The identification of virally encoded microRNAs (miRNAs) has had a major impact on the field of herpes virology. Given their ability to target cellular and viral transcripts, and the lack of immune response to small RNAs, miRNAs represent an ideal mechanism of gene regulation during viral latency and persistence. In this review, we discuss the role of miRNAs in virus latency and persistence, specifically focusing on herpesviruses. We cover the current knowledge on miRNAs in establishing and maintaining virus latency and promoting survival of infected cells through targeting of both viral and cellular transcripts, highlighting key publications in the field. We also discuss potential areas of future research and how novel technologies may aid in determining how miRNAs shape virus latency in the context of herpesvirus infections.

**Introduction**

A fundamental aspect of herpesviruses is their ability to establish a life-long latent infection within the host. Latency is characterized by a restricted gene expression profile, the lack of production of infectious virions with the ability to reactivate at later times, generating new infectious virus (Goodrum *et al.*, 2012). This ability makes herpesviruses highly effective pathogens with seropositivity rates approaching 100% in some populations. Although asymptomatic in most individuals, herpesvirus infections are linked to serious human and veterinary diseases, such as cancer, encephalitis, solid organ and bone marrow transplant rejection, and congenital defects following *in utero* infection. There is currently only one effective vaccine, few effective antiviral therapies and no cures. In many cases, pathogenesis is caused by reactivation of latent virus rather than primary infection (Pellet & Roizman, 2013).

The molecular mechanisms involved in establishing, maintaining and reactivating from latency are poorly understood. However, there are three fundamental aspects that must be achieved for establishment of successful latent infection. Following initial infection and acute replication, the virus must restrict its own gene expression profile, thereby limiting the production of new infectious virus. The virus must both monitor and manipulate the host cell environment to create conditions suitable for appropriate reactivation. Finally, the virus must promote the survival of the infected cell by evading the intrinsically, innate and adaptive responses of the host, as death of the cell would result in loss of the latent genome. Achieving effective latency therefore involves a complex interplay between virus and host factors. Current research would suggest that small regulatory RNAs called microRNAs (miRNAs) play a role in all three aspects of virus latency and persistence (Fig. 1).

In this review, we discuss the role of miRNAs in virus latency and persistence, specifically focusing on herpesviruses. We cover the current knowledge on the role of miRNAs in establishing and maintaining virus latency and promoting survival of infected cells through targeting of both viral and cellular transcripts, highlighting key publications in the field. We also discuss potential areas of future research and how novel technologies may aid in determining how miRNAs shape virus latency in the context of herpesvirus infections.

**Herpesviruses and miRNAs**

miRNAs are small ssRNA species ~20–24 bases in length that regulate gene expression through post-transcriptional mechanisms. As part of the RNA-induced silencing complex (RISC), miRNAs bind to fully or partially complementary sequences, causing mRNA degradation and translational inhibition. Expression of miRNAs is ubiquitous amongst metazoan organisms and these small RNAs play important roles in a variety of biological processes, including development, cellular differentiation, proliferation and apoptosis (Bartel, 2009).

Currently, >300 viral miRNAs have been identified, encoded by multiple virus families, including adenoviruses, polyomaviruses and retroviruses (Kincaid *et al.*, 2013; Kozomara & Griffiths-Jones, 2014). However, the majority of viral miRNAs are expressed by herpesviruses. Herpesviruses comprise a large family of enveloped dsDNA viruses that have coevolved with their hosts since before the mammalian radiation. They are split into three subfamilies,
alpha, beta and gamma, based on sequence homology, with nine known human herpesviruses (HHVs), including herpes simplex virus 1 and 2 (HSV-1 and -2/HHV-1 and -2), varicella zoster virus (HHV-3), Epstein–Barr virus (EBV/HHV-4), human cytomegalovirus (HCMV/HHV-5), HHV-6(A and B) and -7, and Kaposi’s sarcoma virus (KSHV/HHV-8). A number of model viruses and viruses of veterinary importance such as murine gamma herpesvirus 68 (MHV-68), mouse, rat and rhesus CMV, and Marek’s disease virus (MDV) have also been studied in depth (Pellet & Roizman, 2013).

The suggestion that miRNAs play a role in virus latency and persistence is led by a number of observations. First, expression of miRNAs is specifically associated with viruses that establish persistent and latent infections, most likely due to fundamental characteristics of miRNA function (Kozomara & Griffiths-Jones, 2014). Unlike proteins where functional effects can be rapid and robust, effects on protein expression from miRNAs are thought to be modest and require time for protein levels to be reduced. Rapid acute viruses would most likely kill cells before virally encoded miRNAs could have any significant effect. In contrast, the modest effects of miRNAs may be ideal in the environment of latent infections, where cells harbour virus for long periods and subtle effects on gene regulation may be effective. Furthermore, miRNAs are genetically economic and non-immunogenic – two attractive features for persistent viruses.

Second, viral miRNAs form part of the restricted-latency-associated gene expression profile of a number of herpesviruses (Cai et al., 2005; Cosmopoulos et al., 2009; Umbach et al., 2009, 2010). In addition, miRNA genes encoded by alpha and gamma herpesviruses are clustered within regions of the genome associated with latent gene expression (Grundhoff & Sullivan, 2011). A caveat to this pattern are the miRNAs of the beta herpesvirus family which are spread throughout the genome and have not yet been associated with a clear latency gene expression profile (Hook et al., 2014a), although expression has been detected in persistently infected THP-1 cells (Fu et al., 2014; Shen et al., 2014). Whether this suggests that latency or persistence mechanisms of beta herpesviruses differ significantly from alpha and gamma herpesviruses or simply reflect a lesser understanding of beta herpesvirus latency is currently unclear. However, the association of viral miRNAs with latent gene expression led to speculation that they may be involved in viral latency. Current research supports this early supposition and also implicates cellular miRNAs as regulators of virus latency and persistence.

