was somewhat less pronounced, at least for vectors containing the 1874-gB target. PCR-based methods measure all forms of the viral genome (Baigent et al., 2005), including, presumably, genomes that fail to be infectious due to a deficit in, for example, gB protein. Thus, we view inhibition of viral titers as the most relevant quantitative measure of the RNAi effect.

## Discussion

We have demonstrated the use of retroviral vector-based RNAi against MDV, a major pathogenic threat in chickens. Three different shRNA-mir gene constructs, delivered via a replication-competent retroviral vector, successfully reduced viral replication using targets in two very different viral genes, *gB* and *ICP4*. The *gB* glycoprotein gene is known to be essential for MDV replication (Schumacher et al., 2000) and is likely involved in viral spread from cell to cell, whereas the *ICP4* gene, known to be essential in other herpesviruses, acts as a transcription factor. The *ICP4* effect occurs in spite of the fact that this immediate early protein is transported within infectious virions in HSV1 (Yao and Courtney, 1989), which could provide a source of the protein resistant to any RNAi effect. However, the complementation studies of Dargan and Subak-Sharpe (1997) suggest the need for additional *de novo ICP4* synthesis during the viral life cycle after the initial infection.

The results reported here confirm our earlier observation (Chen et al., 2007) and those of others (Boden et al., 2004; Silva et al., 2005; Dickins et al., 2005) that embedding the RNAi target within a pri-miRNA gene transcribed by an RNA polymerase II promoter provides an effective delivery method. Since many pri-miRNA genes are polycistronic (Lagos-Quintana et al., 2001), the possibility exists to express multiple RNAi target sequences within a single transcript (Yu et al., 2003; Chung et al., 2006; Das et al., 2006). Indeed, the endogenous chicken chromosome 4 miRNA cluster that we have modified to deliver three targets originally contained six potential miRNAs, although it has not been proven that all six are expressed. Our results demonstrate that all three target sites in our vector can be functional. However, combining either three different *gB* gene targets or one *ICP4* target and two *gB* targets, each of which was known to inhibit on its own, did not always increase the antiviral effect in an additive fashion. For example, although the 596-gB and 1518-gB targets were effective on their own, they did not significantly enhance the antiviral effect of 1874-gB when added to the multi-target vector (Fig. 3). On the other hand, adding 1518-gB to 1874-gB plus 6170-ICP4 produced a greater reduction in MDV titer than did 1874-gB plus 6170-ICP4 alone [a direct comparison of the 1874–6170-scrambled control result with that of 1874–6170–1518 gave a significant difference ($P<0.05$ using Student's $t$ test), but the difference did not reach

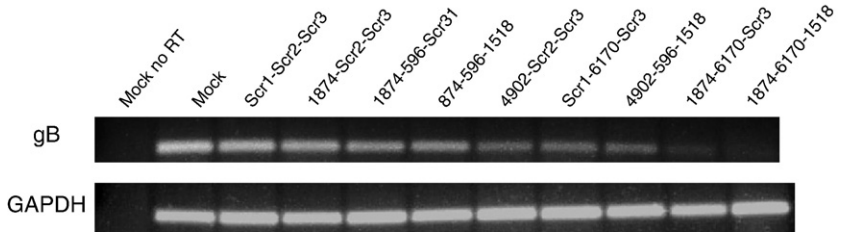


**Fig. 4.** RT-PCR of *gB* mRNA expression from CEF expressing RCASBP(A)3mirs. CEF were infected with 100 pfu of Md5 MDV, and RNA was isolated at 5 dpi. RT-PCR was performed as described in Materials and methods. *GAPDH* mRNA was assayed as a loading control. Mock no RT: RT-PCR was performed using RNA from Mock treatment without added reverse transcriptase. In each case, the first sequence in a series is in the miR-20b site, the second in the miR-19b site and the third in the miR-92-2 site. Scr1, 2 and 3 indicate different scrambled control sequences with similar base compositions to the targets. *gB* targets are 1874, 596 and 1518, whereas *ICP4* targets are 4902 or 6170. Targets are identified by the number of their first nucleotide within the MDV *gB* or *ICP4* coding sequence, respectively (Supplemental Table 1).

significance in the pairwise comparison of all treatment groups using Duncan's test]. Clearly, many factors modulate the effectiveness of any given viral target gene and any specific target sequence within that gene. We are currently exploring the use of additional targets and additional combinations of targets within our multi-target vector to address some of these issues. Regardless of whether multiple targets provide a substantially greater antiviral effect, this approach may also have value in making it more difficult for the virus to evade the RNAi effect through a mutation in the target sequence(s). RNAi escape mutants have been documented for HIV, poliovirus, and hepatitis B and C viruses (reviewed in Leonard and Schaffer, 2006). However viral escape mutations appear to be less frequent in DNA viruses such as MDV, and Palliser et al. (2006) found no mutations in the *UL29* target region in HSV-2 in the course of blocking lethal effects of HSV-2 using RNAi *in vivo*.

