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Characterisation and functional analysis of the murine gammaherpesvirus-68-encoded microRNAs

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Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

The University of Edinburgh

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Declaration

I declare that this thesis has been composed by myself and that all the work included in this thesis is my own, unless otherwise stated. No part of this thesis has been submitted for any other degree.

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Abstract

All mammalian cells encode microRNAs (miRNAs), which are small non-coding RNAs (~ 22 nucleotides) that control numerous physiological processes via regulation of gene expression. A number of viruses, in particular herpesviruses, also encode miRNAs. Gammaherpesviruses such as Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV) are associated with lymphoproliferative disorders and some types of cancer in humans. Gammaherpesvirus-encoded miRNAs are predicted to contribute to pathogenesis and virus life cycle by suppressing host and viral target genes. However, the exact functions of these miRNAs during virus infection in the natural host are largely unknown. Strict species specificity has limited research on the human gammaherpesviruses mainly to in vitro studies.

Murine gammaherpesvirus 68 (MHV-68) encodes at least 15 miRNAs and provides a unique tractable small animal model to investigate in vivo gammaherpesvirus pathogenic features that are difficult to assess in humans. Following intranasal infection of lab mice, the virus undergoes primary lytic infection in the lung epithelial cells and then spreads to the spleen establishing latent infection in splenic B lymphocytes, macrophages, and dendritic cells. The peak of the latent viral load occurs in the spleen at 14 dpi and then it decreases over time, but the virus is not completely eliminated and the latent viral genomes remain in the host cells for lifetime and can reactivate to produce infectious virus under certain conditions.

The aims of my project were to: (1) establish and develop quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays for quantification of the MHV-68 miRNAs, (2) determine the miRNAs expression profiles during the two stages of virus infection (lytic and latent infection), (3) investigate the kinetics of the miRNAs expression during latency in vivo, (4) construct an MHV-68 miRNA mutant virus lacking 9 miRNAs (designated MHV-68.ΔmiRNAs), and (5) carry out thorough phenotypic characterisation of this mutant virus in order to determine the possible functions MHV-68 miRNAs in the context of natural host infection.

It was found that the MHV-68 miRNAs expression pattern varied during different stages of infection, suggesting a differential regulation of the expression of these miRNAs depending on the phase of infection. In order to investigate the kinetics of miRNAs expression during latency *in vivo*, BALB/c mice were infected intranasally with MHV-68 virus and spleens were harvested at days 10, 14, 21, and 32 post infection. The levels of miRNAs expression were determined by qRT-PCR in the splenocytes from infected mice. Interestingly, in contrast to the lytic MHV-68 protein coding genes, the expression of the miRNAs increased over time after 21 dpi, suggesting that the MHV-68-encoded miRNAs may play more fundamental roles during later stages of latent infection.

In order to determine the potential roles of the MHV-68 miRNAs in virus pathogenesis, a miRNA mutant virus lacking the expression of 9 miRNAs, named MHV-68.ΔmiRNAs, was constructed. The miRNA mutant virus replicated with the same kinetics as wild type virus *in vitro* and *in vivo* demonstrating that the deleted MHV-68 miRNAs are dispensable for virus lytic replication. To examine the roles of the miRNAs during virus latency, the MHV-68.ΔmiRNAs virus was characterised throughout a 49-day course of infection.

Although the level of *ex vivo* reactivation of the MHV-68.ΔmiRNAs virus was comparable to that of the WT virus during the establishment of latency and as late as 28 dpi, the reactivation of the MHV-68.ΔmiRNAs virus was approximately 18-times higher than that of the WT virus at 49 dpi despite the similar levels of the genomic viral DNA loads at the same time-point. This suggests that the MHV-68 miRNAs suppress virus reactivation and promote maintenance of long-term latency. Moreover, the lytic viral gene expression levels were higher in splenocytes from the MHV-68.ΔmiRNAs-infected mice than the basal expression levels in the splenocytes from WT MHV-68-infected mice, suggesting that the MHV-68 miRNAs may suppress viral lytic gene expression during long-term latency *in vivo* and thus help the virus lay low.

Lay summary

Human and animal cells produce small molecules called microRNAs (miRNAs). These molecules function as rheostats that make fine-scale adjustments to protein levels inside the cells by switching off gene expression. Therefore, they can regulate almost all the biological processes inside our cells. Interestingly, not only cells express miRNAs, but viruses also produce miRNAs to evade the immune system and turn the conditions within the host to their advantage. However, little is known about the exact functions of the viral miRNAs. Here my research comes in. I try to understand the functions of the miRNAs expressed by a virus called mouse gamma-herpesvirus-68 (MHV-68).

Herpesviruses establish latent infection, which means the virus remains dormant for the lifetime of the host and its gene expression is shut off. However, under certain conditions such as stress or immunosuppression the virus can reactivate and its gene expression is turned on to produce infectious virus. During the latent infection, some human herpesviruses can cause some types of cancer such as Burkett's lymphoma and Kaposi's sarcoma. Unfortunately, we cannot infect lab animals with human herpesviruses because they are not susceptible to the infection with these viruses. The mouse herpesvirus provides a tractable animal model for studying pathologic features that are difficult to assess in humans and thus we can apply the lessons we learn from the mouse to humans.

In my research it was found that the expression levels of MHV-68 miRNAs increase during virus latency, implying that these miRNAs may play a role during latent infection. In order to explore this role, a mutant virus lacking the expression of a number of miRNAs was constructed. The characteristics of this mutant virus were examined in comparison with the intact wild-type virus in order to gain insight into the potential miRNAs functions. During long-term latency, it was found that the levels of viral gene expression and the levels of virus reactivation are higher in the mice infected with the miRNAs mutant virus than in those infected with the wild-type virus. This suggests that the MHV-68 miRNAs inhibit precocious reactivation so that it can maintain the viral latency and help the virus lay low, thus the virus can remain under the radar of the host immune surveillance. These results pave the way for further research that can unravel more about viral miRNAs functions and open doors for establishing novel therapeutic strategies based on targeting these miRNAs.

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Abbreviations

| | |
|-------------------|--|
| 4'-S-EtdU | 2'-deoxy-5-ethyl-beta-4'-thiouridine |
| Ago | Argonaute |
| AIDS | Acquired immunodeficiency syndrome |
| BAC | Bacterial artificial chromosome |
| BART | Bam HI A region rightward transcript |
| BCR | B cell receptor |
| BHK | Baby hamster kidney |
| BHRF1 | Bam HI fragment H rightward open reading frame 1 |
| bp | Base pair |
| BSA | Bovine serum albumin |
| <i>C. elegans</i> | Caenorhabditis elegans |
| CD | Cluster of differentiation |
| cDNA | complementary DNA |
| DNA | Deoxyribonucleic acid |
| DNA pol | DNA polymerase |
| dNTP | Deoxynucleoside triphosphate |
| <i>E. coli</i> | Escherichia coli |
| EBERs | Epstein–Barr virus-encoded small RNAs |
| EBNA | Epstein–Barr virus nuclear antigen |
| EBV | Epstein-Barr Virus |
| eGFP | Enhanced green fluorescence protein |
| FACS | Fluorescence-activated cell sorting |

| | |
|--------------|---|
| FCS | Foetal calf serum |
| FLICE | FADD-like interleukin-1 beta converter enzyme |
| FRET | Fluorescence resonance energy transfer |
| gp | Glycoprotein |
| HCMV | Human cytomegalovirus |
| HIV | Human immunodeficiency virus |
| hpi | Hours post-infection |
| hpt | Hours post-treatment |
| HSUR | Herpesvirus saimiri U RNAs |
| HSV | Herpes simplex virus |
| IE | Immediate-early |
| IFN γ | Interferon gamma |
| IL | Interleukin |
| IM | Infectious mononucleosis |
| I κ B | Inhibitor of nuclear factor kappa B |
| kbp | Kilo base pair |
| KS | Kaposi's sarcoma-associated herpesvirus |
| KSHV | Kaposi's sarcoma-associated herpesvirus |
| LANA | Latency-associated nuclear antigen |
| LCLs | Lymphoblastoid cell lines |
| LMP | Latent membrane protein |
| MCMV | Murine cytomegalovirus |
| MDV | Marek's disease virus |
| MHV-68 | Murine gammaherpesvirus-68 |
| miRNA | MicroRNA |

| | |
|----------------|--|
| mLANA | Murine gammaherpesvirus latency-associated nuclear antigen |
| mM | Milli molar |
| MOI | Multiplicity of infection |
| mRNA | messenger RNA |
| NBCS | New-born calf serum |
| NF- κ B | Nuclear factor kappa B |
| nM | Nano molar |
| NoRT | No reverse transcription |
| NPC | Nasopharyngeal carcinoma |
| NTC | No template control |
| ORF | Open reading frame |
| OvHV-2 | Ovine herpesvirus 2 |
| PCR | Polymerase chain reaction |
| PEL | Primary eusion lymphoma |
| PFU | Plaque forming unit |
| PMA | Phorbol myristate acetate |
| pre-miRNA | Precursor microRNA |
| pri-miRNA | Primary microRNA |
| PTLD | Post-transplant lymphoproliferative disorder |
| qRT-PCR | Quantitative reverse transcription polymerase chain reaction |
| RIN | RNA integrity number |
| RISC | RNA-induced silencing complex |
| RNA | Ribonucleic acid |
| RT | Reverse transcription |
| RTA | Replication and transcription activator |

| | |
|------------|--|
| SDHA | Succinate dehydrogenase complex, subunit A |
| SV40 | Simian virus 40 |
| TMER | tRNA-miRNA-encoded RNA |
| TR | Terminal repeat |
| v/v | Volume per volume |
| vtRNA | Viral transfer RNA |
| VZV | Varicella-zoster virus |
| WT | Wild-type |
| β la | Beta lactamase |

Chapter 1: Introduction

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1 Chapter 1: Introduction

1.1 Herpesviruses

The word herpes is taken from the Greek herpein, meaning to creep or crawl and refers to spreading of lesions characteristic of herpesvirus infections of the skin (Roizman, Campadelli-Fiume et al. 2011). Herpesviruses are now recognized to infect a wide range of tissues and organs in many species and in 2009 the family *Herpesviridae* was elevated to the order *Herpesvirales* following the discovery of fish and molluscs herpes viruses, comprising at least 135 members within 3 families: *Herpesviridae*, *Alloherpesviridae*, and *Malacoherpesviridae*, infecting a wide range of hosts including mammals, birds, fish, frogs, and molluscs (Davison 2010). The various herpesviruses have diverse genomic sequences, but they have similar structure and genome organisation.

All the herpesviruses are able to replicate in the nucleus of the host cell, encode enzymes required for the DNA synthesis and protein processing, and establish latent infection following the primary productive infection with the ability to reactivate at later stage under certain conditions (Roizman and Pellet 2013). The virus particles vary in diameter (120-300 nm) and contain double-stranded linear DNA genomes with sizes ranging from ~120 to 250 kilo base pairs depending on the virus species. The DNA genome is contained in an enveloped icosahedral capsid. A proteinaceous material is present between the lipid bilayer envelope and the capsid, called the tegument. The envelope carries glycoproteins that mediate attachment to the host cell and virus entry. Herpesviruses are important pathogens in both humans and animals. They cause a variety of diseases in humans including cold sores, chicken pox,

shingles, encephalitis, and cancer. In animals they can cause rhinotracheitis, pneumonia, genital infection, mastitis, malignant catarrhal fever, abortion, and cancer.

1.1.1 Classification of the family *Herpesviridae*

Early classification was based on the physical and biological properties such as virion morphology, host range, cell tropism, growth cycle, and pathogenic features. The classification is now based on the genomic organisation and sequence-based phylogeny (Davison 2010). The family *Herpesviridae* is divided into 3 subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Table 1.1 shows a selection of important *Herpesviridae* members and their associated diseases.

1.1.1.1 *Alphaherpesvirinae*

Members of the *Alphaherpesvirinae* subfamily are characterized by short growth cycle, rapid spread, and latency establishment primarily, but not exclusively, in sensory neurons (Roizman and Pellet 2013). The *Alphaherpesvirinae* subfamily includes the genera *Simplexvirus* (e.g. Herpes simplex virus 1 and 2), *Varicellovirus* (e.g. Varicella-zoster virus, Bovine herpesvirus 1, and Feline herpesvirus 1), *Iltovirus* (e.g. Gallid herpesvirus 1 and Psittacid herpesvirus 1), and *Mardivirus* (e.g. Gallid herpesvirus 2 (Marek's disease virus)). The latter virus is different from the other alphaherpesviruses in that it establishes latency in CD4+ T-cells and can cause T-cell lymphomas (Jarosinski, Tischer et al. 2006).

| Subfamily | Genus | Virus name | Host | Disease |
|----------------------------|--------------------------|--|--|--|
| <i>Alphaherpesvirinae</i> | <i>Simplexvirus</i> | Herpes simplex virus 1 and 2 | Human | Labial and genital infections |
| | <i>Varicellovirus</i> | Varicella-zoster virus | Human | Chicken pox and zoster (shingles) |
| | | Bovine herpesvirus 1 | Cattle | Rhinotracheitis, genital infections, abortion, enteritis, conjunctivitis |
| | | Equid herpesvirus 4 | Horses | Rhinopneumonitis |
| | | Feline herpesvirus 1 | Felines | Rhinotracheitis, pneumonia, keratitis, encephalitis |
| | <i>Iltoivirus</i> | Gallid herpesvirus 1 | Chickens | Infectious laryngotracheitis |
| | | Psittacid herpesvirus 1 | Psittacinae | Pacheco's disease |
| <i>Mardivirus</i> | Gallid herpesvirus 2 | Chickens | Marek's disease | |
| <i>Betaherpesvirinae</i> | <i>Cytomegalovirus</i> | Human cytomegalovirus/Human herpesvirus 5 (HHV-5) | Human | Mononucleosis, hepatitis, congenital abnormalities |
| | <i>Muromegalovirus</i> | Murine cytomegalovirus | Mice | Asymptomatic |
| | <i>Roseolovirus</i> | Human herpesvirus 6 and 7 | Human | Roseola |
| | <i>Proboscivirus</i> | Elephant endotheliotropic herpesvirus | Elephants | Death in young calves |
| <i>Gammapherpesvirinae</i> | <i>Lymphocryptovirus</i> | Epstein-Barr virus (EBV)/ Human herpesvirus 4 (HHV-4) | Human | Infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma |
| | <i>Rhadinovirus</i> | Kaposi's sarcoma herpesvirus (KSHV)/ Human herpesvirus 8 (HHV-8) | Human | Kaposi's sarcoma, some types of castleman's disease, primary effusion lymphoma |
| | | Murine herpesvirus 68 (MHV-68)/ Murid herpesvirus 4 (MuHV-4) | Wild rodents | Asymptomatic |
| | | Bovine herpesvirus 4 (BoHV-4) | Cattle | Endometritis, vulvovaginitis, mastitis |
| | | Herpesvirus saimiri | Squirrel monkeys | Asymptomatic in natural host, T-cell lymphoma in other monkey species |
| | <i>Macavirus</i> | Alcelaphine herpesvirus 1 (AIHV-1) | Wildebeest (cattle is dead end host) | Wildebeest-associated malignant catarrhal fever |
| | | Ovine herpesvirus 2 (OvHV-2) | Cattle (susceptible dead end host) and sheep (carrier) | malignant catarrhal fever in cattle, asymptomatic in sheep |

Table 1-1 Selected *Herpesviridae* family members and their medical and veterinary importance.