**Restricting the acute replication programme: viral miRNAs targeting virus transcripts**

A crucial aspect of virus latency is the ability to maintain a homeostatic balance, whereby virus replication and gene expression are restricted, but the potential to reactivate in response to the correct combination of external signals is maintained. Failure to restrict acute replication during establishment and maintenance of latency, or precocious reactivation, could lead to immune clearance or unwanted cytotoxicity; lack of sensitivity to external signals would
reduce the virus’s ability to reactivate and undermine the ability of the virus to spread to new hosts.

A common theme amongst herpesviruses is the expression of major trans-activating proteins that promote virus replication by stimulating acute gene expression (Goodrum et al., 2012). These trans-activating proteins are considered pivotal in regulating the switch between acute and latent infection, and may represent final effectors for both viral and cellular signalling factors that modulate the homeostatic balance of acute/latent infection. miRNAs have the capacity to regulate multiple transcripts, and in the context of virus infection this can include both viral and cellular targets. Expression of miRNAs provides viruses with a potential mechanism to post transcriptionally regulate their own genes during both the acute and latent phases of infection.

Targeting of immediate-early trans-activators by viral miRNAs was first demonstrated in HCMV. Using a comparative bio-informatic strategy, target sites for the HCMV miRNA UL112-1 were identified in the 3’ UTR of the crucial viral trans-activating gene IE72 (Grey et al., 2007). Expression of miR-UL112-1 resulted in decreased expression of IE72 and transfection of cells with a miR-UL112-1 mimic restricted acute replication of the virus. Given the role of IE72 in driving acute replication of HCMV, this study suggests that miR-UL112-1 may play a role in restricting acute gene expression during establishment and maintenance of latency, although this is yet to be shown. A second group independently confirmed this finding, demonstrating that deletion of UL112-1 from the viruses resulted in increased IE72 expression (Murphy et al., 2008).

Further evidence to support the model of latency regulation by viral miRNAs came from an elegant study on KSHV (Bellare & Ganem, 2009). Initial screens demonstrated that KSHV miRNA miR-K12-9* targets the immediate-early trans-activator RTA (replication and transcription activator protein), analogously to the regulation of IE72 by miR-UL112-1. Using a recombinant double-tagged virus in which GFP is expressed during latent infection and red fluorescent protein is expressed in response to reactivation, the authors demonstrated that inhibition of miR-K12-9* resulted in a two- to threefold increase in virus reactivation, confirming the role of viral miRNA targeting of acute trans-activators in the context of virus latency.

Further studies have demonstrated targeting of virus trans-activating genes by virus-encoded miRNAs in each of the herpesvirus subfamilies, indicating this is an evolutionarily conserved mechanism for regulating virus latency (Jung et al., 2014; Lin et al., 2011; Lu et al., 2010b; Riaz et al., 2014; Tang et al., 2008, 2009; Umbach et al., 2008). To what extent this mechanism participates in controlling viral latency remains to be determined. As highlighted by Bellare & Ganem (2009), a two- to threefold increase in reactivation rates is modest compared with the levels of induction by protein trans-activators such as RTA, suggesting that miRNA regulation acts as a ‘safety mechanism’ protecting against promiscuous reactivation events (Bellare & Ganem, 2009). Modest regulation may also raise the threshold for protein expression from target transcripts in response to reactivation signals. This would allow for a regulatory programme that was sensitive to reactivation signals, whilst avoiding promiscuous reactivation from stochastic variations in basal transcript levels (Fig. 2). Introduction of such robustness to the regulatory programme has also been suggested for cellular miRNA function (Ebert & Sharp, 2012).

Restricting the acute replication programme: virus miRNAs targeting cellular transcripts

Whilst restricting acute replication through regulation of virus gene expression is important, regulation of cellular gene expression is equally vital for successful establishment of virus latency. During establishment and maintenance of latency, virally expressed miRNAs can target cellular transcripts and pathways that promote acute replication and virus gene expression. In addition, the virus can manipulate cellular transcription patterns to generate an environment more conducive to reactivation, allowing efficient response to the correct external triggers. The subtle nature of latency combined with the ability of miRNAs to target multiple genes whilst avoiding potential immune responses make miRNAs ideal for this task. Current studies on KSHV provide strong evidence of regulation of latency through targeting of cellular genes by viral miRNA expression.

Although there is general consensus that disrupting KSHV miRNAs interferes with latency maintenance, different studies have identified divergent mechanisms by which this is achieved. Lei et al. (2010) demonstrated that deletion of 10 KSHV miRNAs resulted in increased ORF50 expression, but in contrast to previous studies were unable to detect direct regulation of 3’ UTR ORF50 luciferase reporter constructs. Instead, cells infected with the miRNA deletion mutant displayed lower levels of NFκB signalling than WT-infected cells. As the authors had previously demonstrated a role for NFκB signalling in restricting KSHV acute replication (Ye et al., 2008), regulators of the NFκB pathway were screened for potential targets of KSHV miRNAs. It was found that miR-K12-1 was able to rescue NFκB signalling in cells infected with the knockout virus through targeting the NFκB inhibitor IκBα, resulting in lower ORF50 expression levels.

Another study found that knockdown of Dicer or Argonaute, crucial components of the miRNA pathway, resulted in increased ORF50, suggesting disruption in miRNA function in general promotes virus reactivation (Lu et al., 2010a). Further studies demonstrated that miR-K12-3, 7 and 11 restricted ORF50 expression, and identified NFIB, another inhibitor of the NFκB pathway, as a direct target of miR-K3.