Retroviral-delivered RNAi reduced both the number and size of MDV plaques. These two properties are likely due to the same central effect, since reducing the size of plaques to the point that they are no longer visible will inevitably reduce the titer. In any case, both observations suggest a potentially significant antiviral effect may be obtained via *in vivo* delivery of RNAi against MDV. We are presently engaged in experiments to test this possibility. Although the reductions in titer that we have observed are generally modest, it should be noted that genetic resistance/susceptibility to MDV is a highly multigenic trait (Vallejo et al., 1998; Yonash et al., 1999) involving minor contributions from many polymorphic alleles to achieve, in some cases, high levels of total resistance. Thus, even a partial block in viral replication could significantly lower the frequency and/or the severity of Marek's disease *in vivo*. RNAi inhibition of viral replication should also reduce the rate at which MDV evolves in the field to evade vaccination or to increase virulence (Osterrieder et al., 2006). While replication-competent vectors are unlikely to be of practical value in the field, they provide a useful model system in which to test targets that might later be incorporated into replication-defective retroviruses or other viral vectors. Anti-viral shRNA-mirs could be effectively delivered to chickens via transgenics (Salter et al., 1987; Mozdziak et al., 2003; McGrew et al., 2004) or as a part of a vaccine potentially to create viral-resistant chicken populations (Hu et al., 2002). Additional research will be required to demonstrate an antiviral effect *in vivo* and to overcome the barriers to implementing such a strategy.

## Materials and methods

### *Vector constructions*

The construction of the RCASBP(A)miRNA vector and the corresponding pENTR3C-miR30a entry vector has been described previously (Chen et al., 2007). The pENTR3C-miR30a-sphngo entry vector (Supplementary Fig. 2) was made in an analogous fashion using the overlapping PCR technique of Ho et al. (1989) to insert SphI and NgoMIV restriction sites into the *mir-30a* target region. Primers used in the construction and the resultant insert sequence are shown in Supplemental Fig. 2. The PCR product was cleaved with BamH1 and NotI and inserted into the pENTR3C vector (Invitrogen Corp.) as described previously (Chen et al., 2007).

The pENTR3C-3mir entry vector was constructed using similar methodology. We chose to use the chicken miRNA cluster at 3,968,600–3,970,600 on chromosome 4 of the v2.1 build of the chicken genome sequence (http://genome.ucsc.edu/cgi-bin/hgGateway). The genome sequence in this region includes a gap that can be filled using two overlapping chicken EST sequences (BU384584 and BU337076) that, together, likely correspond to most, but not all, of the pri-miRNA transcribed from this cluster. BU384584 includes miRNAs from the miR-18b, miR-20b, miR-19b and miR-92-2 families and most of a member of the miR-363 family. In addition, there is (at least) a member of the miR-106a family just upstream of the BU384584 cluster. We chose to use the miR-20b, miR-19b and miR-92-2 segment of this cluster, deleting the miR-363 family member, but retaining part of the presumed 3′ end of the pri-miRNA in our construct. The sequence of the resulting insert and the PCR primers used to construct it are shown in Supplementary Fig. 3. Two PCR duplexes were generated that were cleaved with, respectively, HindIII and AvrII and AvrII and NotI, and then were cloned into HindIII–NotI-cut pENTR3C in a three-way ligation. pENTR3C-3mir contains the complete miR-19b family member gene which can be removed and replaced using BspEI/EcoRI digestion and two small filler sequences at the sites of the miR-20b and miR-92-2 family members flanked by unique AvrII and BglII and BamHI and AgeI sites, respectively (Supplementary Figs. 1 and 3).

RNAi target sequences were chosen by computer analysis as described previously (Chen et al., 2007). Target sequences were embedded within oligonucleotides designed to mimic, as nearly as possible, the endogenous flanking and loop miRNA sequence(s) that were obtained in polyacrylamide gel-purified form from commercial sources (Invitrogen Corp. or Integrated DNA Technologies). Duplexes were annealed and ligated into appropriately-digested entry vectors. Oligonucleotide sequences used are listed in Supplementary Table 1. All entry vector inserts were confirmed by DNA sequence analysis, after which the relevant insert was transferred into the RCASBP(A) destination vector using a Gateway® LR reaction according to the manufacturer's recommendations (Invitrogen Corp.). Retroviral vector constructs were verified by restriction digestion using appropriate unique sites within insert cassettes. For the RCASBP(A)shRNA-mir 1874 construct, versions were tested that either contained or lacked an internal U6 promoter in addition to the viral LTR, with only the results of the former shown in Figs. 1 and 2. No significant difference in effect was observed between the two constructs. All other constructs lack the U6 internal promoter.