1.1.1.2 *Betaherpesvirinae*

Betaherpesviruses have a narrower host range and are characterised by long growth cycle, slow spread, latency establishment in myeloid progenitor cells, persistence in epithelial cells of salivary glands and kidneys, and induction of cell enlargement (cytomegalia) (Mocarski, Shenk et al. 2013).

The *Betaa herpesvirinae* subfamily includes the genera *Cytomegalovirus* (e.g. Human cytomegalovirus (HCMV)), *Muromegalovirus* (e.g. Murine cytomegalovirus (MCMV)), *Roseolovirus* (e.g. Human herpesvirus 6 and 7), and *Proboscivirus* (e.g. Elephant endotheliotropic herpesvirus).

1.1.1.3 *Gammaherpesvirinae*

The subfamily *Gammaherpesvirinae* includes the genera *Lymphocryptovirus* (e.g. Epstein-Barr virus (EBV)), *Rhadinovirus* (e.g. Kaposi's sarcoma herpesvirus (KSHV), murine gammaherpesvirus 68 (MHV-68), and herpesvirus saimiri), and *Macavirus* (e.g. Ovine herpesvirus 2 (OvHV-2)). The gammaherpesviruses are characterized by primary productive infection in epithelial cells followed by latency establishment in lymphocytes (Roizman and Pellet 2013). Strict species specificity of gammaherpesviruses precludes infection of animals with the human viruses. MHV-68 (also referred to as γ HV68 and Murid herpesvirus 4 (MuHV-4)) is considered a tractable animal model for studying the gammaherpesviruses pathogenesis.

1.1.2 Herpesvirus structure

All herpesvirus virions consist of 4 major structural elements (Figure 1.1):

- 1- **Genomic DNA core.** It consists of a linear double-stranded DNA genome (120-250 kb).
- 2- **Capsid.** It surrounds the DNA core, 100 nm in diameter, and is composed of 162 capsomeres (150 hexons and 12 pentons), arranged in icosahedral symmetry (triangulation number $T=16$) (Brown and Newcomb 2011). The capsid contains 4 conserved proteins: the major capsid protein, the monomer and dimer proteins of the triplex, and the small capsomere-interacting protein (Roizman and Pellet 2013).
- 3- **Tegument.** It is located between the capsid and the envelope. It contains proteins that facilitate virus infection immediately after entry such as by shutting down host protein synthesis, evading cell defences, and stimulating viral gene expression; in addition, it plays a role in virus assembly and egress (Guo, Shen et al. 2010, Roizman and Pellet 2013).
- 4- **Envelope.** It is the outer layer of the virion. It mainly consists of host-cell derived lipid membranes containing virally encoded glycoproteins that appear in electron micrographs as short spikes embedded in the envelope (Roizman, Campadelli-Fiume et al. 2011).

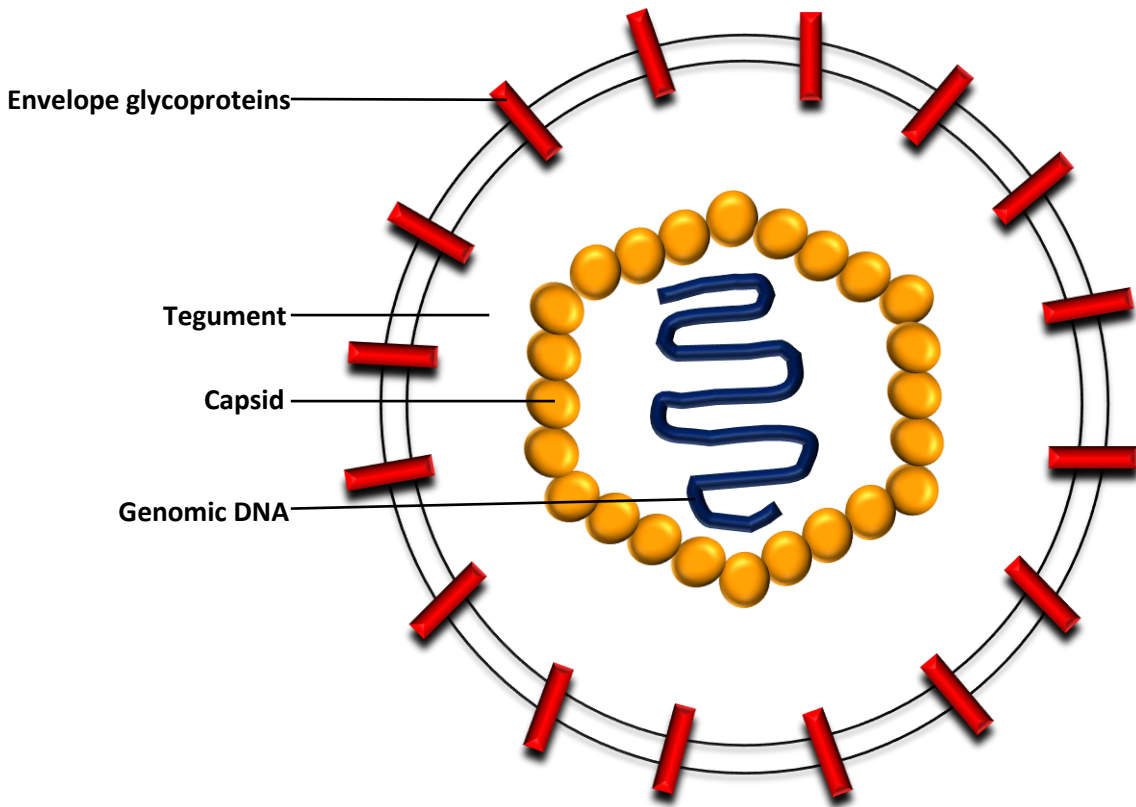


Figure 1.1 Diagram showing herpesvirus structure with major elements denoted.

1.1.3 Herpesvirus life cycle

The herpesvirus life cycle is divided into lytic phase (characterised by production of infectious progeny) and latent phase (characterised by suppression of viral replication resulting in a quiescent state). The establishment of lytic or latent infection in an infected cell in the natural host mainly depends on the type of infected cells and virus tropism. The virus can undergo lytic reactivation from latency; for example during immunosuppression, leading to production of progeny virus that can infect new host. The HSV life cycle is the best understood and has been extensively studied; therefore, its life cycle will be reviewed in detail below.

1.1.3.1 Lytic infection

The purpose of the productive or lytic infection is to produce infectious virions that can infect other cells and spread to other hosts. Lytic infection involves virus attachment and entry into host cells, viral gene expression, viral DNA genome replication, and virion assembly and egress.

1.1.3.1.1 Attachment and entry

The initial step of virus entry into the host cell is virus attachment that involves the binding of the viral envelope glycoproteins to the cell receptors. Herpesviruses infect wide varieties of cell types and therefore they employ multiple cell surface receptors for virus entry. For most herpesviruses the initial attachment involves interactions of viral glycoprotein gB with cell surface heparan sulfate proteoglycans (Roizman and Pellet 2013). Herpes simplex virus glycoproteins gB and gC initially bind to cell surface receptor heparan sulfate followed by binding of the glycoprotein gD to at least one of the three classes of receptors nectins-1,

herpesvirus entry mediator (HVEM), or 3-O sulfated heparan sulfate (Shukla, Liu et al. 1999, Spear 2004, Akhtar and Shukla 2009). The binding of gD to its receptor causes gD conformational changes allowing its interaction with viral glycoproteins gB, gH, and gL forming a complex that mediates fusion of virion envelope with the host cell membrane (Subramanian and Geraghty 2007). After attachment to host cell receptors, herpesviruses enter cells by: fusion of virus envelope with the cell membrane at the cell surface (Roizman and Pellet 2013). This results in release of viral capsids and the associated tegument proteins into the cytoplasm.

Viral capsids are transported to the nuclear pores along the microtubular network via the microtubule motor dynein (Sodeik, Ebersold et al. 1997, Dohner, Nagel et al. 2005, Wolfstein, Nagel et al. 2006, Radtke, Kieneke et al. 2010). The binding of the viral glycoproteins to the host receptors causes immediate rearrangement of host cell cytoskeleton (Lyman and Enquist 2009). For example, binding of the gB glycoprotein of KSHV to the $\alpha 3 \beta 1$ integrin receptor resulted in microtubular stabilisation and the activation of the RhoA and Rac1 GTPases (Naranatt, Krishnan et al. 2005). The inactivation of Rho GTPases by *Clostridium difficile* toxin B led to reduction in microtubular acetylation and decreased delivery of viral DNA to the nucleus. In contrast, the activation of Rho GTPases by *E.coli* cytotoxic necrotising factor resulted in increased nuclear delivery of viral DNA (Naranatt, Krishnan et al. 2005). Hence, activation of the Rho GTPases following receptor binding is a possible trigger for rearrangement of the cytoskeleton following herpesvirus infections.

It has been suggested that the inner tegument proteins enhance capsid transport via the microtubules (Luxton, Haverlock et al. 2005, Wolfstein, Nagel et al. 2006). The

HSV inner tegument proteins VP1/2 and UL37 are required for efficient movement of capsids along the microtubules *in vitro*, whereas capsids without tegument proteins showed no movement (Wolfstein, Nagel et al. 2006). Furthermore, live-cell imaging experiments investigating the retrograde (minus-end) transport of pseudorabies virus capsids fused to monomeric red fluorescent protein and tegument proteins fused to green fluorescent protein in sensory neurons showed that the inner tegument proteins VP1/2 and UL36 were associated with the capsids as they entered the cells and transported to the nucleus, whereas the outer tegument proteins VP13/14, VP16, and VP22 were not associated with the capsids following virus entry and during the transport to the nucleus; however, they underwent anterograde transport and associated with progeny capsids, suggesting that they may direct viral egress (Luxton, Haverlock et al. 2005).

Studies on the temperature-sensitive HSV-1 mutant (tsB7) showed that viral DNA is released at the nuclear pores and that the tsB7 mutation affects the VP1/2 tegument protein, which must be cleaved to allow the release of the viral DNA into nucleus (Jovasevic, Liang et al. 2008, Abaitua, Daikoku et al. 2011, Roizman, Knipe et al. 2013). Interestingly, cellular proteins can also help the docking of viral DNA to nuclear pores such as the nuclear pore complex proteins Nup358/RanBP2 and Nup214/CAN as well as the nuclear factor importin β (Copeland, Newcomb et al. 2009, Padeloup, Blondel et al. 2009, Roizman, Knipe et al. 2013).

1.1.3.1.2 Gene expression

During lytic infection, the viral genes form 3 classes depending on the order of gene expression and the mechanisms that drive them. These are the alpha (α) or immediate-early (IE) genes that do not require new protein synthesis for their

expression and regulate the expression of other viral genes, the beta (β) or early (E) genes whose expression is not dependent on viral DNA synthesis and is dependent on IE gene expression, and the gamma (γ) or late (L) genes whose transcription is dependent on viral DNA synthesis (Roizman and Pellet 2013). The late genes can be divided into 2 groups: (i) γ 1 (also referred to as the leaky-late or early-late (E-L) genes) whose transcription is enhanced by viral DNA synthesis and (ii) the γ 2 (true late) genes whose transcription is totally dependent on viral DNA synthesis. The HSV IE gene expression is transactivated by the HSV virion protein 16 (VP16), a tegument protein that assembles into a transactivator complex with two host cellular proteins, host cell factor 1 (HCF-1) and the octamer binding transcription factor 1 (Oct-1) (Knipe 2015, Roizman and Zhou 2015). This complex binds to IE gene promoters leading to their transcription during lytic infection. Upon the entry of HSV DNA into the nucleus of the neuron, it is circularised and transcription is repressed and there is no DNA replication. As VP16 is a tegument protein that enters the neurons with the infecting virus, little VP16 reaches the nucleus of the neuron, which is a long distance along the axon from the site of infection (Roizman and Zhou 2015). Moreover, neurons contain 2 transcription factors, Zhangfei and Luman, which bind to HCF-1 and prevent formation of active transactivator complexes (Roizman, Knipe et al. 2013). In the absence of IE gene expression, the viral genome remains silent, with the exception of a class of RNAs called latency-associated transcripts (LATs) and a group of microRNAs.

1.1.3.1.3 Viral DNA circularisation

Following the entry into the nucleus, the viral DNA circularises rapidly in the absence of viral protein synthesis (Roizman, Knipe et al. 2013). Using mutant cell

lines and small interfering RNA-mediated knockdown, it has been shown that viral DNA circularisation, formation of endless genomes, and viral DNA replication require the cellular DNA ligase IV/XRCC4 (Muylaert and Elias 2007).

1.1.3.1.4 Association of viral DNA with histones

Herpesviral DNA in the virion is not associated with histones. Chromatin regulation by histone modification plays an important role in lytic or latent infection decision by herpesvirus (Knipe 2015). During HSV infection in epithelial cells, viral proteins minimise association of histones with viral lytic gene promoters and enhance euchromatin histone modification on histones that are associated with viral DNA. In contrast, during latent HSV infection in neurons, viral DNA is loaded with heterochromatin resulting in gene silencing and establishment of latent infection (Knipe and Cliffe 2008, Knipe 2015).

HSV VP16 loads onto IE gene promoters, leading to recruitment of general transcription factors, RNA polymerase II, and chromatin-remodeling enzymes to IE promoters resulting in activation of IE gene transcription (Kristie 2015, Roizman and Zhou 2015). The cellular HCF-1 protein recruits the Set1 histone methyl transferase to IE gene promoters for the euchromatic histone H3 lysine 4 (H3K4) methylation modification, the LSD1 demethylase to remove the heterochromatic H3K9me and H3K9me₂ methylation modification, and the JMJD2 demethylases to demethylate H3K9me₃ (Knipe 2015). Thus, the IE gene promoters-associated histones contain euchromatic histone modifications that correspond to active transcription of IE genes.