Finally, Lu et al. (2010b) found that deletion of KSHV miRNAs from a recombinant virus also resulted in increased ORF50 expression and, as with Lei et al. (2010), did not find strong evidence of direct targeting of ORF50 reporter constructs. Instead, they observed a loss of methylation of
both the cellular and virus genomes. Bioinformatic prediction identified Rbl2, a regulator of DNA methyltransferases, as a target of miR-K4-5p, and demonstrated that miR-K12-4-5p expression could restore methylation of both the viral and cellular genome, although there was no direct evidence to link this to ORF50 regulation and increased acute replication of KSHV.

Interestingly, Ziegelbauer et al. (2009) suggested that targeting of the cellular factor BCLAF1 by KSHV miRNAs miR-K12-5, 9, 10a and 10b may have the opposite effect of sensitizing cells to reactivation stimulus, indicating that some miRNAs may be involved in stabilizing latency, whilst others manipulate the cellular environment to establish conditions conducive for reactivation.

Although in some cases the results of these studies do not necessarily correlate [Bellare & Ganem (2009) only observed effects on reactivation with miR-K12-9*, whereas others identified effects from divergent and non-overlapping miRNAs], this likely reflects the different approaches taken and the particular model of infection used.

In EBV, BART-miR-18-5p has been shown to suppress reactivation from latently infected cell lines (Qiu & Thorley-Lawson, 2014). Qiu & Thorley-Lawson (2014) demonstrated that, in contrast to lymphoblastoid cell lines (LCLs), miR-18-5p is expressed at high levels in resting memory and germinal B-cells. Using microarray analysis, the authors identified and validated the cellular transcript MAP3K2 as a target of miR-18-5p, and demonstrated that suppressing miR-18-5 resulted in increased viral gene expression and virus shedding, whereas ectopic overexpression of miR-18-5p resulted in the opposite effect. These studies suggest that viral miRNAs may contribute to the regulation of virus latency and reactivation through multiple mechanisms, targeting both cellular and viral transcripts.

Restricting the acute replication programme: role of cellular miRNAs

Following infection of a cell, viruses must contend with numerous host miRNAs, some of which are expressed at high levels and have the potential to target virus transcripts directly. Although it has been suggested that cellular miRNAs directly target viruses as an antiviral mechanism, this is unlikely due to the ability of viruses to rapidly escape through mutation (Bogerd et al., 2014). However, cellular miRNAs may still have antiviral roles in more indirect ways, and studies have confirmed that viruses have evolved specific defences against such antiviral miRNAs (Cazalla et al., 2010; Lee et al., 2013; Libri et al., 2012; Marcinowski et al., 2012). Viruses have also evolved to take advantage of cellular miRNAs and their ability to regulate cellular pathways important for virus latency.

Studies in EBV-infected cells have demonstrated that a cellular miRNA family called miR-200 can induce reactivation through targeting cellular factors zinc finger E-box binding homeobox 1 and 2 (ZEB1 and 2) (Ellis-Connell et al., 2010; Lin et al., 2010). ZEB proteins are normally involved in the transition from epithelial cells to mesenchymal cells, although in EBV-infected cells they have been shown to stabilize latency by negatively regulating the major trans-activating protein Zta (Ellis et al., 2010). As miR-200 miRNAs have been shown to target ZEB1 and 2 their role in EBV latency was investigated. Ellis-Connell et al. (2010) and Lin et al. (2010) both demonstrated a negative correlation of ZEB proteins and miR-200 miRNAs in epithelial and B-cell lines infected with EBV. Ectopic overexpression of miR-429, a miR-200 family member, resulted in downregulation of ZEB with a subsequent increase in Zta expression. Interestingly, activation of B-cells by antibody cross-linking, a potent mechanism for reactivating EBV, resulted in decreased miR-200 expression,
implicating these miRNAs in the signal cascade from external stimulus to viral gene expression and ultimately reactivation (Lin et al., 2010).

The miR-200 family has also been implicated in the control of HCMV latency. As with other herpesviruses, HCMV expresses two major trans-activating proteins involved in driving acute virus replication. As discussed above, viral miRNA UL112-1 targets IE72. However, a recent study identified target sites for miR-200 miRNAs within the 3’ UTR of the second major trans-activating protein IE86 (O’Connor et al., 2014). Expression of miR-200b resulted in downregulation of a luciferase reporter construct containing the 3’ UTR of IE86 and reduced expression of IE86 protein during virus infection. A modest reduction in virus replication following infection of fibroblast cells over-expressing miR-200b was also observed. Although the precise site of HCMV latency has not been fully confirmed, studies suggest CD34+ haematopoietic progenitor cells may represent a potential site of virus persistence (Sinclair & Reeves, 2013). Crucially, mutation of the miR-200b target sites within the 3’ UTR of IE86 resulted in increased levels of IE72 and higher levels of viral DNA detected in the supernatant of HCMV-infected CD34+ cells, suggesting a loss of miR-200b regulation increased acute replication of the virus. Activation of CD34+ cells with 12-O-tetradecanoylphorbol-13-acetate resulted in a decrease in miR-200b levels suggesting, like EBV infection of B-cells, that miR-200 miRNAs may play a role in the signalling cascade that leads to virus reactivation.

Modulation of cellular miRNAs during infection of CD34+ progenitor cells has also been suggested to play a role in maintaining HCMV latency. Using miRNA microarray analysis, Poole et al. (2011) observed a two- to threefold reduction in miR-92a expression following infection of CD34+ cells. Reduction in miR-92a resulted in a corresponding increase in the cellular target, GATA2, and subsequent increases in IL-10 expression. IL-10 expression was suggested to contribute to maintenance of HCMV genome levels in CD34+ cells and protect the cells from apoptosis, supporting the concept that modulation of cellular miRNAs can promote a cellular environment conducive to HCMV latency.