### *Cells and viruses*

DF-1 cells were maintained in Leibowitz's L-15 and McCoy 5A media (1:1) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals), 50 μg/ml of gentamicin (Invitrogen), and 0.25 μg/ml of fungizone at 39 °C. CEF cells were maintained in the same media supplemented with 4% calf serum (Invitrogen Corp.). SOgE cells were maintained in Dulbecco's modified Eagle's medium with 7.5% FBS with selection in 1 mg/ml of G418 (Invitrogen Corp.) once every five passages (Schumacher et al., 2002). The serum content for MDV- or HVT-infected cells was reduced to 1% when cells reached confluence. Md11 (80 passages in CEF) and Md5 (42 passages in CEF) MDV strains and the FC126 strain of HVT were obtained from Richard Witter and Mohammad Heidari, USDA-ARS, Avian Disease and Oncology Lab (ADOL). The Md11 MDV used to infect SOgE cells was passed in these cells four times prior to use.

### *Delivery of RCAS and RCAN vectors into cell culture*

Propagation of RCASBP(A) vectors in DF-1 cells was initiated by transfection of plasmid DNA containing the retroviral provirus using SuperFect Transfection Reagent (Qiagen, Inc.) according to the manufacturer's protocol. Viral spread was monitored by assaying culture supernatants for ALV capsid protein by ELISA (Smith et al., 1979). RCASBP(A) stocks were generated from cell supernatants by centrifugation at 1500 ×*g* for 10 min at 4 °C and stored in aliquots at −80 °C. RCASBP(A) vectors were delivered into CEF cells by infection of virus grown on DF-1 cells. Briefly, $10^6$ CEF were seeded on 10 cm dishes and allowed to attach overnight. Viral stocks were added to DF-1 media at a MOI of 1.0. CEF were then incubated in virus-containing media with 4 μg/ml polybrene for 24 h before being replaced with fresh medium. Sample supernatants were collected after one passage and infection was monitored by ELISA as described above. Propagation of RCASBP(A) vectors in SOgE cells was initiated by transfection of

plasmid DNA using a Nucleofactor Device with the manufacturer-supplied Basic Fibroblast Kit (Amaxa Biosystems). RCASBP(A) spread was monitored using a Becton-Dickinson FACSCalibur (BD Biosciences) machine using a subgroup(A) ALV-specific antibody (RAV-1, a gift from Lucy Lee, ADOL). GFP expression from the RCASBP(A)-GFP vector was also detected by fluorescence microscopy as described previously (Chen et al., 2007).

*Plaque assays*

DF-1 cells expressing shRNA-mir constructs were infected by FC126 HVT as follows: $5.0\times10^5$ cells were seeded on 6-well plates and allowed to attach overnight. Four hundred plaque forming units (pfu) were added in DF-1 media followed by incubation for up to 4 dpi. Plaques were observed and counted under a LEICA DM IRB/E inverted microscope (Leica Wetzlar), and images were captured by a DEL-750CE digital system. Plaque assays were repeated four times (shRNA-mir30a) or three times (shRNA-mir30a-sphngo), each time in duplicate (Table 1). CEF and SOgE cells expressing shRNA-mir constructs were infected with 100 to 400 pfu of Md11 or Md5 strains of MDV in a similar fashion, except that plaques were counted at 5 dpi. Plaque assays employing RCASBP(A)miRNA (Fig. 1) were repeated twice (CEF cells) or three times (SOgE cells). Plaque assays employing RCASBP(A)3mirs (Fig. 3) were repeated four times.

*Immunofluorescence assay*

DF-1 cell monolayers (4 dpi with 400 pfu of FC126 strain HVT) were fixed with ice-cold 40% ethanol and 60% acetone and incubated with the primary L78 antibody (gift from Lucy Lee, ADOL) for 30 min as described (Lee et al., 1983). Cells were washed three times with phosphate-buffered saline (PBS) and incubated with the secondary antibody, FITC-labeled goat anti-mouse IgG (MP Biomedicals) for 30 min. After three washes with PBS, cells were examined under the fluorescent microscope. CEF cells were infected with Md11 strain MDV at 100 or 150 pfu and incubated up to 5 days. Plaques were stained in a similar manner except that the primary antibody was IAN86 (gift from Lucy Lee, ADOL).