A previous published study on HSV revealed that there are higher levels of acetylated H3 histone associated with the LAT promoter as compared to the key transcriptional activator infected cell protein 0 (ICP0) viral gene, suggesting that the active chromatin is specifically associated with the LAT gene (Kubat, Amelio et al. 2004). Moreover, during the establishment of HSV latency, the lytic-gene promoters become increasingly associated with chromatin that contains modified histones, which are associated with heterochromatin, particularly dimethyl H3K9me2 (Wang, Zhou et al. 2005). The methylation of the HSV DNA cannot be detected during latency and hence the lytic genes are likely silenced by heterochromatin rather than by methylation of DNA (Dressler, Rock et al. 1987, Kubat, Tran et al. 2004). Thus, the lytic genes are associated with heterochromatin, whereas the LAT gene is associated with euchromatin.

Concurrent with the viral DNA association with histones, the cellular nuclear domain 10 (ND10) or promyelocytic leukemia nuclear bodies (PML), which are associated with multiple functions such as epigenetic regulation of gene expression and transcriptional activation, are localised adjacent to the newly entered DNA (Roizman, Knipe et al. 2013). The role of ND10 during HSV infection is controversial. Chee et. al. suggested that wild-type HSV replicated at similar levels in murine PML^{-/-} and PML^{+/+} cells and that IFN- α or $-\gamma$ have a major effects in PML^{+/+} cells and minimal effects in PML^{-/-} cells; however, it was reported that the replication of an ICP0 mutant virus is 3-fold higher in PML^{-/-} cells as compared to PML^{+/+} cells (Chee, Lopez et al. 2003). On the other hand, Everett et. al. reported that ICP0 co-localises with ND10 and causes PML degradation and that an ICP0 mutant virus was 3- to 5-fold more efficient in plaque formation when PML was depleted by short interfering RNA (Everett, Rechter et al. 2006). Recently, it has

been reported that HSV-1 at multiplicity of infection of 5 plaque forming units (pfu) per cell replicates to equal levels in the parental human epithelial cells and in PML^{-/-} cells, which were generated by transfection of clustered regularly interspaced short palindromic repeats (CRISPR)/cas9 cassette targeting exon 1 of PML, but surprisingly, at low multiplicity of infection (0.1 pfu/cell), the virus replicated in the parental cells 100-fold higher than PML^{-/-} cells (Xu, Mallon et al. 2016), suggesting that PML can also be beneficial to virus replication.

1.1.3.1.5 Viral genome replication

Herpesvirus DNA replication occurs in the nucleus and it is dependent on the presence of the viral early proteins, the DNA replication proteins, and sufficient biosynthetic precursors. Like other viruses, herpesviruses rely on the host cell metabolic activity to provide building blocks and energy for viral replication. Some herpesviruses can alter the cellular metabolic pathways in order to facilitate viral replication. For example, HSV enhances pyrimidine biosynthesis (Vastag, Koyuncu et al. 2011), whereas HCMV manipulates the tricarboxylic acid cycle to enhance lipid biosynthesis (Munger, Bennett et al. 2008, Vastag, Koyuncu et al. 2011, Yu, Clippinger et al. 2011).

1.1.3.1.6 Virus assembly and egress

The different stages of virion assembly occur initially in the nucleus and then the cytoplasm. In the nucleus, the newly formed capsids are assembled on temporary scaffolds, then filled with newly replicated viral genome in an energy dependent manner (Mettenleiter, Klupp et al. 2009, Roizman and Pellet 2013). The first main

barrier during virus egress is the nuclear membrane. The viral nucleocapsids are too large to pass through the nuclear pores, which exhibit no gross perturbation during viral movement across the nuclear membrane (Hofemeister and O'Hare 2008). Instead, the nucleocapsids become enveloped by budding at the inner nuclear membrane into the perinuclear space (primary envelopment). This process is followed by de-envelopment of the virus particles, involving membrane fusion between the outer nuclear membrane and the virus envelope, thereby the capsids are delivered into the cytoplasm (Johnson and Baines 2011). It has been shown recently that in the absence of regulated nuclear egress, the alphaherpesvirus pseudorabies virus was able to exit the nucleus after virus-induced nuclear envelope breakdown (Schulz, Klupp et al. 2015), suggesting that both mechanisms (envelopment-de-envelopment pathway and nuclear envelope breakdown) can occur simultaneously under certain conditions.

The newly formed virions that enter the cytoplasm consist of nucleocapsids and inner tegument proteins. Further assembly steps involve addition of tegument proteins and some RNAs (cytoplasmic tegumentation) followed by acquisition of mature envelope (secondary envelopment) during budding into cytoplasmic membranes, producing infectious virus. Then the enveloped virions are transported to the cell surface via the cytoplasmic vesicles followed by fusion between virion envelope and the plasma membrane resulting in virus release (Mettenleiter 2006, Guo, Shen et al. 2010).

1.1.3.2 Latent infection

A common feature of the herpesviruses is their ability to establish and maintain latent infection within the natural host. In latently infected cells, the viral genome takes the form of a circular molecule from which a very limited number of viral genes are expressed and therefore no infectious virus can be detected. The latent virus genome is able to reactivate and undergo lytic infection cycle and produce infectious virions under certain conditions such as immunosuppression. Latent infection is different from chronic or persistent infection in that the infectious virus is not produced during latency. Different herpesviruses establish latent infection in different cell types. For example, HSV establishes latency in the neurons of sensory ganglia, whereas EBV establishes latency mainly in B lymphocytes (Roizman and Pellet 2013).

Latency is a complex process that involves interaction between viral and host cellular factors. The exact molecular mechanisms behind the establishment of and the reactivation from latency are not fully understood. Latent infection is usually established following the primary lytic infection and it involves: (i) suppression of lytic infection and restriction of lytic gene expression while maintaining latent gene expression, (ii) enhanced survival of the latently infected cells through evasion of the immune response and prevention of apoptosis, and (iii) prevention of undesired reactivation whilst keeping the virus ability to reactivate under the appropriate conditions.

In addition to viral proteins, which play crucial roles in latency, virally encoded microRNAs (miRNAs) also play important roles during latent infection. The miRNAs are small non-coding RNAs that regulate gene expression post-transcriptionally. They are present in all mammalian cells and play various roles in

different biological processes such as cellular differentiation, proliferation, development, apoptosis, and cell cycle regulation (Bartel 2004). The miRNAs encoded by herpesviruses can be elegant tools for promoting viral latency because they are non-immunogenic and therefore can evade the innate and adaptive immunity. Many studies have shown that the miRNAs encoded by herpesviruses play various roles in promoting latent infection such as inhibition of lytic gene expression, enhancing the proliferation and survival of latently infected cells, and evading the immune response (reviewed in section 1.6).

Almost all herpesviruses that have been studied in-depth encode miRNAs with the exception of Varicella Zoster Virus (VZV), a human herpesvirus that causes chicken pox and shingles (Umbach, Nagel et al. 2009). However, the other animal Varicelloviruses such as Bovine Herpesvirus-1 and Suid Herpesvirus-1 do encode miRNAs (Glazov, Horwood et al. 2010, Anselmo, Flori et al. 2011). This suggests that VZV latency is different from the latency of other herpesviruses in terms of viral miRNA utilisation.

1.2 Important human gammaherpesviruses

1.2.1 Epstein-Barr virus (EBV)

In 1964, EBV was discovered by Anthony Epstein and his colleague Yvonne Barr using electron microscopy in cells cultured from Burkitt's lymphoma biopsies (Epstein, Henle et al. 1965). A few years later, Werner and Gertrude Henle discovered that EBV is responsible for infectious mononucleosis, a self-limiting lymphoproliferative disease characterised by proliferation of infected B lymphocytes followed by their destruction by T cytotoxic lymphocytes (Henle, Henle et al. 1968). After its discovery, EBV was shown to be associated with several neoplastic diseases

such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, oral hairy leukoplakia, and extranodal NK-T-cell lymphoma (Pope, Horne et al. 1968, zur Hausen, Schulte-Holthausen et al. 1970, Cohen, Wang et al. 1989, Bignon, Bernard et al. 1990, Herbst, Niedobitek et al. 1990, Brocksmith, Angel et al. 1991, Bellas, Mampaso et al. 1993, Nicolas, Marechal et al. 1997).

EBV is a highly successful virus that persists in over 90% of the human adult population where most primary infections occur in childhood with no obvious symptoms (Longnecker, Kieff et al. 2013). EBV infection is asymptomatic in immunocompetent individuals, but during immunosuppression the virus causes lymphoproliferative disorders and can be associated with some types of cancer (Young and Rickinson 2004). EBV infects B lymphocytes through binding of the viral glycoprotein gp350 to the CD21 receptor of the B cells in addition to binding of gp42 to human leukocyte antigen class II molecules as a co-receptor (Nemerow, Mold et al. 1987, Borza and Hutt-Fletcher 2002). EBV can also infect epithelial cells; however, at less efficiency than B cells and through different pathways (Borza and Hutt-Fletcher 2002). Interestingly, EBV is able to transform resting B lymphocytes into latently infected lymphoblastoid cell lines *in vitro* (LCLs) and therefore they are considered the dominant *in vitro* model for studying EBV latency and virus-induced B cell proliferation and transformation (Young and Rickinson 2004).

1.2.1.1 EBV Latency

Like other herpesviruses, EBV gene expression is highly restricted during latent infection. There are 2 sets of EBV proteins produced in the latently infected B lymphocytes: Epstein-Barr nuclear antigens (EBNAs) and latent membrane proteins (LMPs). There are 6 distinct EBNAs (EBNA-1, -2, -3A, -3B, -3C, and LP) resulting from differential splicing of mRNAs from long primary transcripts, whereas there are 3 LMPs: LMP-1, -2A, and -2B (Rowe, Lear et al. 1992). Fascinatingly, EBV makes use of the normal B cell biology to establish life-long latent infection in memory B cells through activation of naïve B cells to become proliferating blasts and consequently differentiate into resting memory B cells through the process of the germinal-center reaction (Figure 1.2) (Thorley-Lawson and Gross 2004). Therefore, the difference between the antigen-activated B cell blast and the virus-infected B cell blast is that viral genes, not antigens, provide the signals required for differentiation of the B cell blasts into memory B cells.

The EBV infects naïve B cells where viral gene expression exhibits latency III pattern (growth programme) with expression of all EBNA proteins and LMP proteins as well as Epstein-Barr virus-encoded RNAs (EBERs), BamHI A rightward transcript (BART) miRNAs, and BamHI H reading frame 1 (BHRF1) miRNAs (Table 1.2). The EBNA-1 protein plays a central role in latency by maintaining the viral genome as an extrachromosomal episome in dividing cells (Yates, Warren et al. 1984, Yates, Warren et al. 1985, Kang and Kieff 2015). The EBNA-2 activates EBV-infected naïve B cells and trans-activates the expression of LMP-1 and LMP-2A proteins (Wang, Tsang et al. 1990, Zimber-Strobl, Kremmer et al. 1993). LMP-2A mimics antigen-independent B cell receptor (BCR) signaling through constitutive activation of the ERK/MAPK

pathway, therefore EBV-infected naïve B cells undergo germinal-centre reaction (Caldwell, Wilson et al. 1998, Anderson and Longnecker 2008, Kang and Kieff 2015). LMP-1 mimics the constitutive active form of CD40, resulting in B cell growth and differentiation (Kilger, Kieser et al. 1998).

It has been suggested that the virus drives the infected naïve B cells into the memory state by switching the viral transcription programme from the growth programme (latency III) to the default programme (latency II) (Thorley-Lawson and Gross 2004, Longnecker, Kieff et al. 2013) (Figure 1.2 and table 1.2). At this stage of latency, the virus expresses EBNA-1 and LMP proteins as well as EBERs and BART miRNAs (Babcock and Thorley-Lawson 2000, Longnecker, Kieff et al. 2013). The latently infected memory B cells shut down viral protein expression and circulate in the peripheral blood, entering latency 0 programme where only the EBERs can be detected in the infected cells (Hohaus, Santangelo et al. 2011, Longnecker, Kieff et al. 2013). However, EBNA-1 along with EBERs and BART miRNAs are expressed in dividing latently infected memory B cells (latency programme/latency I) (Hochberg, Middeldorp et al. 2004, Longnecker, Kieff et al. 2013). When memory B cells differentiate into plasma cells, the latent virus is reactivated and replicates so that it can spread to a new host and infect new naïve B cells within the same host (Laichalk and Thorley-Lawson 2005).

The type III latency is established in EBV-positive cells during the post-transplant lymphoproliferative disorder and in human B cells immortalised *in vitro* by EBV infection (LCLs), whereas biopsies from Hodgkin's lymphomas and nasopharyngeal

carcinomas exhibit type II latency and the cells derived from Burkitt's lymphoma and gastric carcinoma exhibit type I latency (Table 1.2).

Besides EBV proteins, the miRNAs encoded by EBV play roles in promoting latency. For example, although mutant EBV viruses that lack BHRF1 miRNAs expression were able to generate LCLs following infection of naïve B cells, the initial proliferation and transformation capacity of LCLs were reduced as compared to the WT virus due to reduction in cell cycle progression and increased apoptosis (Seto, Moosmann et al. 2010, Feederle, Linnstaedt et al. 2011). It has been shown that the miRNA, BART-miR-18-5p, targets the cellular MAP3K2, which is important for initiation of lytic viral cycle, resulting in suppression of reactivation in latently infected memory B cells (Qiu and Thorley-Lawson 2014). Moreover, it has been demonstrated that BART miRNAs target multiple pro-apoptotic cellular mRNAs in latently infected epithelial cells derived from nasopharyngeal carcinoma, resulting in enhanced cell survival and robust long-term latent infection (Kang, Skalsky et al. 2015).

The most abundant EBV encoded non-coding RNAs, EBERs, play various roles during latency. They augment colony formation and enhance cell growth (Komano, Maruo et al. 1999, Houmani, Davis et al. 2009, Repellin, Tsimbouri et al. 2010) and play roles in enhancing cell survival through inhibition of PKR-dependent apoptosis (Nanbo and Takada 2002). In addition, they play roles in modulating innate immunity (Samanta, Iwakiri et al. 2006, Iwakiri, Zhou et al. 2009).