Perhaps the most striking example of how herpesviruses have evolved to regulate latency through the activity of cellular miRNAs comes from a recent study on HSV-1. A key characteristic of herpesviruses is their ability to trigger acute replication in one cell type whilst establishing latency in another. For example, infection of skin epithelial cells with HSV results in robust virus replication, whereas infection of neuronal cells ultimately results in restricted virus gene expression and latency. The cellular factors that contribute to restriction of virus replication that allow establishment of latency in neuronal cells are not well understood. A number of cellular miRNAs are expressed in a tissue-specific manner, especially within neuronal cells.

Pan et al. (2014) identified two potential target sites for the neuronal-specific miR-138 miRNA within the 3’ UTR of the HSV trans-activator ICP0 (Pan et al., 2014). ICP0 is the functional equivalent of RTA of EBV and KSHV, and IE72 of HCMV. Transfection of a miR-138 mimic reduced levels of ICP0 protein from an expression construct and binding of miR-138 to the two target sites within the ICP0 3’ UTR was confirmed by PAR-CLIP (photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation) analysis on HSV-infected 293 cells overexpressing miR-138. Mutation of the identified target sites in a recombinant HSV-1 virus resulted in increased expression of acute-associated genes following infection of a human neuronal cell line, although replication of the virus was unaffected.

Unlike other HHVs, HSV does not adhere to strict species specificity, allowing in vivo studies to be performed in mice. Infection of the cornea leads to acute replication in the eye followed by replication in the enervating transgenic ganglia before acute virus clearance and establishment of latency within the neuronal cells. Mutation of the miR-138 target sites resulted in increased expression of acute-associated gene expression in the eye and the ganglia, although this did not translate to increased virus levels. Crucially, infection with the mutant virus resulted in fourfold higher levels of morbidity and mortality than in mice infected with WT virus, indicating the loss of miR-138 regulation of ICP0 caused a significant phenotype. Although technically difficult, levels of acute virus gene expression were measured by quantitative real-time PCR and suggest that latent stability may be reduced by the loss of miR-138 regulation.

**Viral miRNAs promote the survival and proliferation of latently infected cells**

Herpesviruses have evolved diverse strategies that allow long-term maintenance of their genomes without requiring acute replication or generation of new virions. HSV achieves this by infecting neuronal cells that do not divide and are thought to persist for the lifetime of the host (Roizman & Whitley, 2013). In contrast, gamma herpesviruses infect lymphatic and epithelial cells with finite lifespans that have the capacity to divide. Without mechanisms that promote cell survival, proliferation and the transfer of virus genomes to newly divided cells, latency would fail and the infection would be cleared. In addition to suppressing acute replication, viral miRNAs have been shown to play a role in promoting the survival and proliferation of latently infected cells.

*In vitro* infection of naive B-cells by EBV results in transformation and proliferation, whilst expression of the viral EBNA1 protein maintains the viral genome by tethering to the cellular chromosome. This ensures a significant population of latently infected cells and contributes to the oncogenic nature of the virus (Frappier, 2012). Recent studies have defined the role of virus-encoded miRNAs in the transformation of B-cells. Using bacterial artificial chromosome (BAC) technology, two groups generated recombinant EBV viruses that lacked expression of BHRF1...
miRNAs (Feederle et al., 2011; Seto et al., 2010). Both control and knockout viruses also lacked the majority of BART miRNAs due to a large deletion in the B98.5 BAC strain used. Although these mutant viruses were able to generate LCLs following infection of naive B-cells, initial activation and outgrowth of LCLs was reduced significantly. The reduced efficiency of LCL outgrowth was attributed to a reduction in the active cell cycle and an increase in levels of apoptosis. BART miRNAs expressed by EBV have also been demonstrated to play a role in lymphomagenesis. EBV genomes can be cleared from Burkitt’s lymphoma cells using a dominant-negative EBNA1 construct. The loss of the EBV genome resulted in increased death of cells due to apoptosis. The negative impact of genome loss on Burkitt’s lymphoma growth was partially rescued by ectopic expression of BART miRNAs (Vereide et al., 2014). This rescue was due in part to protection against apoptosis potentially through targeting of caspase-3 by two of the EBV miRNAs, BART16 and BART 1-3p. Previous studies have identified cellular targets of BART miRNAs that block or inhibit apoptosis (Choy et al., 2008).

The ability of EBV miRNAs to promote virus infection was further demonstrated in vivo using a humanized mouse model. Infection of immune-deficient mice reconstituted with human foetal stem cells resulted in systemic infection and detection of EBV genomes by quantitative PCR in the peripheral blood of mice (Wahl et al., 2013). Deletion of BHRF1-1, 2 and 3 miRNAs resulted in ~2 week delay in the detection of viral genomes, indicating deletion of miRNAs resulted in a significant growth defect. However, deletion of the miRNAs had no effect on the generation of tumours, questioning the de novo oncogenic nature of the viral miRNAs.