*Plaque size measurement*

DF-1 cells previously treated with RCASBP(A)miRNA-sphngo were infected by 400 pfu of FC126 HVT. DF-1 cells monolayers (4 dpi) in 6 well plates were then fixed with ice-cold 40% ethanol and 60% acetone. Plaque images were captured using a DEL-750CE digital system. CEF previously treated with RCASBP(A)miRNA were infected by 100 to 400 pfu of Md11 strain MDV in a similar fashion, except that plaques images were captured at 5 dpi. For size comparisons, 8 to 15 randomly chosen plaques (a sufficient number to achieve statistical significance according to pilot experiments) for each treatment were analyzed using ImageJ (freeware from the National Institutes of Health; http://rsb.info.nih.gov/ij/index.html). To ensure random sampling, all plaques in a 10 mm diameter circle in the center of the plate were measured; if fewer than 8 plaques were found in this area, the diameter of the circle was increased to 15 mm. Plaque images were first processed using ImageJ to increase the picture quality. Plaque size measurement was performed according to the Measuring and Counting Objects Instruction (http://rsb.info.nih.gov/ij/docs/index.html). The unit and scale were set at 1.058 pixels/μm based on a known micrometer ruler. Both HVT and MDV plaques were measured from 3 independent experiments.

*Flow cytometry*

For flow cytometric analysis of RCASBP(A) propagation in SOgE cells, cells were incubated with the primary antibody, RAV-1, for 30 min at 4 °C. Cells were washed three times with DMEM containing 1% FBS followed by centrifugation (1000 ×*g*, 5 min). Cells were then incubated with the secondary antibody, FITC-labeled goat anti-mouse IgG (MP Biomedicals), for 30 min at 4 °C. After three washes, cells were analyzed using a Becton-Dickinson FACScaliber flow cytometer and CellQuest Pro software (BD Bioscience).

*Reverse transcriptase PCR (RT-PCR)*

CEF expressing various RCASBP(A)-3mirs were challenged with 100 pfu of Md5 strain MDV. RNA was collected at 5 dpi and treated with DNase I using the RNeasy kit according to manufacturer's instructions (Qiagen, Inc.). One microgram of RNA was reverse transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Corp.) in a 20-μl reaction volume. Amplifications of *gB* and chicken *GAPDH* sequences were carried out in separate reactions using the following primers: *GAPDH* forward, TGACAAGTCCCTGAAAATTGTCA; *GAPDH* reverse, CAAGGGTGCCAGGCAGTT; *gB* forward, CCAGTGGGTTCAACCGTGA; and *gB* reverse, CGGTGGCTTTTCTAGGTTCG (gifts from Robert Silva, ADOL). PCR was done in 50 μl containing 25 μl 2× PCR master mix (Promega Corp.), 2 μL cDNA, and primers at 2.5 pmol each for 2 min at 95 °C followed by 20 cycles (24 cycles for *gB*) of 1 min at 95 °C, 30 s at 52.5 °C (57 °C for *gB*) and 30 s at 72 °C. cDNA prepared from mock infection minus reverse transcriptase (no −RT Mock) and from CEF without Md5 (CEF) were used as negative controls.

*Quantitative real time PCR (qPCR) assay for viral genomes*

CEF expressing various RNAi constructs were challenged with 100 pfu of Md5 strain MDV. DNA was collected at 5 dpi using the DNeasy Blood & Tissue kit and treated with RNase I according to manufacturer's instructions (Qiagen, Inc.). DNA was diluted to a concentration of 20 ng/μl for use in the qPCR assay. pCRBlunt (Invitrogen Corp.) plasmids (gifts from Dr. Robert Silva, ADOL) containing MDV *gB* and chicken *GAPDH* genes were used to construct standard curves. A series of 10-fold dilutions of plasmid DNA in 5 ng/μl sheared salmon sperm DNA (Sigma), from $10^6$ to 10 copies per μl, was used to generate standard curves. Amplifications of *gB* and chicken *GAPDH* sequences were carried out in separate reactions using the following primers and probes: *gB* forward, CGGTGGCTTTTCTAGGTTCG; *gB* reverse, CCAGTGGGTTCAACCGTGA; *gB* probe, FAM-CATTTTCGCGGCGGTTCTAGACGG-TAMRA; *GAPDH* forward, ACAGAGGTGCTGCCCAGAA; *GAPDH* reverse, ACTTTCCCCACAGCCTTAGCA; *GAPDH* probe, VIC-TCATCCCAGCGTCCACT-TAMRA (gifts from Dr. Robert Silva, ADOL). Real-time qPCR assays were performed using an ABI 7900 HT machine (Applied Biosystems). Each reaction contained 0.5 μM of each primer and 0.2 μM of the corresponding probe, 7.5 μl TaqMan® Universal PCR Master Mix (Applied Biosystems) and 3 μl DNA template in a total reaction volume of 15 μl. The cycling parameters consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Viral loads were expressed as the copy number of *gB* divided by that of *GAPDH*. Reactions were done in duplicate. The correlation coefficients ($R^2$ values) of the standard curves were 0.999 (*GAPDH*) and 0.997 (*gB*). PCR amplification efficiency was >0.96.