1.2.1.2 EBV types

To date, there are approximately 83 strains of EBV that have been completely sequenced (Palser, Grayson et al. 2015). Based on the genomic variation, there are

two types of EBV, type 1 and type 2, which differ mainly in the sequences of EBNA-2 and EBNA-3 genes (Palser, Grayson et al. 2015). However, there are variations in other viral genes, mainly latent genes, but this variation does not correspond to the type 1/type 2 classification. The major phenotypic difference between the two EBV types is that type 1 is more efficient in transforming primary B cells (Palser, Grayson et al. 2015, Young, Yap et al. 2016). It has been shown recently that in contrast to type 1, Type 2 can latently infect T cells, inducing cell proliferation and cytokine production (Coleman, Wohlford et al. 2015). However, the impact of genomic variation on the biology of EBV infection is not clear.

1.2.1.3 EBV latent transcripts

1.2.1.3.1 The EBV nuclear antigens

1.2.1.3.1.1 EBNA-1

EBNA-1 maintains the viral genome as extrachromosomal episome in proliferating cells through binding to multiple sites within the plasmid latent origin of replication (oriP); thus, it segregates the daughter genomes into each of the two progeny cells during mitotic division (Yates, Warren et al. 1984, Rawlins, Milman et al. 1985, Kanda, Otter et al. 2001). The EBNA-1 protein carries a glycine-alanine repeat sequence that stabilises the protein through blocking the protein proteasomal degradation as well as preventing antigen processing by inhibition of the MHC class I-restricted presentation, thereby inhibiting CD8⁺ T-cell cytotoxic response against EBV-infected cells (Levitskaya, Coram et al. 1995, Young and Rickinson 2004).

| Latency programme | Viral gene expression | Type of infected B cell | Programme function | Disease examples |
|--------------------------|---|--|--|---|
| Latency III (growth) | EBNA-1, -2, -3A, -3B, -3C, -LP LMP-1, -2A, -2B EBERs BART miRNAs BHRF1 miRNAs | Naïve B cells | B cell activation | Post-transplant lymphoproliferative disease |
| Latency II (default) | EBNA-1 LMP-1, -2A, -2B EBERs BART miRNAs | Germinal centre B cells | Differentiation of activated B cells into memory B cells | Nasopharyngeal carcinoma Hodgkin's lymphoma T-cell lymphoma |
| Latency I (latency) | EBNA-1 EBERs BART miRNAs | Dividing peripheral blood memory B cells | Maintaining viral genome in dividing latently infected cells | Burkitt's lymphoma Gastric carcinoma |
| Latency 0 | EBERs | peripheral blood memory B cells | Facilitate life-long latency | |
| Lytic | All lytic genes | Plasma cells | Virus lytic replication | |

Table 1-2 EBV transcription programmes and associated diseases.

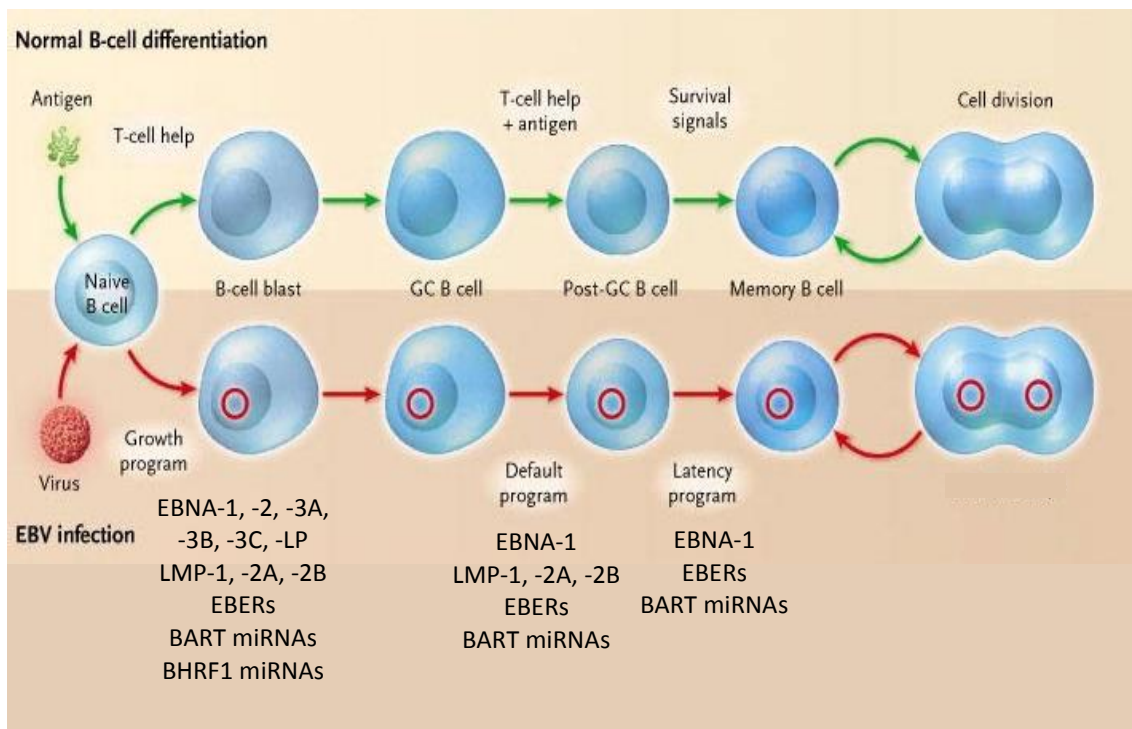


Figure 1.2 EBV resembles normal B cell differentiation in order to drive naïve B cells into memory B cell pool.

Following infection of naïve B cells, EBV activates these cells to become proliferating B cell blasts that undergo germinal centre reaction using the growth programme in a manner similar to that of antigen-mediated activation of naïve B cells. The antigen-specific T help signals lead to survival of activated B cells and entry into memory B cell pool. The virus uses the default programme in order to induce these survival signals in the latently infected B cells driving them into becoming memory B cells. These memory cells then exit cell cycle and enter the peripheral circulation and exhibit no viral protein expression. Occasionally, memory B cells divide as part of normal B cell homeostasis. If the dividing memory B cell is infected with EBV, it expresses EBNA-1 protein, allowing the division of the latent viral genome along with the cell. The latently infected memory B cells may differentiate into plasma cells, resulting in virus reactivation and infectious virus production. Gene expression during each latency phase is indicated. Figure adapted from (Thorley-Lawson and Gross 2004).

On the other hand, EBNA-1 is consistently recognised by CD4⁺ T cells and gains access to MHC II processing by autophagy and undergo lysosomal degradation (Paludan, Schmid et al. 2005).

EBNA-1 acts as transcriptional activator, regulating its own expression as well as the expression of other viral latency genes e.g. EBNA-2 and -3 and LMP-1 (Levitskaya, Coram et al. 1995, Young and Rickinson 2004). It has been shown that EBNA-1 is highly expressed in nasopharyngeal carcinoma tissues and it plays a role in inducing epithelial to mesenchymal transition in these tissues through regulation of the signalling pathways involving transforming growth factor- β 1 (TGF- β 1), microRNA 200a (miR-200a) and miR-200b, and zinc finger E-box binding homeobox 1 (ZEB1) and ZEB2 (Wang, Tian et al. 2014). EBNA-1 can play a role in facilitating latency through induction of let-7a miRNA which in turn reduces the cellular protein Dicer level, resulting in inhibition of reactivation (Mansouri, Pan et al. 2014).

1.2.1.3.1.2 EBNA-2

EBNA-2 plays a crucial role in B lymphocyte transformation (Cohen, Wang et al. 1989). Restoration of the EBNA-2 gene into an EBV strain, P3HR-1, lacking the EBNA-2 gene confirmed the role of EBNA-2 protein in B immortalisation (Rabson, Gradoville et al. 1982, Hammerschmidt and Sugden 1989). EBNA-2 acts as a transcriptional activator of cellular and viral genes; it upregulates the expression of B cell antigens such as CD21 and CD23 and the viral LMP-1 and -2; in addition, it transactivates the Cp promoter resulting in a switch from Wp to Cp during B-cell infection (Wang, Gregory et al. 1990, Gahn and Sugden 1995, Altmann, Pich et al. 2006). EBNA-2 does not bind DNA directly, but rather it activates gene expression

by interacting with the cellular proteins such as RBP-J κ (Grossman, Johannsen et al. 1994). Both EBNA-2 and EBNA-LP associate the transcription factor bound to upstream DNA elements of cMyc, forming a long-range DNA looping, resulting in cell cycle entry and proliferation (Alfieri, Birkenbach et al. 1991, Zhao, Zou et al. 2011, Portal, Zhou et al. 2013).

1.2.1.3.1.3 EBNA-3 family

The EBNA-3 family of proteins comprises EBNA-3A, B, and C. It was shown that EBNA-3A and EBNA-3C are essential for B-cell transformation *in vitro*. In contrast, EBNA-3B is not required for transformation, but it acts as oncogenic suppressor (Tomkinson and Kieff 1992, Tomkinson, Robertson et al. 1993, White, Ramer et al. 2012). EBNA-3 proteins bind to the transcription factor RBP-J κ and disrupt its binding to EBNA-2, inhibiting EBNA-2 mediated transactivation (Waltzer, Perricaudet et al. 1996, Cludts and Farrell 1998). It has been recently shown that EBNA-3 proteins interact with the USP46/USP12 deubiquitination complexes to regulate LCLs growth (Ohashi, Holthaus et al. 2015).

1.2.1.3.1.4 EBNA-LP

EBNA-LP is transcribed along with EBNA-2 at the start of infection in primary B cells and co-operates with EBNA-2 in upregulating viral and cellular gene expression (Harada and Kieff 1997, Nitsche, Bell et al. 1997) and in inducing B cell proliferation (Alfieri, Birkenbach et al. 1991, Zhao, Zou et al. 2011, Portal, Zhou et al. 2013).

1.2.1.3.2 The EBV latent membrane proteins

1.2.1.3.2.1 Latent membrane protein 1 (LMP-1)

LMP-1 is a constitutively active tumour necrosis factor receptor (TNFR) mimic and it plays a key role in transforming primary B cells into LCLs (Kaye, Izumi et al. 1993, Mosialos, Birkenbach et al. 1995). LMP-1 is a major EBV oncogene that mimics CD40 signalling and activates several signalling pathways such as the NF- κ B transcription factor pathway, the JAK/STAT pathway, and the phosphatidylinositol 3-kinase (PI3-K) pathway resulting in induction of anti-apoptotic cytokine genes, cell survival, actin polymerisation, and cell motility (Gires, Kohlhuber et al. 1999, Eliopoulos, Davies et al. 2002, Dawson, Tramountanis et al. 2003).

1.2.1.3.2.2 Latent membrane protein 2 (LMP-2)

There are 2 LMP-2 proteins, LMP-2A and LMP-2B. The 2 proteins only differ in that the first exon of LMP-2A encodes 119 extra N-terminal cytoplasmic amino acids, whereas the LMP-2B first exon is non-coding. LMP-2A mimics a B cell receptor (BCR) signal and thus inhibits normal B-cell signal transduction through interaction of the N-terminal cytoplasmic domain with tyrosine kinases, resulting in inhibition of virus reactivation from latency in B cells (Miller, Lee et al. 1994, Schaadt, Baier et al. 2005). Therefore, LMP-2A can counterbalance deficient B cell receptors in mouse model or human B cells, promoting B cell survival (Caldwell, Wilson et al. 1998, Mancao and Hammerschmidt 2007). In addition, LMP-2A has been reported to inhibit recognition of B cells by EBV specific CD8⁺ T cells (Rancan, Schirrmann et al. 2015). LMP-2B was found to negatively regulate LMP-2A function (Rechsteiner, Berger et al. 2008). In general, LMP proteins stimulate the normal activation of B cells i.e. mimic the normal antigen-mediated B cell

differentiation in order to allow the latently infected B cells to enter the long lived memory B cell pool.

1.2.1.3.2.3 EBV-encoded RNAs (EBERs)

EBV expresses 2 non-polyadenylated non-coding RNAs named EBER-1 and EBER-2, which are highly expressed in EBV latently-infected cells (Arrand and Rymo 1982). EBERs form stem-loop structures giving rise to double-stranded RNA-like molecules (Young, Yap et al. 2016). Although they are present in all forms of EBV latency, they are not essential for the EBV-induced B cell transformation (Swaminathan, Tomkinson et al. 1991). EBERs are present in exosomes released from EBV-transformed cells and can interact with cellular proteins, exerting various functions during EBV infection such as promoting cell growth (Houmani, Davis et al. 2009, Repellin, Tsimbouri et al. 2010), inhibiting apoptosis (Nanbo and Takada 2002), and modulating the innate immune response (Samanta, Iwakiri et al. 2006, Iwakiri, Zhou et al. 2009, Young, Yap et al. 2016).

1.2.1.3.2.4 EBV miRNAs

EBV miRNAs were the first virally encoded miRNAs discovered (Pfeffer, Zavolan et al. 2004). EBV encodes at least 25 primary miRNAs giving rise to at least 44 mature miRNAs: 4 primary miRNAs are encoded from the BHRF1 cluster and the rest are encoded from the BART clusters (Cai, Schafer et al. 2006, Grundhoff, Sullivan et al. 2006, Zhu, Pfuhl et al. 2009, Chen, Chen et al. 2010). Barth *et al.* demonstrated that EBV-miR-BART2 miRNA, which is expressed antisense to the 3' UTR of the viral DNA polymerase BALF5, inhibits BALF5 expression and results in

20% reduction in virus production, suggesting that EBV-miR-BART2 promotes latency by suppression of active virus replication (Barth, Pfuhl et al. 2008).

BART cluster 1 miRNAs were reported to target the 3'UTR of the EBV oncogene LMP-1, which is a crucial protein for transformation, leading to inhibition of LMP-1 expression (Lo, To et al. 2007). This effect seems paradoxical, but very high levels of LMP-1 are toxic to the cells and modulating the LMP-1 levels may prevent this toxicity; in addition, modulating the LMP-1 level can be an immunoevasion mechanism by which the virus may avoid immunological attack when the LMP-1 is expressed on the cell surface. Expression of LMP-2 can also be inhibited by another miRNA, EBV-miR-BART22 that is expressed at high levels in NPC (Lung, Tong et al. 2009). Suppression of LMP-1 and LMP-2 expression by EBV miRNAs can serve as a mechanism to prevent their toxic effect or to evade the specific immune response targeting these proteins.