Studies investigating the oncogenesis of KSHV have been limited by the lack of tractable models of transformation in human cells. Recently, a model using rat mesenchymal cells has been developed which demonstrates effective transformation following infection with KSHV (Jones et al., 2013). Deletion of KSHV miRNAs resulted in a loss of focal cell formation and increased contact growth inhibition following infection of rat mesenchymal cells, suggesting a role for KSHV miRNAs in promoting cellular transformation (Moody et al., 2013). The transforming ability of the mutant virus was rescued by complementation with a construct containing the deleted region from the virus or through ectopic expression of individual KSHV miRNAs, miR-K12-1, 4 and 11. Similar to miRNA knockout of EBV miRNAs, loss of KSHV miRNAs resulted in slower growth of transformed cells and higher levels of apoptosis. The authors previously demonstrated that lxBz was a cellular target of miR-K12-1 and demonstrated that the miRNA knockout phenotype could be rescued by small interfering RNA knockdown of lxBz alone, suggesting this gene plays a central role in the phenotypic effects of KSHV miRNAs (Lei et al., 2010). Indeed, expression of an lxBz construct lacking its endogenous 3’UTR in WT-infected cells resulted in increased apoptosis and reduced cell cycle. Perhaps the most striking aspect of this study is the ability of KSHV miRNAs to function in rat cells. Given the general lack of homology amongst herpesvirus miRNAs, it would be expected that miRNAs from a human virus would fail to function in cells of another species. The authors confirmed that the identified miR-K1 target site in human lxBz is conserved and functional in rat lxBz. The fact that multiple KSHV miRNAs could rescue the deletion phenotype individually indicates that conserved sites exist for these miRNAs as well, begging the question as to why viral miRNAs do not demonstrate higher levels of sequence conservation.

The ideal model for investigating viral miRNAs is within the natural host. For obvious reasons this is not possible for human viruses; however, models using evolutionarily-related viruses in their natural hosts exist. One such model is MHV-68. MHV-68 was isolated from a free-living rodent population and provides a small-animal model for gamma herpesvirus studies (Blaskovic et al., 1980; Sunil-Chandra et al., 1992). MHV-68 also shares many of the pathological features of human gamma herpesviruses, such as lymphotropism and the ability to induce lymphoproliferative disease (Sunil-Chandra et al., 1994). As with other gamma herpesviruses, MHV-68 expresses multiple miRNAs clustered within a genomic region associated with latent gene expression (Péiffer et al., 2005; Zhu et al., 2010).

In a recent study, all 14 MHV-68 miRNAs were mutated to either abolish or reduce their expression (Feldman et al., 2014). Whilst deletion of the miRNAs had no effect on the ability of the virus to replicate in tissue culture or during acute replication in the lungs of mice, deletion of the miRNAs did cause a modest phenotype in the context of latent infection. Using limiting dilution and nested quantitative PCR, the authors demonstrated a 2.4-fold reduction in latent genome levels. Ex vivo culture of splenocytes also demonstrated a reduced ability to reactivate, although this phenotype was only observed at a lower infectious dose (100 p.f.u.) with no discernible difference observed at a higher infectious dose.

Interestingly, similarly to EBV and KSHV, deletion of the viral miRNAs may have affected the normal progression of B-cell infection. Infection of naive B-cells with MHV-68 results in initial proliferation followed by establishment of latently infected memory B-cells. A reporter gene fused to the viral latency protein ORF73 allows monitoring of latently infected B-cells following infection of mice. At16 days post-infection, splenocytes were harvested, stained for virus infection, and sorted into naive, germinal and memory B-cell populations. Deletion of the miRNAs resulted in a modest twofold reduction in the MHV-68 positive memory B-cell population. Crucially, deletion of the miRNAs also resulted in an overall reduction in virus fitness as determined by a loss of morbidity following infection of IFN-γ knockout mice when compared with WT virus, confirming the importance of the viral miRNAs to overall pathogenesis.
Inducing proliferation of infected cells through the miR-155 cellular pathway

Although virus-encoded miRNAs display little in the way of evolutionary conservation, a number of viral miRNAs do share sequence homology with cellular miRNAs, suggesting they act as functional orthologues by targeting the same cellular transcripts. The most prominently studied is the KSHV-encoded miRNA miR-K12-11, which shares sequence homology with the cellular miRNA, miR-155. miR-155 plays a central role in the regulation of lymphocyte activation and development during normal physiological conditions, whereas overexpression is associated with a number of cancers, especially those of lymphoid origin (Eis et al., 2005; Rodriguez et al., 2007). Overexpression of miR-155 in transgenic mice also results in abnormal proliferation of B-cells (Costinean et al., 2006). Given the sequence similarity, it was suggested that miR-K12-11 aids KSHV-induced proliferation of infected cells through targeting miR-155-regulated cellular pathways. Initial studies confirmed miR-K12-11 and miR-155 regulate a common set of cellular genes (Gottwein et al., 2007; Skalsky et al., 2007), with a series of elegant in vivo experiments demonstrating that miR-K12-11 expression resulted in B-cell transformation in humanized mice models and that miR-K12-11 could rescue miR-155 knockout mice (Boss et al., 2011; Sin et al., 2013). These data suggest miR-K12-11 plays a central role in the ability of KSHV to induce proliferation of infected cells, ensuring the effective establishment of long-term viral latency within the host. The lack of effective in vivo models for KSHV infection makes confirmation of this theory difficult.

However, the existence of a miR-155 orthologue in a chicken herpesvirus has allowed such studies to be performed in the context of a natural host. MDV is a lymphotropic alpha herpesvirus that induces aggressive tumour formation in infected birds. Deletion of the miR-155 orthologue, miR-M4, from MDV resulted in significant reduction of tumour formation following infection of susceptible chickens (Zhao et al., 2011). Tumour formation could also be rescued by inserting a miR-155 expression cassette into the miR-M4 deletion virus, although this was delayed significantly, suggesting miR-155 could not fully rescue the function of miR-M4. A recent publication identified targets of miRNAs in cells co-infected with MDV-1 and 2. Comparative analysis identified nine transcripts that were targeted by miR-155, miR-M4 and miR-K11. However, further analysis is required to determine whether these transcripts play a critical role in the function of miR-155 and its viral orthologues (Parnas et al., 2014).