*Statistical analysis*

The reduction in HVT and MDV titer due to RNAi constructs was analyzed with a simple general linear model in which the RNAi treatment was treated as a fixed variable. The statistical analysis was accomplished with the SAS for Windows v9.1.3 (SAS Institute Inc., 2004). The differences in average viral titers among the RNAi treatments were compared with Duncan's multiple range tests. For convenient visual evaluation, however, the relative average virus titer for each of the RNAi treatments was calculated by dividing each by the average titer of the negative control, mock infection. The standard

errors for the relative average HVT or MDV titers were estimated from the variances and covariances corresponding to the average titer ratios between the RNAi treatments and the negative control (mock) following an approximate procedure as described by van Kempen and van Vliet (2000). The reduction in HVT and MDV plaque sizes by RNAi constructs was also analyzed using SAS with a simple general linear model in which the RNAi treatment was treated as a fixed variable. The differences in average plaque sizes among the RNAi treatments were compared with Tukey's Studentized Range tests.

## Acknowledgments

This work was supported in part by the USDA NRI Competitive Grants Program (2004-35204-14780). We thank Lucy Lee, Robert Silva, Taejoong Kim, Mohammad Heidari and Richard Witter of the USDA-ARS Avian Disease and Oncology Laboratory (East Lansing, MI) for gifts of antibodies, PCR primers and virus stocks and assistance in viral plaque assays.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.03.019.