It was reported that expression of the chemokine CXCL11/I-TAC, whose 3'UTR can be targeted by BHRF1-3 miRNA, was inhibited following expression of BHRF1-3 miRNA and this effect was reversed by transfection of an antisense oligonucleotide to BHRF1-3 (Xia, O'Hara et al. 2008). EBV miRNAs can enhance cell survival through exerting anti-apoptotic functions. For example, EBV-miR-BART5 inhibits the expression of p53-up-regulated modulator of apoptosis (PUMA) (Choy, Siu et al. 2008). Another study demonstrated that BART cluster 1 miRNAs can inhibit the expression of the pro-apoptotic BIM protein as well as PUMA (Marquitz, Mathur et al. 2011). A recent study has identified the mRNA targets of BART miRNAs in NPC cell line using photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP), where 10 pro-apoptotic mRNA targets were

validated by indicator assays and ectopic expression of the miRNAs (Kang, Skalsky et al. 2015), suggesting that EBV BART miRNAs promote cell survival of the latently infected epithelial cells and thus enhance long-term infection.

EBV miRNAs can promote latent infection through suppression of reactivation. For instance, BART-miR-18-5p has been demonstrated to inhibit the expression of the cellular protein MAP3K2 leading to increased viral lytic gene expression and virus production (Qiu and Thorley-Lawson 2014). In addition to their roles in promoting cell survival and latency, EBV miRNAs can contribute to the virus-induced oncogenesis and to B cell proliferation (Seto, Moosmann et al. 2010, Babu, Ponia et al. 2011, Vereide, Seto et al. 2014). It has been recently shown that EBV-miR-BART1 targets the tumour suppressor, phosphatase and tensin homologue (PTEN), leading to activation of PTEN-dependent pathways and induction of epithelial–mesenchymal transition and metastasis in nasopharyngeal carcinoma (Cai, Ye et al. 2015).

1.2.1.4 EBV associated diseases

EBV is usually asymptomatic in immunocompetent individuals, but it can be associated with lymphoproliferative disorders and some types of cancers, particularly in immunocompromised individuals.

1.2.1.4.1 Infectious mononucleosis (IM)

IM mainly occurs during primary infection in immunocompetent young adults with symptoms including mild fever, pharyngitis, lymphadenopathy, and fatigue

(Williams and Crawford 2006). IM is an immunopathological disease resulting from increased CD8⁺ T cell cytotoxic response targeting EBV-infected B cells for destruction following EBV-induced B cell proliferation (Williams and Crawford 2006).

1.2.1.4.2 Post-transplant lymphoproliferative disorder (PTLD)

This disease mainly occurs due to therapeutic immunosuppression following organ transplantation where EBV infection causes uncontrolled B lymphocyte proliferation (Singavi, Harrington et al. 2015). It is thought that the impaired T cell response leads to failure in eliminating EBV-infected cells and hence the uncontrolled B cell proliferation (Thorley-Lawson and Gross 2004).

1.2.1.4.3 Burkitt's lymphoma (BL)

BL is a cancer in the lymphatic system involving B lymphocytes and is associated with EBV infection. It was named after Denis Parsons Burkitt, an Irish physician who first described the disease in 1958 while working in Uganda and saw a young boy with jaw swelling that was proved to be lymphoma tumour (Burkitt 1958). This lymphoma is the most common childhood cancer in equatorial Africa. In 1964, Anthony Epstein and Yvonne Barr studied lymphoma biopsies sent from Uganda and discovered via electron microscopy herpesvirus particles, named Epstein-Barr virus, in cells cultured from the tumour biopsies (Epstein, Henle et al. 1965).

There are 3 clinical variants of BL (Molyneux, Rochford et al. 2012): (i) The endemic variant (also referred to as African variant) occurs in children living in malaria endemic regions (such as equatorial Africa, Brazil, and Papua New Guinea)

and the EBV infection is found in almost all patients and the disease involves jaw bones, distal ileum, kidney, ovaries, cecum, or breast (Molyneux, Rochford et al. 2012); (ii) The non-African BL (sporadic BL) is more common in places where malaria is not holoendemic and it is rarely associated with EBV infection and the jaw is less commonly affected, whereas the ileocecum is the most commonly affected site (Brady, MacArthur et al. 2007, Molyneux, Rochford et al. 2012), (iii) Immunodeficiency-associated BL is most commonly associated with human immunodeficiency virus (HIV) infection (Bellan, Lazzi et al. 2003).

All BL variants are characterised by translocation between the c-myc gene and one of the immunoglobulin heavy or light chain loci and thus facilitate tumourigenesis (Brady, MacArthur et al. 2007). EBV virus infection plays different roles in BL tumour cells such as promoting cell survival and the malignant phenotype (Takada 2001, Brady, MacArthur et al. 2007).

1.2.1.4.4 Hodgkin's lymphoma (HL)

HL was first described in 1832 by Thomas Hodgkin (Hodgkin 1832). The characteristic mononucleated Hodgkin's cells and the multinucleated Reed-Sternberg (RS) cells were first described by Dorothy Reed and Carl Sternberg (Kuppers 2009). There are 4 histologic subtypes of the classical HL (Roizman and Pellet 2013): (i) Nodular sclerosing HL: it is the most common subtype and characterised by large tumour nodules with varying degree of fibrosis; (ii) Mixed cellularity HL: it is most commonly associated with EBV infection and it consists of classic RS cell mixed with various inflammatory cells without fibrosis; (iii) Lymphocyte-rich HL: it is rare type and has the most favourable prognosis; (iv) Lymphocyte depleted HL: it is very

rare subtype composed of large number of pleomorphic RS cells with few reactive lymphocytes.

EBV contributes to the pathogenesis of HL through several mechanisms such as protecting growth-transformed germinal centre B cells from apoptosis (Bechtel, Kurth et al. 2005, Chaganti, Bell et al. 2005, Mancao, Altmann et al. 2005, Mancao and Hammerschmidt 2007) and development of HRS cells from germinal centre B cells with Ig V gene mutations (Brauninger, Schmitz et al. 2006).

1.2.1.4.5 Nasopharyngeal carcinoma (NPC)

NPC is a cancer of squamous epithelium that is among the most common cancers in southern China, but rare in Europe and North America. It is divided into different types depending on the degree of tumour cells differentiation. Undifferentiated NPC are associated with EBV infection (Vasef, Ferlito et al. 1997). The viral encoded IL-10 was shown to be highly expressed in NPC and to be associated with increased IL-1 α and IL-1 β expression by epithelial cells and CD4⁺ T cells and thereby contribute to tumour growth and immune evasion (Huang, Sheen et al. 1999).

1.2.2 Kaposi's sarcoma-associated herpesvirus (KSHV)

Kaposi's sarcoma (KS) was first described in the 19th century by Moritz Kaposi in patients with rare skin pigmentation (Kaposi 1872). Initially considered a non-aggressive disease of old men in the Mediterranean, after emergence of the HIV pandemic in the 1980s, KS was given more attention as KS became associated with acquired immune deficiency syndrome (AIDS) (Gottlieb, Ragaz et al. 1981). In 1994 Chang *et al.* identified viral DNA sequences in KS lesions but not in normal skin

using representational difference analysis (a PCR-based technique) and it was found that these sequences were homologous to capsid and tegument genes of the gammaherpesviruses EBV and HVS, leading to KSHV (also referred to as human herpesvirus 8, HHV8) discovery (Chang, Cesarman et al. 1994).

1.2.2.1 KSHV latency

KSHV is strictly human virus and therefore *in vivo* studies in the natural host is not possible. The knowledge of KSHV latency depends on the latently infected cell lines such as primary effusion lymphoma (PEL) cell lines from AIDS patients with advanced disease and biopsy samples. During latency, KSHV genome is maintained as a circular extrachromosomal episome within the nucleus of infected cells with most viral genes being silenced except a few latency-associated genes. Lytic infection can be triggered from latently infected cells by treatment with chemicals or the viral trans-activator RTA that can initiate lytic cycle, allowing for virus stock production and gene expression analysis. KSHV can latently infect several cell lines such as human fibroblasts, endothelial, and epithelial cells. Fibroblasts and epithelial cells have not been found to be infected *in vivo*, while B-cells, which are the site of latency *in vivo*, have been difficult to infect *in vitro* (Damania and Cesarman 2013).

PEL cells predominantly harbour latent infection where most transcripts are expressed from a region at the far right end of the genome (Damania and Cesarman 2013). Latency-associated nuclear antigen-1 (LANA-1), vCyclin and vFLIP are transcribed through a program of alternative splicing and internal translational initiation. The kaposin locus gives rise to a family of proteins; kaposin A, B, and C, as well as miRNAs (Damania and Cesarman 2013). In KS lesions, the main

proliferating cell is called the spindle cell because it has spindle-like shape and it is thought to be of endothelial lineage because it expresses markers of endothelial origins (Gessain and Duprez 2005, Damania and Cesarman 2013). It has been demonstrated that the viral FLIP induces the spindle cell phenotype in the KSHV infected endothelial cells and to up-regulate cytokine production (Matta, Surabhi et al. 2007). Interestingly, viral gene expression is different between latent PEL and latent KS cells. For example, LANA-2/ viral interferon regulatory factor 3 (vIRF3) is a B-cell specific latency gene that is expressed in latently infected PEL, but not detected in KS tumours (Rivas, Thlick et al. 2001). In contrast, vIRF1 is expressed in latently infected KS cells but not in latently infected PEL (Dittmer 2003). EBV has at least four latent viral gene transcription patterns. Hence, it is not surprising that KSHV also has a complex latent gene expression pattern.

Hughes *et. al.* have recently shown that the ubiquitin-proteasome system is important for KSHV latency and lytic activation (Hughes, Wood et al. 2015). The authors used a small molecule inhibitor in order to inhibit the NEDDylation cascade in PEL cells. NEDDylation is an ubiquitination pathway that is dependent on the ubiquitin-like protein, NEDD8 (Xirodimas 2008). Following NEDDylation inhibition, the cells underwent cytotoxicity and exhibited lytic gene expression without viral DNA replication. This phenotype was partly attributed to the inhibition of NF- κ B signalling, which is required for KSHV latency, as a result of blocking the proteasomal degradation of I κ B α , an inhibitor of NF- κ B transcription factors. Furthermore, the authors demonstrated that suppression of NEDDylation prevented the viral pre-replication complex recruitment to the origin of lytic DNA replication (OriLyt).

In addition to the cellular factors and viral proteins, KSHV encoded miRNAs play important roles during KSHV latency such as inhibition of lytic gene expression (Lei, Bai et al. 2010, Lin, Liang et al. 2011, Plaisance-Bonstaff, Choi et al. 2014), promoting cell survival (Lei, Bai et al. 2010), and cancer development (Gottwein, Mukherjee et al. 2007, Forte, Raja et al. 2015). These roles are discussed in detail in section 1.2.2.2.5.

1.2.2.2 Latency transcripts

1.2.2.2.1 Latency-associated nuclear antigen (LANA)

LANA, the product of ORF73, is a large immunogenic protein expressed in all latently infected cells where it localises in the nucleus (Kedes, Lagunoff et al. 1997, Kellam, Boshoff et al. 1997, Rainbow, Platt et al. 1997). It is a multifunctional protein with DNA replication, anti-apoptotic, cell cycle and gene regulatory functions, most of which are exerted by interactions with other proteins (Kaul, Verma et al. 2007). It is essential for the establishment and maintenance of the latent viral episome in the nucleus. It activates the semiconservative replication of the episome by binding to DNA sequences within the terminal repeats; thus, showing functional resemblance to EBV EBNA-1 that binds to the *oriP* sequence (Hu, Garber et al. 2002, Grundhoff and Ganem 2003).

Furthermore, LANA tethers the viral episome to host chromosomes during cell division, allowing for efficient segregation of the viral DNA to both daughter cells (Ballestas, Chatis et al. 1999, Cotter and Robertson 1999). It is also involved in the regulation of multiple pathways affecting cell cycle and survival; nevertheless,

LANA itself is not sufficient for transformation (Watanabe, Sugaya et al. 2003). Lan *et al.* demonstrated that LANA inhibits lytic replication through negative regulation of the expression of the lytic trans-activator RTA and antagonising its function (Lan, Kuppers et al. 2004).

Two groups demonstrated that LANA interacts with the cellular KRAB-associated protein 1 (KAP1) leading to down-regulation of lytic gene expression in order to promote latency (Sun, Liang et al. 2014, Zhang, Zhu et al. 2014). LANA has been shown to promote oncogenesis by interacting with the MH2-M, the LANA-binding domain of bone morphogenetic protein (BMP)-activated pSmad1 (Liang, Hu et al. 2014).

1.2.2.2.2 Viral Cyclin

vCyclin, a product of ORF72, is a homologue of the cellular protein cyclin D and acts in a similar manner in regulating the cell cycle. It forms functional complexes with the cellular cyclin-dependent kinase 6 (cdk6), leading to phosphorylation of pRB and thereby stimulating cell cycle progression (Van Dross, Yao et al. 2005). However, there are differences between vCyclin and the cellular cyclin D. For example, vCyclin has a wider range of substrates such as histone H1 (Godden-Kent, Talbot et al. 1997) and origin recognition complex 1 (Laman, Coverley et al. 2001), suggesting that vCyclin may regulate multiple cell-cycle phases. It has been suggested that vCyclin mediates phosphorylation of nucleophosmin (NPM) via its association with CDK6 and facilitates NPM-LANA interaction and recruitment of HDAC1, enhancing KSHV latency (Sarek, Jarviluoma et al. 2010).

1.2.2.2.3 Viral FLIP

vFLIP (also referred to as K13), a product of ORF71, is the viral homologue of the cellular FLIP (FLICE (FADD like interleukin1 beta-converting enzyme, now called caspase8) inhibitory protein). vFLIP up-regulates the anti-apoptotic transcription factor NF κ B by interacting with the inhibitory κ B kinase complex (Liu, Eby et al. 2002). Activation of the NF κ B signalling pathway has several effects, including inhibition of apoptosis by up-regulation of several anti-apoptotic molecules and enhancing cell proliferation (Guasparri, Keller et al. 2004, Chugh, Matta et al. 2005).

In addition, vFLIP-mediated activation of NF κ B signalling pathway results in suppression of the AP-1 pathway, which is critical for KSHV lytic replication and thus vFLIP can inhibit productive replication and promote latency (Ye, Zhou et al. 2008). Consistent with the previous findings, Zhao *et. al.* demonstrated that vFLIP is able to antagonise RTA and inhibit lytic protein expression leading to inhibition of infectious virus production (Zhao, Punj et al. 2007). On the other hand, during lytic reactivation, RTA suppresses the vFLIP-mediated NF κ B activation through vFLIP proteasomal degradation (Ehrlich, Chmura et al. 2014).

vFLIP has also been reported to induce the spindle cell phenotype seen in KSHV infected endothelial cells and to up-regulate cytokine production (Sun, Matta et al. 2006, Matta, Surabhi et al. 2007).