Although EBV does not express a miR-155 orthologue, it has been found to induce expression of cellular miR-155 following infection, at least in part through expression of the latency-associated gene LMP1 (Lu et al., 2008; Yin et al., 2008). Similarly to KSHV and MDV, induction of cellular miR-155 is required for the initial proliferation of infected B-cells, and inhibition of miR-155 results in reduced cell cycle and increased apoptosis (Linnstaedt et al., 2010). How miR-155 generates a strong survival signal is not fully understood, although it has been suggested that targeting of the bone morphogenic signalling pathway could block reactivation of EBV, thereby stabilizing latency (Yin et al., 2010). However, other studies have found inhibiting miR-155 reduces EBV genome copy number, arguing against this model (Lu et al., 2008).

Immune evasion

Despite limiting their exposure to immune recognition through a variety of mechanisms, including restricting expression of antigenic virus proteins, herpesviruses face an ongoing and robust immune response from the host. Initial host responses included innate mechanisms such as IFN, apoptosis and NK cells, whilst the adaptive arm of the immune response includes antibodies and cytotoxic T-cells. Herpesviruses have developed many strategies to subvert and avoid these host mechanisms, allowing persistent infection to be maintained (Feng et al., 2013; Noriega et al., 2012). Studies have implicated viral miRNAs as potential immune evasion effectors, particularly in beta and gamma herpesviruses.

In 2007, Stern-Ginossar et al. identified the MHC class I-related chain B (MICB) as a target for the HCMV miRNA UL112-1 using a bioinformatics approach. MICB is a stress-induced ligand that is targeted by NK cells through the NKG2D ligand. The authors demonstrated that miR-UL112-1 was able to inhibit expression of MICB and reduce the cytotoxic effects of NK cells. Further studies demonstrated that MICB is targeted by miRNAs expressed by EBV and KSHV, suggesting this may be a common mechanism of herpesvirus immune evasion (Nachmani et al., 2009). This is the only current example of viral miRNAs targeting cell-mediated immune responses and as yet there are no convincing data to support interference with adaptive cellular immune responses by viral miRNAs. A report has suggested the HCMV miRNA miR-US4-1 targets ERAP1 to restrict viral peptide loading on MHC class I (Kim et al., 2011). However, deep-sequencing data suggest the miR-US4-1 mimic sequence was incorrect, casting doubt on the validity of the study (Stark et al., 2012).

In addition to targeting receptors for cell-mediated immune responses, HCMV miRNAs also inhibit secretion of pro-inflammatory cytokines, which in turn may reduce immune cell recruitment to sites of infection. Through RISC immunoprecipitation and bioinformatic analysis, Hook et al. (2014b) identified a number of cellular targets of HCMV miRNAs involved in membrane organization. Deletion of miR-UL112-1, US5-1 and US5-2 resulted in a defect in membrane reorganization following infection, causing a failure in formation of the virus assembly compartment and increased secretion of inflammatory cytokines TNF-α and IL-6. miR-UL148D-1 has also been shown to target the chemokine RANTES (regulated on activation, normal T-cell expressed and secreted) (Kim et al., 2012; Xia et al., 2008).
Studies have demonstrated KSHV miRNAs also target genes that restrict inflammatory cytokines, suggesting this may represent a functional theme of viral miRNAs. TWEAKR (TNF-like weak inducer of apoptosis receptor) was identified as a target of KSHV miRNA miR-K12-10a following genome-wide microarray screens (Abend et al., 2010). Targeting of TWEAKR by miR-K12-10a resulted in reduced expression of the pro-inflammatory cytokines IL-8 and MCP-1. Using a similar strategy, Abend et al. (2012) also demonstrated that miR-K12-9 reduced NFκB signaling through combined targeting of IRAK1 and MyD88, resulting in reduced expression of inflammatory cytokines IL-6 and IL-8.

Conclusions and future studies

Although we are only just beginning to understand the diverse role played by miRNAs in the context of virus infections, studies suggest two overarching themes during herpesvirus latency. First, both viral and cellular miRNAs are involved in restricting the acute replication of herpesviruses to aid establishment and maintenance of latency, and could potentially provide robustness during reactivation in response to triggering signals (Fig. 1, Table 1). Second, cellular and viral miRNAs contribute to overall cell survival signals generated by herpesviruses during the establishment and maintenance of latency.

Studies have identified virus-encoded miRNAs that target crucial trans-activator proteins, thought to be pivotal in controlling latency establishment and reactivation, in all three herpesvirus subfamilies (Bellare & Ganem, 2009; Grey et al., 2007; Jung et al., 2014; Lin et al., 2011; Lu et al., 2010b; Tang et al., 2008, 2009; Umbach et al., 2008). This would suggest an evolutionarily conserved mechanism of latency control, and cell culture experiments support a functional role of viral miRNAs in maintaining latency, albeit at subtle levels.

As yet, there is little in vivo data to support the role of viral miRNAs in directly regulating reactivation from latency, but this may reflect the technical difficulties in demonstrating such a scenario. Studies using HSV miRNA knockout viruses failed to detect establishment and reactivation phenotypes associated with loss of regulation of acute trans-activators in mouse or guinea pig (Kramer et al., 2011; Tang et al., 2011). However, associated phenotypes may be context dependent and require sensitive assays, and although HSV infects mice, spontaneous reactivation does not occur and methods for accurately quantifying spontaneous reactivation in vivo are lacking. Genome-wide screens of viral miRNA targets also failed to identify significant targeting of virus genes, suggesting cellular transcripts are the predominant targets of viral miRNAs (Gottwein et al., 2011; Haecker et al., 2012; Riley et al., 2012; Skalsky et al., 2012). Again, such studies were performed in in vitro models of latency, which may not accurately reflect the context in which viral miRNAs control reactivation. Further studies are therefore required to demonstrate convincingly the potential for viral miRNA regulation of latency through direct targeting of trans-activators.