## References

Afonso, C.L., Tulman, E.R., Lu, Z., Zsak, L., Rock, D.L., Kutish, G.F., 2001. The genome of turkey herpesvirus. J. Virol. 75 (2), 971–978.
Baigent, S.J., Smith, L.P., Currie, R.J., Nair, V.K., 2005. Replication kinetics of Marek's disease vaccine virus in feathers and lymphoid tissues using PCR and virus isolation. J. Gen. Virol. 86 (Pt 11), 2989–2998.
Berkhout, B., Haasnoot, J., 2006. Interplay between virus infection and the cellular RNA interference machinery. FEBS Lett. 580 (12), 2896–2902.
Bhuyan, P.K., Kariko, K., Capodici, J., Lubinski, J., Hook, L.M., Friedman, H.M., Weissman, D., 2004. Short interfering RNA-mediated inhibition of herpes simplex virus type 1 gene expression and function during infection of human keratinocytes. J. Virol. 78 (19), 10276–10281.
Boden, D., Pusch, O., Silbermann, R., Lee, F., Tucker, L., Ramratnam, B., 2004. Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. Nucleic Acids Res. 32 (3), 1154–1158.
Bumstead, N., Sillibourne, J., Rennie, M., Ross, N., Davison, F.J., 1997. Quantification of Marek's disease virus in chicken lymphocytes using the polymerase chain reaction with fluorescence detection. J. Virol. Methods 65 (1), 75–81.
Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B.C., Green, P.J., Jain, N., Isaacs, G., Morgan, R.W., 2006. Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript. J. Virol. 80 (17), 8778–8786.
Calnek, B.W., Witter, R.L., 1997. Marek's disease, In: Calnek, B.W. (Ed.), Diseases of Poultry, 10th ed. Iowa State University Press, Ames, Iowa, pp. 369–413.
Cantello, J.L., Anderson, A.S., Morgan, R.W., 1994. Identification of latency-associated transcripts that map antisense to the ICP4 homolog gene of Marek's disease virus. J. Virol. 68 (10), 6280–6290.
Cantello, J.L., Parcells, M.S., Anderson, A.S., Morgan, R.W., 1997. Marek's disease virus latency-associated transcripts belong to a family of spliced RNAs that are antisense to the ICP4 homolog gene. J. Virol. 71 (2), 1353–1361.
Chang, Y., Chang, S.S., Lee, H.H., Doong, S.L., Takada, K., Tsai, C.H., 2004. Inhibition of the Epstein–Barr virus lytic cycle by Zta-targeted RNA interference. J. Gen. Virol. 85 (Pt. 6), 1371–1379.
Chang, K., Elledge, S.J., Hannon, G.J., 2006. Lessons from nature: microRNA-based shRNA libraries. Nat. Methods 3 (9), 707–714.
Chen, M., Granger, A.J., VanBrocklin, M.W., Payne, W.S., Hunt, H., Zhang, H., Dodgson, J.B., Holmen, S.L., 2007. Inhibition of avian leukosis virus replication by vector-based RNA interference. Virology 365 (2), 464–472.
Chung, K.H., Hart, C.C., Al-Bassam, S., Avery, A., Taylor, J., Patel, P.D., Vojtek, A.B., Turner, D.L., 2006. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. Nucleic Acids Res. 34 (7), e53.
Compel, P., DeLuca, N.A., 2003. Temperature-dependent conformational changes in herpes simplex virus ICP4 that affect transcription activation. J. Virol. 77 (5), 3257–3268.
Cullen, B.R., 2006. Viruses and microRNAs. Nat. Genet. 38, S25–S30 (Suppl.).
Dargan, D.J., Subak-Sharpe, J.H., 1997. The effect of herpes simplex virus type 1 L-particles on virus entry, replication, and the infectivity of naked herpesvirus DNA. Virology 239 (2), 378–388.
Das, R.M., Van Hateren, N.J., Howell, G.R., Farrell, E.R., Bangs, F.K., Porteous, V.C., Manning, E.M., McGrew, M.J., Ohyama, K., Sacco, M.A., Halley, P.A., Sang, H.M., Storey, K.G., Placzek, M., Tickle, C., Nair, V.K., Wilson, S.A., 2006. A robust system for RNA interference in the chicken using a modified microRNA operon. Dev. Biol. 294 (2), 554–563.
DeLuca, N.A., McCarthy, A.M., Schaffer, P.A., 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. J. Virol. 56 (2), 558–570.
Dickins, R.A., Hemann, M.T., Zilfou, J.T., Simpson, D.R., Ibarra, I., Hannon, G.J., Lowe, S.W., 2005. Probing tumor phenotypes using stable and regulated synthetic microRNA precursors. Nat. Genet. 37 (11), 1289–1295.
Dixon, R.A., Schaffer, P.A., 1980. Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein VP175. J. Virol. 36 (1), 189–203.
Dorange, F., Tischer, B.K., Vautherot, J.F., Osterrieder, N., 2002. Characterization of Marek's disease virus serotype 1 (MDV-1) deletion mutants that lack UL46 to UL49 genes: MDV-1 UL49, encoding VP22, is indispensable for virus growth. J. Virol. 76 (4), 1959–1970.
Federspiel, M.J., Hughes, S.H., 1997. Retroviral gene delivery. Methods Cell Biol. 52, 179–214.
Feederle, R., Kost, M., Baumann, M., Janz, A., Drouet, E., Hammerschmidt, W., Delecluse, H.J., 2000. The Epstein–Barr virus lytic program is controlled by the co-operative functions of two transactivators. EMBO J. 19 (12), 3080–3089.