1.2.2.2.4 Kaposins

The kaposin proteins are encoded by alternative splicing of ORF12 giving rise to at least three proteins, kaposin A, B, and C (Sadler, Wu et al. 1999). Kaposin A is a

transmembrane protein that can transform rodent fibroblasts (Muralidhar, Pumfery et al. 1998). Kaposin A-transformed cells exhibit enhanced activation serine/threonine kinase pathways, some of which are involved in cell proliferation (Muralidhar, Veytsmann et al. 2000). Kliche *et. al.* demonstrated that kaposin A interacts with cytohesin-1, a guanine nucleotide exchange factor for ADP Ribosylation Factor (ARF) GTPases and a cell adhesion regulator, resulting in activation of the ERK-1/2 MAP kinase signalling pathway, which is thought to be involved in transformation (Kliche, Nagel et al. 2001).

Kaposin B was reported to enhance the expression of cytokines by inhibiting the degradation of their mRNAs (McCormick and Ganem 2005). Cytokine mRNAs contain AU-rich elements (AREs) in the 3'UTR that makes them unstable and exposed to degradation. Kaposin B stabilises the cytokine mRNAs by activating the kinase MK2, a target of the p38 mitogen-activated protein kinase signalling pathway and an inhibitor of ARE-mRNA decay (McCormick and Ganem 2005). Kaposin B contributes to the chronic inflammatory environment through activation of the proto-oncogene STAT3 and inactivation the STAT3 transcriptional repressor TRIM28 in MK2 –dependent manner (King 2013).

1.2.2.2.5 KSHV miRNAs

KSHV encodes at least 12 primary miRNAs located in the KSHV latency-associated region (KLAR) of the genome (Cai, Lu et al. 2005, Pfeffer, Sewer et al. 2005, Samols, Hu et al. 2005). KSHV miRNAs play important roles in maintaining latent infection. miR-K12-7 and 9 inhibit the expression of the latent-lytic molecular switch protein RTA that is crucial for initiation of lytic viral replication (Bellare and Ganem

2009, Lin, Liang et al. 2011). miR-K12-4-5p inhibits the expression of retinoblastoma (Rb)-like protein 2 (Rbl2), which is an inhibitor of DNA methyltransferases (DNMT)-1-3a and -3b whose activity maintains methylation of the RTA promoter and inhibit its transcription (Lu, Stedman et al. 2010). Furthermore, miR-K12-1 inhibits expression of I κ B α , a suppressor of NF- κ B complexes, enhancing NF- κ B-dependent viral latency and cell survival (Lei, Bai et al. 2010). miR-K12-3 can also promote KSHV latent infection via targeting the nuclear factor I/B, an activator of RTA promoter (Lu, Li et al. 2010). In contrast, miR-K12-5 and 9 inhibit the Bcl-2-associated factor (BCLAF1), resulting in an increase in lytic replication (Ziegelbauer, Sullivan et al. 2009).

miR-K12-11 is an orthologue of the cellular miR-155, which plays key roles in physiological lymphocyte development and activation but its overexpression is associated with lymphoid cancer development (Eis, Tam et al. 2005, Gottwein, Mukherjee et al. 2007, Rodriguez, Vigorito et al. 2007, Skalsky, Samols et al. 2007). It was demonstrated that miR-K12-11 and miR-155 target a common set of mRNAs and that miR-K12-11 plays a role in B cell transformation in humanised mice and can complement the deficiency of miR-155 *in vivo* (Gottwein, Mukherjee et al. 2007, Skalsky, Samols et al. 2007, Boss, Nadeau et al. 2011, Sin, Kim et al. 2013).. A KSHV mutant virus lacking a cluster of 10 primary miRNAs revealed that the KSHV miRNAs are crucial for cellular transformation and oncogenesis in primary rat mesenchymal cells as well as for cell survival and apoptosis inhibition (Moody, Zhu et al. 2013).

1.2.2.3 KSHV associated diseases

1.2.2.3.1 Kaposi's sarcoma

KS lesions occur in several tissues, but are most commonly located in the skin where they form brownish-purple raised nodules. The disease has been reported in four epidemiological forms: classic, endemic, immunocompromised, and HIV-associated KS. The classic form usually appears mostly in the toes and soles of elderly men from Mediterranean or eastern European regions (Fenig, Brenner et al. 1998, Iscovich, Boffetta et al. 1998, Damania and Cesarman 2013). Endemic KS has originally been described in young African people from sub-Saharan Africa. This form is aggressive and not related to HIV (Cook-Mozaffari, Newton et al. 1998, Olsen, Chang et al. 1998). The immunosuppression-associated KS form usually appears during immunosuppression or post-transplantation (Qunibi, Al-Furayh et al. 1998, Luppi, Barozzi et al. 2000). The HIV-associated KS occurs in AIDS patients and is presented with cutaneous lesions that can spread to other organs and it is more common among AIDS patients than renal transplant recipients (Beral, Peterman et al. 1990).

Histologically KS lesions are different from other classical tumours and are more complex. The main proliferating cell is called the spindle cell because it has spindle-like shape and it is thought to be of endothelial lineage because it expresses markers of endothelial origins (Damania and Cesarman 2013). KSHV is required for the development of KS. Various observations support this, including the correlation of seroprevalence and KS risk and the presence of KSHV DNA in all KS tumours where almost all spindle cells are latently infected (Boshoff and Weiss 2001).

1.2.2.3.2 Primary effusion lymphoma (PEL)

PEL is commonly present as a lymphomatous effusion in the body cavity and is more common in male HIV patients; however, HIV-negative males and females can also develop PEL (Damania and Cesarman 2013). B cells within the tumour undergo malignant clonal expansion and every tumour cell is KSHV-positive (Boshoff and Weiss 2001). Tumour cells can be co-infected with EBV; however, PEL is not present without KSHV infection (Damania and Cesarman 2013). KSHV-infected PEL cells express latent viral proteins and viral IL-6 (Parravicini, Chandran et al. 2000) that can be involved in oncogenesis. vFLIP and vIRF-3 are required for proliferation and survival of the cultured PEL cells (Guasparri, Keller et al. 2004, Wies, Mori et al. 2008)

1.2.2.3.3 Multicentric Castleman's disease (MCD)

MCD is a rare systematic lymphoproliferative disorder characterised by hyperactivation of the immune system, proliferation of lymphocytes, excessive cytokine production, and multiple organ impairment (Fajgenbaum, van Rhee et al. 2014). MCD occurs frequently in AIDS patients associated with KSHV infection; whereas only 40-50% of HIV-negative cases are associated with KSHV (Boshoff and Weiss 2001). During MCD, the plasmablasts localised in the mantle zones surrounding the germinal centres harbour KSHV virus (Dupin, Diss et al. 2000, Du, Liu et al. 2001).

It is suggested that KSHV infects naïve B-cells and induces their differentiation into plasmablasts, bypassing the germinal centre reaction (Du, Liu et al. 2001). It has been shown that vIL-6 is expressed constitutively in the plasmoblasts surrounding the lymphoid follicles, suggesting that the systematic symptoms observed in the MCD can be due to the excessive vIL-6 cytokine production (Du, Liu et al. 2001).

1.3 Gammaherpesviruses of veterinary importance

Several gammaherpesviruses are of veterinary and scientific importance. They can cause huge economic loss, particularly in the livestock industry. MHV-68 provides a unique amenable small animal model for studying gammaherpesvirus pathogenesis in the context of natural virus-host interaction. MHV-68 is introduced in section 1.5.

One of the important gammaherpesviruses in the veterinary field is the ovine herpesvirus-2 (OvHV-2). OvHV-2 is the causative agent of malignant catarrhal fever (MCF), a fatal lymphoproliferative disorder of cattle and some wild ruminants that is characterised by fever, ocular and nasal discharge, bilateral keratitis, and corneal opacity with death occurring a few days to several weeks after the onset of clinical symptoms (Russell, Stewart et al. 2009, Li, Cunha et al. 2014). Sheep are the reservoir hosts in which OvHV-2 infection is asymptomatic, but they can spread the infection to susceptible cattle (Russell, Stewart et al. 2009, Li, Cunha et al. 2014).

OvHV-2 predominantly infects CD2⁺ T cells in sheep and cattle, but only cattle develop IL-2 dependent T cell proliferation and MCF disease (Schock, Collins et al. 1998, Meier-Trummer, Ryf et al. 2010). Immortalised latently infected T cell lines can be cultured from infected cattle large granular lymphocytes (Reid, Buxton et al. 1989). The mechanism(s) behind the development of MCF pathogenesis is poorly understood.

OvHV-2 has been predicated to encode at least 45 candidate miRNAs using next-generation sequencing, from which 35 miRNAs have been validated by northern blot

or qRT-PCR in an OvHV-2-immortalised T cell line (Levy, Hopkins et al. 2012, Nightingale, Levy et al. 2014). Riaz *et. al.* demonstrated that a number of OvHV-2 miRNAs target viral mRNAs involved in latency and reactivation (Riaz, Dry et al. 2014), suggesting that these miRNAs may play roles in facilitating OvHV-2 latent infection.

Other animal gammaherpesviruses such as bovine herpesvirus-4 and equid herpesvirus-2 are associated with a variety of clinical symptoms in cattle and horses, respectively. However, the exact molecular mechanisms behind their pathogenesis are still largely unknown.

1.4 Murine gammaherpesvirus-68 (MHV-68)

MHV-68 (also referred to as murid herpesvirus-4 (MuHV-4) and γ HV-68) was originally isolated from bank voles (*Myodes glareolus*) and yellow-necked field mice (*Apodemus flavicollis*) captured in Slovakia (Blaskovic, Stancekova et al. 1980). Other closely related viruses were also isolated from field voles (*Microtus agrestis*), wood mice (*Apodemus sylvaticus*), and white-toothed shrews (*Crocidura russula*) (Chastel, Beaucournu et al. 1994, Hughes, Kipar et al. 2010). In the United Kingdom, a survey showed that MHV-68 is endemic in wood mice, whereas it is not endemic in bank voles, suggesting that wood mice are the major MHV-68 reservoir (Blasdell, McCracken et al. 2003). Laboratory mice (*Mus musculus*) can be infected with MHV-68 where the virus can establish lifelong latent infection mainly in B cells, macrophages, and splenic dendritic cells (Sunil-Chandra, Efstathiou et al. 1992, Weck, Kim et al. 1999, Flano, Husain et al. 2000).

MHV-68 is genetically related to and has similar pathogenic features as EBV and KSHV (Virgin, Latreille et al. 1997, Barton, Mandal et al. 2011). Due to strict species specificity of human gammaherpesviruses, it is difficult to study the different aspects of their pathogenesis *in vivo*; therefore, MHV-68 is considered a valuable small animal model for studying the pathogenesis of gammaherpesviruses. In addition, cloning of the MHV-68 genome into a bacterial artificial chromosome (BAC) can allow for studying viral genes functions by generating virus mutants (Adler, Messerle et al. 2000).

1.4.1 MHV-68 genome

The complete MHV-68 genome has been sequenced and aligned to other gammaherpesviruses sequences (Virgin, Latreille et al. 1997). The genome comprises a unique sequence of approximately 118 bp flanked by several copies of 1,213 bp terminal repeat and contains 2 internal repeats: 100 bp and 40 bp internal repeats (Virgin, Latreille et al. 1997). The MHV-68 genome is co-linear with primate gammaherpesviruses genomes (Figure 1.3) and it encodes at least 80 open reading frames (ORFs), most of which are homologous to other gammaherpesviruses or cellular genes; however, there are a number of unique MHV-68 ORFs whose sequences are not related to viral or cellular genes, termed M1, M2, M3, and M4 and located at the left end of the genome (Figure 1.3) (Virgin, Latreille et al. 1997). In addition, this site of the genome also encodes unique 8 tRNA-like molecules associated with at least 15 miRNAs (see sections 1.5.4.6.1 and 1.5.4.6.2)

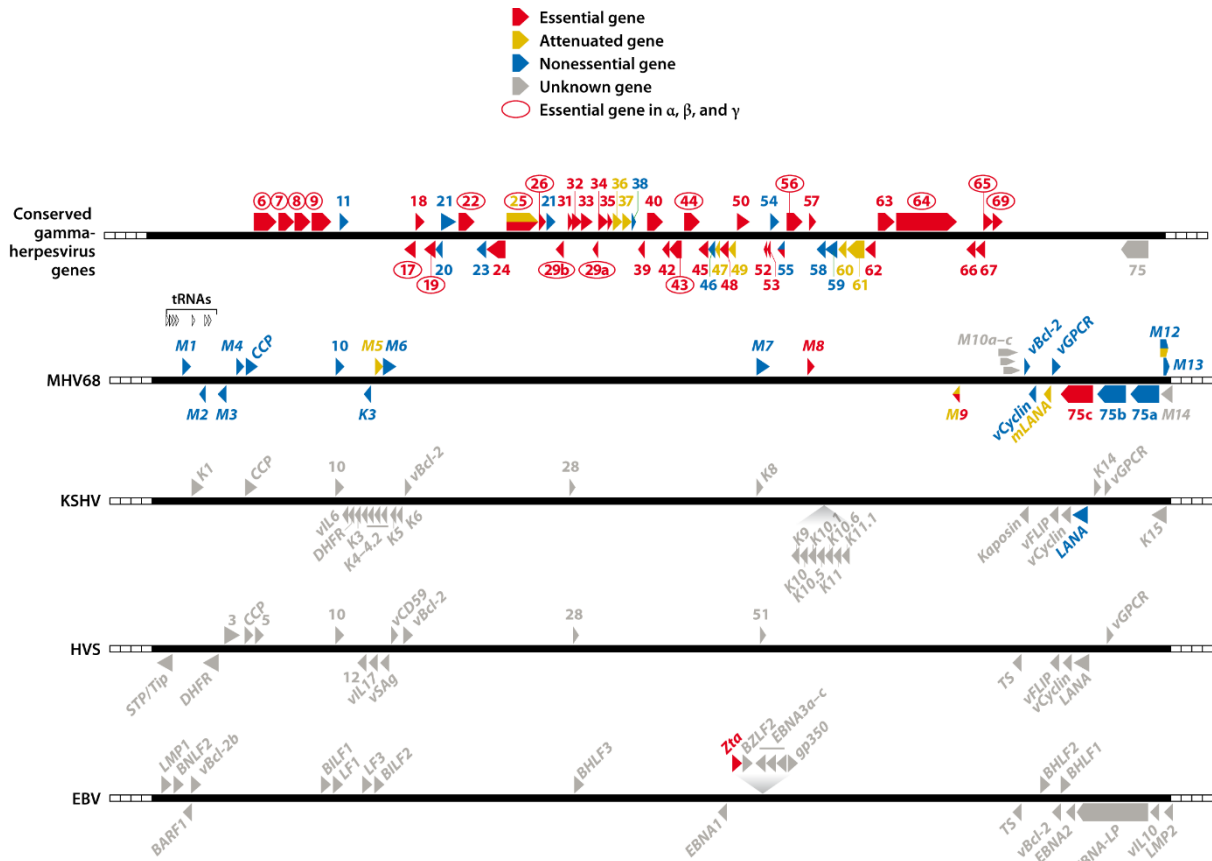


Figure 1.3 Schematic diagram of gammaherpesvirus genomes showing their conserved and unique genes. Conserved genes are shown at the top. Essential genes that play a critical role in MHV-68 lytic replication in fibroblasts are indicated (based on signature-tagged transposon mutagenesis (Moorman, Lin et al. 2004, Song, Hwang et al. 2005)). Figure adapted from (Barton, Mandal et al. 2011).