In vivo evidence for direct targeting of virus trans-activators by cellular miRNAs is more convincing. In contrast to viral miRNAs, cellular miRNAs tend to be conserved, allowing for in vivo studies of human viruses in animal models. Targeting of HSV ICP0 transcript by miR-138 displayed a clear in vivo phenotype when the target site was disrupted, demonstrating an evolutionary strategy for using cellular miRNAs to restrict acute replication in a cell-specific manner (Pan et al., 2014). Another study suggests this occurs in HCMV indicating this phenomenon may be common amongst herpesviruses (O’Connor et al., 2014).

Multiple studies have demonstrated reduced growth of infected cells, and an increase in apoptosis due to disruption of viral and cellular miRNAs following initial infection (Feederle et al., 2011; Linstraet al., 2010; Moody et al., 2013; Pfeffer et al., 2005; Seto et al., 2010; Vereide et al., 2014; Zhu et al., 2010). Although viral and cellular miRNAs can directly block apoptosis, the increase in apoptosis in these studies may reflect an end point due to the loss of miRNA regulation of multiple other cellular signal pathways that eventually result in apoptosis (Abend et al., 2012; Choy et al., 2008; Suffert et al., 2011). Characterizing these pathways will be an important part of future studies defining the role of viral and cellular miRNAs in shaping herpesvirus latency.

The development of new strategies and technologies for both the investigation of herpesvirus latency and for the identification of miRNA targets will be paramount to achieving this goal. HSV represents an attractive model for studying miRNAs with the availability of both acute and latent cell culture models as well as small-animal models. However, it could be argued that cellular targets of HSV miRNAs may not necessarily be conserved – a problem for both in vivo model and in vitro models, which rely on rat neuronal cultures. Yet, the recent KSHV study would suggest that at least some cellular targets are well conserved and the role of cellular miRNAs in infection has clearly been demonstrated (Moody et al., 2013).

Model systems for CMV latency are only now being developed and there is no naturally occurring small-animal model for HCMV. It is probably fair to say that latency is least well understood for beta herpesviruses. However, latency models using CD34+ stem cells and derived cell lines are becoming more prominent (Goodrum et al., 2012). Interestingly, a recent study identified a short variant transcript encoding only exon 4 of IE72 in an in vitro CD34+ latency model (Tarrant-Elorza et al., 2014). This protein was shown to tether the virus genome to the cellular chromatin, enabling genome maintenance during cell division. The 3′ UTR of this transcript has not been mapped, so it is currently unclear whether this latency transcript would be a potential target of miR-UL112-1. In addition to cell culture systems, there are multiple animal viruses related to HCMV with well-studied and tractable models, such as
rhesus, rat and mouse CMV (Buck et al., 2007; Hancock et al., 2012; Meyer et al., 2011). HCMV and chimpanzee CMV represent one of the few examples of conserved herpesvirus miRNAs (Grey et al., 2005). Together this provides a wealth of models for evolutionary studies that may not be available to the same extent with alpha and gamma herpesviruses.

Humanized mouse models also exist for HCMV infection and it will be interesting to see if these will play a role in future studies on HCMV miRNAs (Smith et al., 2010).

Finally, gamma herpesviruses represent the best studied in the context of miRNAs. The existence of multiple latent cell lines for both EBV and KSHV have proved a valuable resource, whilst the recent generation of infectious BACs has greatly improved the scope of possible studies, reflected by the numerous studies of knockout viruses with both EBV and KSHV (Abend et al., 2012; Choy et al., 2008; Suffert et al., 2011). Studies on MDV and the recent study on MHV-68 demonstrate that animal models of gamma herpesviruses will also contribute valuable data on the role of miRNAs in latency, and the use of humanized mice has also proved effective in evaluating the function of human herpesvirus miRNAs in the context of in vivo environments (Feldman et al., 2014; Zhao et al., 2011).

Defining the targets of miRNAs is crucial for both understanding observed phenotypes related to miRNAs and also providing clues to their functional role. Although bioinformatic searches provided an initial strategy for target identification of viral miRNAs, genome-wide biochemical screens