Godfrey, A., Anderson, J., Papanastasiou, A., Takeuchi, Y., Boshoff, C., 2005. Inhibiting primary effusion lymphoma by lentiviral vectors encoding short hairpin RNA. Blood 105 (6), 2510–2518.
Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., Pease, L.R., 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77 (1), 51–59.
Hu, W.Y., Myers, C.P., Kilzer, J.M., Pfaff, S.L., Bushman, F.D., 2002. Inhibition of retroviral pathogenesis by RNA interference. Curr. Biol. 12 (15), 1301–1311.
Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J., Sutrave, P., 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. 61 (10), 3004–3012.
Jia, Q., Sun, R., 2003. Inhibition of gammaherpesvirus replication by RNA interference. J. Virol. 77 (5), 3301–3306.
Kingham, B.F., Zelnik, V., Kopacek, J., Majerciak, V., Ney, E., Schmidt, C.J., 2001. The genome of herpesvirus of turkeys: comparative analysis with Marek's disease viruses. J. Gen. Virol. 82 (Pt. 5), 1123–1135.
Lagos-Quintana, M., Rauhut, R., Lendeckel, W., Tuschl, T., 2001. Identification of novel genes coding for small expressed RNAs. Science 294 (5543), 853–858.
Lee, L.F., Liu, X., Witter, R.L., 1983. Monoclonal antibodies with specificity for three different serotypes of Marek's disease viruses in chickens. J. Immunol. 130 (2), 1003–1006.
Leonard, J.N., Schaffer, D.V., 2006. Antiviral RNAi therapy: emerging approaches for hitting a moving target. Gene Ther. 13 (6), 532–540.
Li, D.S., Pastorek, J., Zelnik, V., Smith, G.D., Ross, L.J., 1994. Identification of novel transcripts complementary to the Marek's disease virus homologue of the ICP4 gene of herpes simplex virus. J. Gen. Virol. 75 (Pt. 7), 1713–1722.
Li, D., O'Sullivan, G., Greenall, L., Smith, G., Jiang, C., Ross, N., 1998. Further characterization of the latency-associated transcription unit of Marek's disease virus. Arch. Virol. 143 (2), 295–311.
Mallanna, S.K., Rasool, T.J., Sahay, B., Aleyas, A.G., Ram, H., Mondal, B., Nautiyal, B., Premraj, A., Sreekumar, E., Yadav, M.P., 2006. Inhibition of Anatid Herpes Virus-1 replication by small interfering RNAs in cell culture system. Virus Res. 115 (2), 192–197.
McGrew, M.J., Sherman, A., Ellard, F.M., Lillico, S.G., Gilhooley, H.J., Kingsman, A.J., Mitrophanous, K.A., Sang, H., 2004. Efficient production of germline transgenic chickens using lentiviral vectors. EMBO Rep. 5 (7), 728–733.
McKie, E.A., Ubukata, E., Hasegawa, S., Zhang, S., Nonoyama, M., Tanaka, A., 1995. The transcripts from the sequences flanking the short component of Marek's disease virus during latent infection form a unique family of 3′-coterminal RNAs. J. Virol. 69 (2), 1310–1314.
Mozdziak, P.E., Pophal, S., Borwornpinyo, S., Petitte, J.N., 2003. Transgenic chickens expressing beta-galactosidase hydrolyze lactose in the intestine. J. Nutr. 133 (10), 3076–3079.
Nair, V., 2005. Evolution of Marek's disease—a paradigm for incessant race between the pathogen and the host. Vet. J. 170 (2), 175–183.
Nakahara, K., Carthew, R.W., 2004. Expanding roles for miRNAs and siRNAs in cell regulation. Curr. Opin. Cell Biol. 16 (2), 127–133.
Niikura, M., Dodgson, J.B., Cheng, H.H., 2006. Direct evidence of host genome acquisition by the alphaherpesvirus Marek's disease virus. Arch. Virol. 151 (7), 537–549.
Ohashi, K., O'Connell, P.H., Schat, K.A., 1994. Characterization of Marek's disease virus BamHI-A-specific cDNA clones obtained from a Marek's disease lymphoblastoid cell line. Virology 199 (2), 275–283.
Osterrieder, N., Kamil, J.P., Schumacher, D., Tischer, B.K., Trapp, S., 2006. Marek's disease virus: from miasma to model. Nat. Rev., Microbiol. 4 (4), 283–294.
Palliser, D., Chowdhury, D., Wang, Q.Y., Lee, S.J., Bronson, R.T., Knipe, D.M., Lieberman, J., 2006. An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. Nature 439 (7072), 89–94.
Pavlova, I.V., Virgin 4th, H.W., Speck, S.H., 2003. Disruption of gammaherpesvirus 68 gene 50 demonstrates that Rta is essential for virus replication. J. Virol. 77 (10), 5731–5739.
Preston, C.M., 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. J. Virol. 29 (1), 275–284.
Root, D.E., Hacohen, N., Hahn, W.C., Lander, E.S., Sabatini, D.M., 2006. Genome-scale loss-of-function screening with a lentiviral RNAi library. Nat. Methods 3 (9), 715–719.
Ross, N., O'Sullivan, G., Coudert, F., 1996. Influence of chicken genotype on protection against Marek's disease by a herpesvirus of turkeys recombinant expressing the glycoprotein B (gB) of Marek's disease virus. Vaccine 14 (3), 187–189.
Salter, D.W., Smith, E.J., Hughes, S.H., Wright, S.E., Crittenden, L.B., 1987. Transgenic chickens: insertion of retroviral genes into the chicken germ line. Virology 157 (1), 236–240.
SAS Institute Inc., 2004. SAS 9.1.3 Help and Documentation. SAS Institute Inc., Cary, NC.