(Virgin, Latreille et al. 1997, Zhu, Strehle et al. 2010). The genes that play a key role in MHV-68 replication were identified by transposon mutagenesis analyses and it was found that the MHV-68 unique genes are not involved in replication and thus they are suggested to be latency associated genes (Moorman, Lin et al. 2004, Song, Hwang et al. 2005) (Figure 1.3).

1.4.2 MHV-68 pathogenesis

The natural route of MHV-68 infection is unknown. However, the respiratory route is thought to be the natural route of infection (Nash, Dutia et al. 2001). Attempts to transmit the virus horizontally between laboratory mice by cage contact were unsuccessful (Aligo, Brosnan et al. 2014), therefore the route of virus transmission in natural host is not clear.

When laboratory mice are infected intranasally with MHV-68 virus, the virus undergoes lytic replication in the lung where it replicates in the alveolar epithelial cells and reach peak lytic replication at 5 days post infection and clearance of this lytic replication occurs at 10 days after infection (Sunil-Chandra, Efstathiou et al. 1992); however, long-term virus persistence in lung epithelial cells was also reported (Stewart, Usherwood et al. 1998). From the lung, the virus spreads to the mediastinal lymph nodes (MLN) and then to the spleen where virus latency is established predominately in B lymphocytes; however, latent virus also exists in macrophages and dendritic cells (Weck, Kim et al. 1999, Flano, Husain et al. 2000, Nash, Dutia et al. 2001, Flano, Kim et al. 2002, Kim, Flano et al. 2003, Willer and Speck 2003).

MHV-68 latency is a dynamic process that involves viral components working in concert with cellular factors. The latent virus amplification in the spleen reaches peak

levels at 14 days post-infection (dpi) (acute phase of latent infection) accompanied by enlargement of the spleen (splenomegaly) due to B cell proliferation that is driven by CD4⁺ T cell response (Sunil-Chandra, Efstathiou et al. 1992, Ehtisham, Sunil-Chandra et al. 1993, Usherwood, Ross et al. 1996). By day 21 post infection, the splenomegaly starts to resolve as the activated CD8⁺ T cells increase in numbers in peripheral blood, killing MHV-68-infected cells by cytotoxicity (Tripp, Hamilton-Easton et al. 1997). This pathology (splenomegaly and activated CD8⁺ T cells in the peripheral blood) is reminiscent of the EBV-induced IM. The MHV-68 pathogenesis is summarised in figure 1.4

1.4.3 MHV-68 life cycle

1.4.3.1 MHV-68 primary lytic infection

MHV-68 undergoes primary lytic infection in alveolar epithelial cells in the lung following intranasal infection of lab mice (Sunil-Chandra, Efstathiou et al. 1992). The host immune defence controls acute MHV-68 replication via CD8⁺ T cells, CD4⁺ T cells, and interferon gamma (IFN γ) by reducing the numbers of infected cells (Stevenson and Doherty 1998, Sparks-Thissen, Braaten et al. 2005). Antiviral agents such as thiopyrimidine analogue 2'-deoxy-5-ethyl-beta-4'-thiouridine (4'-S-EtdU) can target viral thymidine kinase resulting in inhibition of virus replication *in vitro* and *in vivo* without affecting latency establishment (Barnes, Dyson et al. 1999).

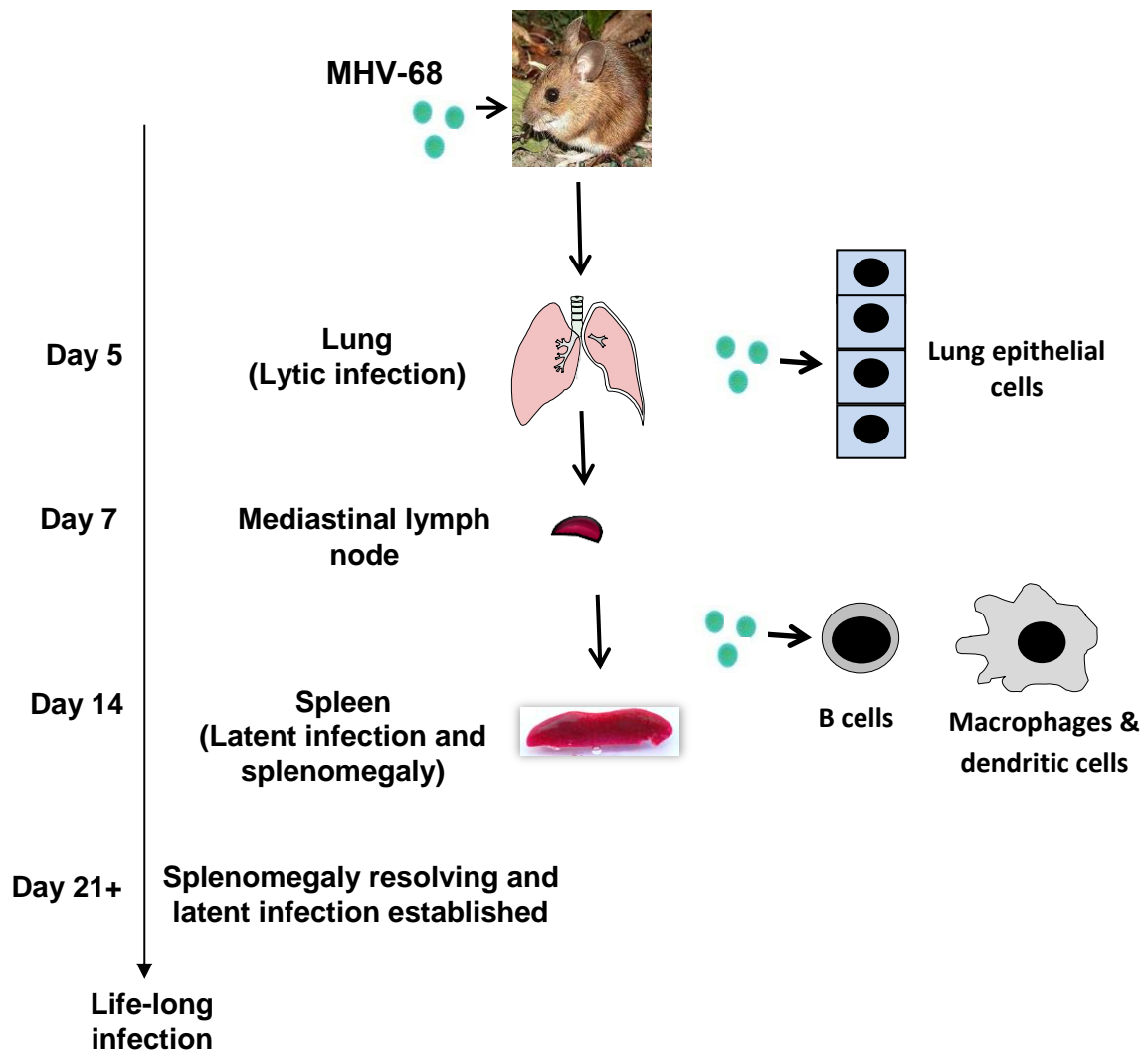


Figure 1.4 MHV-68 pathogenesis. The virus causes primary lytic infection in the lung epithelial cells with peak of virus replication at 5 days post infection (dpi) before it spreads to the spleen via the mediastinal lymph node to establish latent infection in B cells, macrophages, and dendritic cells with the peak of latent viral titre occurring at 14 dpi and associated with clonal B cell expansion and splenomegaly. After 3 weeks post infection, the splenomegaly resolves and the latent infection is established for life.

The immediate-early protein encoded by ORF50, the replication and transcription activator (RTA), plays a key role in initiating virus lytic replication during *de novo* infection and reactivation from latency resulting in release of infectious virus (Wu, Usherwood et al. 2000, Wu, Tong et al. 2001, Pavlova, Virgin et al. 2003, Boname, Coleman et al. 2004). RTA is conserved among all known gammaherpesviruses and has a DNA binding domain at its N-terminus and a transactivation domain at its C-terminus allowing for acting as a transcriptional activator of many viral genes (Wu, Tong et al. 2001). Like MHV-68 RTA, the RTA of KSHV acts as a molecular switch essential for initiation of complete virus lytic cycle and for reactivation (Sun, Lin et al. 1998, Lukac, Kirshner et al. 1999). In EBV, RTA in association with another immediate-early protein, ZTA (also referred to as ZEBRA or Z), initiates the viral lytic replication through activation of lytic gene expression (Gruffat and Sergeant 1994, Liu, Sista et al. 1996).

ORF49 was reported to interact with the RTA negative regulator, poly (ADP-ribose) polymerase-1 (PARP-1), resulting in derepression of the PARylated RTA and thus promote RTA transactivation, leading to enhanced viral replication (Noh, Cho et al. 2012). In addition, ORF49 can be packaged into virions as an inner tegument protein, allowing ORF49 to exert its function during the early phase of *de novo* lytic infection (Noh, Cho et al. 2012)

1.4.3.2 MHV-68 latent infection

After clearance of lytic virus from the lung a low level of virus persists in epithelial cells and during long-term latency in B cells (Stewart, Usherwood et al. 1998, Flano, Kim et al. 2003). The lung has been reported to be a major site for virus persistence

(Stewart, Usherwood et al. 1998). The virus spreads from lung to the mediastinal lymph nodes where it establishes latency in B cells, macrophages, and dendritic cells (Nash, Dutia et al. 2001) followed by latency establishment in splenic B cells, macrophages, and dendritic cells at 10-14 days post-infection (dpi) and the latent virus load reaches peak levels at 14 dpi (Sunil-Chandra, Efstathiou et al. 1992, Weck, Barkon et al. 1996, Flano, Husain et al. 2000). During latency establishment in the spleen, the B cells undergo CD4⁺ T cell-dependent proliferation resulting in an increase in numbers of infected B cells and splenomegaly (Usherwood, Ross et al. 1996). The numbers of latently infected cells decrease to low levels during long-term latency as a result of cell-mediated host immune response and the long-term latency is maintained in germinal centre and memory B cells (Flano, Kim et al. 2002, Willer and Speck 2003, Evans, Moser et al. 2008, Nealy, Coleman et al. 2010, Jennings, Grayson et al. 2014).

Three weeks after infection, the CD4⁺ T cells decrease in number and the CD8⁺ T cells increase in numbers leading to destruction of large numbers of infected cells (Tripp, Hamilton-Easton et al. 1997). These pathological features are reminiscent of the IM of EBV (Williams and Crawford 2006). Therefore, there are at least 2 stages of latency in the spleen: the early latency (acute latency), during which the viral latent load is maximum and the expression of a number of latency-associated genes can be readily detected, and the long-term latency, during which the viral latent load is very low and viral gene expression is rarely detected and the virus remains latent for life within the host with occasional spontaneous reactivation, which possibly helps boost latent infection by infecting naïve B cells; thus, the long-term latent infection depends on a precise balance between latency and reactivation. The exact

molecular mechanisms behind the establishment and maintenance of latency are not fully understood.

Recently, it has been reported that MHV-68 miRNAs are dispensable for productive infection nor establishment of latency *in vivo* , but can modulate reactivation in a virus dose-dependent manner and increase the numbers of MHV-68-infected memory B cells *in vivo*, and promote virus-induced lethal pneumonia in immunodeficient mice (Feldman, Kara et al. 2014, Diebel, Oko et al. 2015).

1.4.3.3 MHV-68 reactivation from latency

Periodic reactivation from latency is a crucial process that can contribute to the homeostatic maintenance of long-term MHV-68 infection through re-seeding latency reservoirs; however, the balance between latency and reactivation has to be tightly regulated in order to guarantee successful virus infection. Spontaneous reactivation of MHV-68 virus during long-term latent infection was reported in previous published studies (Liu, Flano et al. 1999, Hwang, Wu et al. 2008).

The molecular mechanisms governing MHV-68 reactivation control and induction are not completely understood. Viral and cellular influences can modulate reactivation. For example, the cellular CD8⁺ T cell response can control virus reactivation through long-term IFN γ production (Evans, Moser et al. 2008). The viral protein RTA encoded by ORF50 is a major key player in virus lytic reactivation as it can induce lytic gene expression and initiate complete virus lytic cycle (Wu, Usherwood et al. 2000). It has been shown that there is differential expression of the RTA transcript in spleen cell subpopulations, for example, RTA mRNA was detected

in macrophages and dendritic cells, but not in B cells during the establishment of latent infection (Marques, Efstathiou et al. 2003), indicating that the mechanism of latency establishment can vary depending on the infected cell type. Alternatively, this may suggest that macrophages and dendritic cells may not support latent infection.

Reactivation from latency is different from primary productive infection for many reasons: (1) the primary lytic infection cycle starts by attachment cell surface and entry into host cells, whereas initiation of reactivation is from inside of the latently infected cells; (2) primary lytic infection occurs in lung epithelia cells, whereas reactivation occurs mainly in peripheral lymphoid organs; (3) the immune response is stimulated following the primary lytic infection and therefore virus reactivation occurs after the specific antibodies and T cell response have been generated. This suggests that primary lytic infection and virus reactivation may require different molecular mechanisms.