Table 1. Viral and cellular miRNAs with potential roles in latency

<table>
<thead>
<tr>
<th>Role in latency</th>
<th>Virus or host</th>
<th>Virus miRNAs</th>
<th>miRNA</th>
<th>Targets</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Establishment and reactivation</td>
<td>Viral miRNAs</td>
<td>HSV-1</td>
<td>miR-H2-3p and 6</td>
<td>ICP0 and ICP4</td>
<td>Umbach et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HSV-2</td>
<td>miR-H2, 3 and 4</td>
<td>ICP0 and ICP34.5</td>
<td>Tang et al. (2009, 2011)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HCMV</td>
<td>miR-UL112-1</td>
<td>IE72</td>
<td>Grey et al. (2007), Murphy et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EBV</td>
<td>miR-BART-20-5p</td>
<td>BZLF1 and BRLF1</td>
<td>Jung et al. (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>miR-BART-18-5p</td>
<td>MAP3K2</td>
<td>Qiu &amp; Thorley-Lawson (2014)</td>
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<tr>
<td></td>
<td></td>
<td>KSHV</td>
<td>miR-K12-7-5p</td>
<td>RTA</td>
<td>Lin et al. (2011)</td>
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<tr>
<td></td>
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<td></td>
<td>miR-K12-9*</td>
<td>RTA</td>
<td>Bellare &amp; Ganem (2009)</td>
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<td></td>
<td></td>
<td></td>
<td>miR-K12-1</td>
<td>IxBz</td>
<td>Lei et al. (2010)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>miR-K12-3, 7 and 11</td>
<td>NFIB</td>
<td>Lu et al. (2010a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>miR-K12-4-5p</td>
<td>Rb12</td>
<td>Lu et al. (2010b)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>miR-K12-5, 9, 10a and 10b</td>
<td>BCLAF1</td>
<td>Ziegelbauer et al. (2009)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ovhv2-miR-5</td>
<td>ORF50</td>
<td>Riaz et al. (2014)</td>
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<tr>
<td></td>
<td></td>
<td>Ovine herpesvirus-2</td>
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<tr>
<td>Cellular miRNAs</td>
<td>HSV</td>
<td>miR-138</td>
<td>ICP0</td>
<td>Pan et al. (2014)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HCMV</td>
<td>miR-200b</td>
<td>IE86</td>
<td>O’Connor et al. (2014)</td>
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<td></td>
<td></td>
<td>miR-92a</td>
<td>GATA2</td>
<td>Poole et al. (2011)</td>
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<tr>
<td></td>
<td>EBV</td>
<td>miR-429</td>
<td>ZEB1 and 2</td>
<td>Ellis-Connell et al. (2010), Lin et al. (2010)</td>
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</tr>
<tr>
<td>Survival and maintenance</td>
<td>Viral miRNAs</td>
<td>EBV</td>
<td>miR-BHRF1, 2 and 3</td>
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<td>Feederle et al. (2011), Seto et al. (2010), Wahl et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>miR-BARTs</td>
<td>Multiple</td>
<td>Vereide et al. (2014)</td>
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<tr>
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<td></td>
<td></td>
<td>miR-BART5</td>
<td>PUMA</td>
<td>Choy et al. (2008)</td>
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<tr>
<td></td>
<td></td>
<td>KSHV</td>
<td>miR-K12-1, 4 and 11</td>
<td>IxBz</td>
<td>Moody et al. (2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>miR-K12-11 (miR-155 orthologue)</td>
<td>Multiple</td>
<td>Boss et al. (2011), Gottwein et al. (2007), Sin et al. (2013), Skalsky et al. (2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MHV-68</td>
<td>Multiple</td>
<td>Unknown</td>
<td>Feldman et al. (2014)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDV</td>
<td>miR-M4</td>
<td>Multiple</td>
<td>Zhao et al. (2011)</td>
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<td></td>
<td></td>
<td></td>
<td>miR-155</td>
<td>Multiple</td>
<td>Linnstaedt et al. (2010), Lu et al. (2008), Yin et al. (2008, 2010)</td>
</tr>
<tr>
<td>Immune evasion</td>
<td>Viral miRNAs</td>
<td>HCMV</td>
<td>miR-UL112-1</td>
<td>MICB</td>
<td>Stern-Ginossar et al. (2007)</td>
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<td></td>
<td></td>
<td></td>
<td>miR-UL112-1, US5-1 and 2</td>
<td>Multiple</td>
<td>Hook et al. (2014b)</td>
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<td></td>
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<td>miR-UL148D-1</td>
<td>RANTES</td>
<td>Kim et al. (2012)</td>
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<tr>
<td></td>
<td></td>
<td>EBV</td>
<td>miR-BART2-5p</td>
<td>MICB</td>
<td>Nachmani et al. (2009)</td>
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<td></td>
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<td>KSHV</td>
<td>miR-K12-7</td>
<td>MICB</td>
<td>Nachmani et al. (2009)</td>
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<td></td>
<td></td>
<td></td>
<td>miR-K12-9</td>
<td>IRAK1 and MyD88</td>
<td>Abend et al. (2012)</td>
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</tbody>
</table>
are becoming more powerful approaches for the generation of reliable and accurate target information. Techniques such as RIP-CHIP (RNA-binding protein immunoprecipitation microarray), HTS-CLIP (high-throughput sequencing cross-linking and immunoprecipitation) and PAR-CLIP have all contributed large amounts of data on potential targets of virus and cellular miRNAs in the context of virus infection (Dölken et al., 2010; Gottwein et al., 2011; Haecker et al., 2012; Pavelin et al., 2013; Riley et al., 2012; Skalsky et al., 2012). Proteomics analysis has also proved effective, and as the level of sensitivity increases such approaches are likely to play a larger role in miRNA functional analysis (Gallaher et al., 2013; Lee et al., 2012). CLASH (cross-linking, ligation and sequencing of hybrids) technology, the most recent biochemical screen for miRNA targets, promises to generate the most accurate target information to date, leading the way in the generation of high confidence target datasets which will be invaluable for future studies (Grosswendt et al., 2014; Helwak et al., 2013).

In conclusion, although there is much still to be learned, it is clear that miRNAs play an important role during virus latency. Both viral and cellular miRNAs are important for restricting the acute replication of viruses, whilst promoting proliferation and survival of infected cells. This is achieved through multiple mechanisms, including direct and indirect targeting of crucial viral trans-activators, recognition of cell-specific miRNAs, tapping into cellular miRNA signal pathways through functional orthologues, and manipulation of the cellular environment. Although this review has focused on latent infection, it is clear that viral miRNAs can also have potent effects during acute replication (Hook et al., 2014b). Future areas of research may include studying dynamically regulation of viral miRNAs during latency, and how this impacts on establishment, maintenance and reactivation. Recent studies have also demonstrated that viruses express RNAs that target and degrade cellular miRNAs that have antiviral functions (Cazalla et al., 2010; Lee et al., 2013; Libri et al., 2012; Marcinowski et al., 2012). It will be interesting to define whether viruses also regulate their own miRNAs using the same mechanism or whether other post-transcriptional regulation occurs.

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