Schaefer-Klein, J., Givol, I., Barsov, E.V., Whitcomb, J.M., VanBrocklin, M., Foster, D.N., Federspiel, M.J., Hughes, S.H., 1998. The EV-O-derived cell line DF-1 supports the efficient replication of avian leukosis–sarcoma viruses and vectors. Virology 248 (2), 305–311.

Schumacher, D., Tischer, B.K., Fuchs, W., Osterrieder, N., 2000. Reconstitution of Marek's disease virus serotype 1 (MDV-1) from DNA cloned as a bacterial artificial chromosome and characterization of a glycoprotein B-negative MDV-1 mutant. J. Virol. 74 (23), 11088–11098.

Schumacher, D., Tischer, B.K., Reddy, S.M., Osterrieder, N., 2001. Glycoproteins E and I of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J. Virol. 75 (23), 11307–11318.

Schumacher, D., Tischer, B.K., Teifke, J.P., Wink, K., Osterrieder, N., 2002. Generation of a permanent cell line that supports efficient growth of Marek's disease virus (MDV) by constitutive expression of MDV glycoprotein E. J. Gen. Virol. 83 (Pt. 8), 1987–1992.

Silva, J.M., Li, M.Z., Chang, K., Ge, W., Golding, M.C., Rickles, R.J., Siolas, D., Hu, G., Paddison, P.J., Schlabach, M.R., Sheth, N., Bradshaw, J., Burchard, J., Kulkarni, A., Cavet, G., Sachidanandam, R., McCombie, W.R., Cleary, M.A., Elledge, S.J., Hannon, G.J., 2005. Second-generation shRNA libraries covering the mouse and human genomes. Nat. Genet. 37 (11), 1281–1288.

Smith, E.J., Fadly, A., Okazaki, W., 1979. An enzyme-linked immunosorbent assay for detecting avian leukosis–sarcoma viruses. Avian Dis. 23 (3), 698–707.

Tang, G., 2005. siRNA and miRNA: an insight into RISCs. Trends Biochem. Sci. 30 (2), 106–114.

Tischer, B.K., Schumacher, D., Messerle, M., Wagner, M., Osterrieder, N., 2002. The products of the UL10 (gM) and the UL49.5 genes of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J. Gen. Virol. 83 (Pt. 5), 997–1003.

Trang, P., Kilani, A., Kim, J., Liu, F., 2000. A ribozyme derived from the catalytic subunit of RNase P from *Escherichia coli* is highly effective in inhibiting replication of herpes simplex virus 1. J. Mol. Biol. 301 (4), 817–826.

Trang, P., Lee, J., Kilani, A.F., Kim, J., Liu, F., 2001. Effective inhibition of herpes simplex virus 1 gene expression and growth by engineered RNase P ribozyme. Nucleic Acids Res. 29 (24), 5071–5078.

Tulman, E.R., Afonso, C.L., Lu, Z., Zsak, L., Rock, D.L., Kutish, G.F., 2000. The genome of a very virulent Marek's disease virus. J. Virol. 74 (17), 7980–7988.

Valencia-Sanchez, M.A., Liu, J., Hannon, G.J., Parker, R., 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev. 20 (5), 515–524.

Vallejo, R.L., Bacon, L.D., Liu, H.C., Witter, R.L., Groenen, M.A., Hillel, J., Cheng, H.H., 1998. Genetic mapping of quantitative trait loci affecting susceptibility to Marek's disease virus induced tumors in F2 intercross chickens. Genetics 148 (1), 349–360.

van Kempen, G.M., van Vliet, L.J., 2000. Mean and variance of ratio estimators used in fluorescence ratio imaging. Cytometry 39 (4), 300–305.

Wiebusch, L., Truss, M., Hagemeier, C., 2004. Inhibition of human cytomegalovirus replication by small interfering RNAs. J. Gen. Virol. 85 (Pt. 1), 179–184.

Witter, R.L., 1997. Increased virulence of Marek's disease virus field isolates. Avian Dis. 41 (1), 149–163.

Xie, Q., Anderson, A.S., Morgan, R.W., 1996. Marek's disease virus (MDV) *ICP4*, *pp38*, and *meq* genes are involved in the maintenance of transformation of MDCC-MSB1 MDV-transformed lymphoblastoid cells. J. Virol. 70 (2), 1125–1131.

Yao, F., Courtney, R.J., 1989. A major transcriptional regulatory protein (ICP4) of herpes simplex virus type 1 is associated with purified virions. J. Virol. 63 (8), 3338–3344.

Yonash, N., Bacon, L.D., Witter, R.L., Cheng, H.H., 1999. High resolution mapping and identification of new quantitative trait loci (QTL) affecting susceptibility to Marek's disease. Anim. Genet. 30 (2), 126–135.

Yoon, J.S., Kim, S.H., Shin, M.C., Hong, S.K., Jung, Y.T., Khang, I.G., Shin, W.S., Kim, C.C., Paik, S.Y., 2004. Inhibition of herpesvirus-6B RNA replication by short interference RNAs. J. Biochem. Mol. Biol. 37 (3), 383–385.

Yoshida, S., Lee, L.F., Yanagida, N., Nazerian, K., 1994. The glycoprotein B genes of Marek's disease virus serotypes 2 and 3: identification and expression by recombinant fowlpox viruses. Virology 200 (2), 484–493.

Yu, J.Y., Taylor, J., DeRuiter, S.L., Vojtek, A.B., Turner, D.L., 2003. Simultaneous inhibition of GSK3alpha and GSK3beta using hairpin siRNA expression vectors. Mol. Ther. 7 (2), 228–236.

Zeng, Y., Wagner, E.J., Cullen, B.R., 2002. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol. Cell 9 (6), 1327–1333.