1.4.4 MHV-68 latent genes

1.4.4.1 ORF73 (mLANA)

MHV-68 ORF73 encodes a homolog of KSHV LANA (referred to as mLANA) that comprises the conserved C-terminal domain and lacks the N-terminal domains and the long internal repeat present in the LANA encoded by KSHV (Russo, Bohenzky et al. 1996, Virgin, Latreille et al. 1997). The MHV-68 mutant viruses lacking mLANA expression are deficient in establishment of latency after intranasal infection, which is consistent with the role of the mLANA in maintaining the viral

genome as an extrachromosomal episome in the nuclei of the dividing cells (Fowler, Marques et al. 2003, Moorman, Willer et al. 2003). However, a mLANA-null mutant virus was able to establish latent infection when the virus was inoculated intraperitoneally or when immunocompromised mice were used in the study (Paden, Forrest et al. 2010). In addition to the critical role played by mLANA during the establishment and maintenance of latency, it plays a role in acute lytic replication and it is essential for virus reactivation from latency (Forrest, Paden et al. 2007, Paden, Forrest et al. 2010). mLANA-null mutant MHV-68 virus exhibited an increase in viral lytic gene expression and induction of rapid cell death, a phenotype that is suggested to be due to the role played by mLANA in stabilising p53 (Forrest, Paden et al. 2007).

1.4.4.2 M1

The left end of MHV-68 genome encodes 4 unique proteins, designated M1-4, that are suggested to play roles during latent infection. The M1 secreted protein has sequence homology (~25%) to another MHV-68 protein, M3, that functions as a chemokine binding protein, and to the poxvirus serine proteinase inhibitor (serpin), SPI-1, that is involved in regulating apoptosis and in regulating T cell cytotoxicity in conjunction with another poxvirus serpin, SPI-2 (Ali, Turner et al. 1994, Brooks, Ali et al. 1995, Macen, Garner et al. 1996, Virgin, Latreille et al. 1997, van Berkel, Preiter et al. 1999). Despite the sequence homology between M1 and SPI-1, M1 lacks sequence homology to the conserved functionally important hinge domain and reactive site loop existing in SPI-1 and other serpins (Potempa, Korzus et al. 1994). A mutant MHV-68 virus lacking M1 expression exhibited hyper-reactivation phenotype, indicating a role of M1 in suppressing virus reactivation, possibly

through induction of V β 4+ CD8+ T cell response and long-term IFN γ production in (Clambey, Virgin et al. 2000, Evans, Moser et al. 2008).

1.4.4.3 M2

M2 is a membrane-associated protein that can be expressed during latent infection *in vitro* in the latently infected B lymphoma cell line S11 and *in vivo* in spleen (Husain, Usherwood et al. 1999, Virgin, Presti et al. 1999). M2 was reported to contain an actively recognised CD8+ T cell epitope and to be important in the establishment of and reactivation from latency (Jacoby, Virgin et al. 2002, Macrae, Usherwood et al. 2003, Simas, Marques et al. 2004, Herskowitz, Jacoby et al. 2005). It has been reported that M2 interacts with the cellular Vav proteins and thus modulates the B cell receptor signalling pathway in order to promote latency through enhancing B cell activation, proliferation, and survival (Madureira, Matos et al. 2005).

M2 is not involved in MHV-68-induced B cell clonal expansion nor V β 4+ CD8+ T cell expansion, but it is required for differentiation of B cells into memory B cells and plasma cells (Macrae, Usherwood et al. 2003, Simas, Marques et al. 2004, Herskowitz, Jacoby et al. 2005, Liang, Collins et al. 2009). M2 expression in primary murine B cells resulted in increased proliferation and survival of the cells that was IL-10 dependent (Siegel, Herskowitz et al. 2008). Moreover, the use of M2-transgenic mice revealed that M2 is required for regulation of antibody responses in a T-cell dependent manner and for inhibition of apoptosis in the infected B cells (de Oliveira, Almeida et al. 2013).

1.4.4.4 M3

M3 is a secreted protein that is highly expressed during lytic infection and it is also expressed during establishment of a latent infection, but not during long-term latency in the spleen (Simas, Swann et al. 1999, van Berkel, Preiter et al. 1999, Virgin, Presti et al. 1999, Marques, Efstathiou et al. 2003, Martinez-Guzman, Rickabaugh et al. 2003). It functions as a broad-spectrum chemokine binding protein that inhibits chemokine action (Parry, Simas et al. 2000, van Berkel, Barrett et al. 2000).

Disruption of M3 expression by insertion of a β -galactosidase expression cassette into the M3 had little effect on lytic replication in the lung, but significantly compromised latency establishment in mice infected with this mutant virus (Bridgeman, Stevenson et al. 2001). Another study that used M3 null virus generated by insertion of a stop codon and frame shift near the 5' end of M3 ORF, designated M3.stop, revealed that the M3.stop mutant virus had similar levels of latency establishment and reactivation to that of its wild-type counterpart (van Berkel, Levine et al. 2002). However, this mutant virus was 100-fold less virulent than the wild-type virus following intracerebral virus inoculation into 3 weeks old mice (van Berkel, Levine et al. 2002). The lack of M3 expression caused significant reduction in latency and impaired germinal centre reaction in M3.stop-infected wood mice (Hughes, Kipar et al. 2011).

1.4.4.5 M4

M4 is a secreted glycosylated protein expressed by the M4 gene (Evans, Moorman et al. 2006). M4 is expressed during productive infection in the lung and during the establishment of latency in the spleen, but not during long-term latency (Marques, Efstathiou et al. 2003, Townsley, Dutia et al. 2004, Evans, Moorman et al. 2006).

The role(s) of M4 in MHV-68 pathogenesis is not completely understood. However, it is suggested that M4 can modulate lytic and latent infection *in vivo* (Townsend, Dutia et al. 2004, Evans, Moorman et al. 2006, Geere, Ligertwood et al. 2006). M4 is believed to be a chemokine-binding protein (Dutia *et. al.*, unpublished).

1.4.4.6 v-Cyclin (ORF72)

MHV-68 v-Cyclin is encoded by ORF72 and it is a homologue of the cellular cyclin D and is conserved among rhabdoviruses. Transgenic mice expressing MHV-68 v-Cyclin under the control of the lymphocyte-specific protein tyrosine kinase (LCK) proximal promoter showed development of T cell lymphoma (van Dyk, Hess et al. 1999). The crystal structure of MHV-68 v-Cyclin revealed that it interacts with cyclin-dependent kinase 1 (CDK1) and CDK2, thereby regulating cell cycle progression (Card, Knowles et al. 2000). Furthermore, the crystal structure showed that v-Cyclin is resistant to the inhibition by the CDK inhibitor CKI p27^{Kip1} (Swanton, Card et al. 1999).

v-Cyclin is expressed during both lytic and latent infections (Allen, Dickerson et al. 2006). An MHV-68 mutant virus lacking v-Cyclin expression and carrying a β -galactosidase expression cassette that was inserted in place of the v-cyclin ORF, showed a defect in acute virus replication and reactivation from latency (Hoge, Hendrickson et al. 2000). Further studies using v-Cyclin null MHV-68 and v-Cyclin CDK binding mutants demonstrated that these viruses exhibited a defect in lytic replication in the lung following low-dose intranasal inoculation, which was more severe as compared to that observed following intraperitoneal inoculation (Upton and Speck 2006). The same study showed that none of the mutant viruses induced

splenomegaly. The authors also found that although the v-Cyclin null mutant virus was attenuated for reactivation from peritoneal exudate cells, none of the CDK binding mutants were defective in reactivation from these cells, suggesting that v-Cyclin has CDK-independent functions that are important for reactivation from the peritoneal macrophages.

The role of v-Cyclin during lytic replication was further characterised in the rat lung epithelial cell line, RL-65, showing that v-Cyclin plays a role in virus lytic replication and virus egress from the infected cells (Scott and Speck 2014).

1.4.4.7 vBcl-2 (M11)

vBcl-2 is encoded by the MHV-68 M11 gene and it is a homologue of the cellular Bcl-2. All known gammaherpesviruses encode at least one Bcl-2 homologue. M11 transcripts can be detected during lytic and latent infections (Virgin, Presti et al. 1999, Roy, Ebrahimi et al. 2000, Marques, Efstathiou et al. 2003). Similar to the cellular anti-apoptotic Bcl-2 family members, the MHV-68 vBcl-2 contains a hydrophobic BH3 domain-binding groove which is important for vBcl-2 anti-apoptotic function during latent infection *in vivo* (Loh, Huang et al. 2005). This domain is also important for binding to the pro-autophagic protein Beclin-1 and was able to suppress autophagy more efficiently than cellular Bcl-2 (Ku, Woo et al. 2008). This autophagy inhibition has been shown to be important for MHV-68 latency *in vivo* and it is distinguished from the vBcl-2 anti-apoptotic function (E, Hwang et al. 2009). Coleman *et. al.* have used vBcl-2 mutant MHV-68 viruses to demonstrate that vBcl-2 is required for inhibiting B cell receptor-mediated apoptosis in immature and transitional B cells, but not in mature B cells, suggesting that vBcl-2

is important for survival of and viral latency in developing B cell (Coleman, McGraw et al. 2014).

1.4.4.8 MHV-68 non-coding RNAs

1.4.4.8.1 MHV-68 tRNAs

MHV-68 encodes 8 viral tRNA-like molecules (vtRNAs) that are associated with at least 15 miRNAs (Bowden, Simas et al. 1997, Pfeffer, Sewer et al. 2005, Zhu, Strehle et al. 2010). The miRNAs are located immediately downstream of the vtRNAs. The vtRNAs and their associated miRNAs, also referred to as tRNA-miRNA-encoded RNAs (TMERs) (Feldman, Kara et al. 2014, Diebel, Oko et al. 2015), are clustered in the leftmost end of the genome between nucleotide position 127 to 5585, dispersed between the two unique open reading frames, M1 and M2. The vtRNAs can be detected during lytic and latent infection. They are predicted to have cloverleaf-like secondary structure and they contain RNA polymerase III (pol III) type 2 promoter elements that drive their expression (Bowden, Simas et al. 1997). Although the vtRNAs are processed into mature tRNAs with post-transcriptional addition of 3' CCA termini, which indicates that these vtRNAs are recognised as tRNAs by cellular machinery, they are not aminocylated by the cellular aminoacyl-tRNA synthetases and therefore they are unlikely involved in translation (Bowden, Simas et al. 1997).

It has been demonstrated that the vtRNAs are selectively packaged within the virions indicating that they may constitute an integral part of the infectious virus and that they may be required immediately upon entry into the cells (Cliffe, Nash et al. 2009).

A recent study has shown that MHV-68 virus lacking all the vtRNAs and miRNAs exhibited attenuation in lethal pneumonia in IFN γ knock-out mice, whereas expression of one vtRNA (vtRNA1) in the absence of all other vtRNAs and miRNAs partially reversed this attenuation in virulence, suggesting that at least one vtRNA may promote viral pathogenesis in immunocompromised mice (Feldman, Kara et al. 2014, Diebel, Oko et al. 2015). MHV-68 microRNAs are reviewed in section 1.6.

1.5 microRNAs

1.5.1 Discovery

miRNAs are short non-coding RNAs (~19-25 nucleotides) that regulate gene expression at the post-transcriptional level in multicellular organisms and play key roles in diverse biological processes (Zamore and Haley 2005, Bartel 2009). The first miRNA was discovered in 1993 during studies of genes controlling developmental timing of the nematode *C. elegans* (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993). It was found that the *lin-4* gene of *C. elegans* is not a protein coding gene, but rather it encodes small RNAs that had sequence complementarity to the 3' UTR of *lin-14* leading to interaction between the *lin-4* small RNA and the 3' UTR of *lin-14*, resulting in reduction of *lin-14* protein levels without affecting mRNA levels (Lee, Feinbaum et al. 1993, Wightman, Ha et al. 1993). Since then these small RNAs (miRNAs) have been found in almost all multicellular organisms as well as viruses and they affect different biological processes such as cellular differentiation, proliferation, apoptosis, development, and oncogenesis (Bartel 2004, Bartel 2009). The first virally encoded miRNAs were discovered in EBV and subsequent work identified miRNAs expressed by almost all herpesviruses as well as by viruses from

other families (Pfeffer, Zavolan et al. 2004, Cullen 2011, Kincaid and Sullivan 2012).

1.5.2 Biogenesis

Most cellular and viral miRNAs are transcribed by RNA polymerase II in the form of long primary stem-loop miRNAs (pri-miRNAs), containing one or multiple precursor(s) of imperfectly complementary stem-loop hairpin structure (pre-miRNAs) (Figure 1.6) (Winter, Jung et al. 2009, Bogerd, Karnowski et al. 2010, Kincaid and Sullivan 2012, Rosewick, Momont et al. 2013). The pre-miRNAs are freed from the pri-miRNAs in the nucleus by a microprocessor complex that consists of Drosha and DGCR8 (Slezak-Prochazka, Durmus et al. 2010). The pre-miRNAs are then exported out of the nucleus into the cytoplasm via Exportin 5 (XPO5) (Figure 1.6) (Lee, Jeon et al. 2002, Kincaid and Sullivan 2012).

The pre-miRNAs are cleaved in the cytoplasm by the endonuclease Dicer, resulting in a transient small RNA duplex (~ 22 nucleotides), of which one strand (guide strand) is stably incorporated into the RNA-induced silencing complex (RISC) and interact with its target mRNA by base pairing, whereas the other strand, called the star (*) or passenger strand, is less likely to associate with RISC and is usually found at lower levels than the guide strand (Winter, Jung et al. 2009). Argonaute protein (Ago) is a key component of the RISC complex and the Ago-loaded miRNAs (miRISC) bind to target transcripts and repress gene expression via mRNA degradation or translational inhibition (Winter, Jung et al. 2009). GW182 proteins are another group of proteins important for miRNA-induced downregulation (Liu, Rivas et al. 2005). Both GW182 and Ago proteins inhibit protein synthesis when

artificially tethered to the 3' UTR of the mRNA, suggesting that the RISC proteins act as effectors in the repression, while the miRNAs function mainly as guides that identify the target mRNAs (Pillai, Artus et al. 2004, Rehwinkel, Behm-Ansmant et al. 2005, Behm-Ansmant, Rehwinkel et al. 2006).

Some viral miRNAs use non-canonical biogenesis pathway. For example, the MHV-68 pri-miRNAs are transcribed from larger tRNA-like molecules by RNA pol III and are cleaved by the by host tRNase Z, but not Drosha, to liberate the pre-miRNAs (Bogerd, Karnowski et al. 2010). The BLV pre-miRNAs are transcribed directly by RNA pol III and do not require the Drosha cleavage step (Figure 1.6) (Cullen 2012, Rosewick, Momont et al. 2013). The herpesvirus saimiri (HVS) expresses Sm class U RNAs (HSURs) in the latently infected marmoset T cells. HSV does not utilise the microprocessor complex, but instead uses the host integrator complex to generate the 3' end of the HSURs and the pre-miRNA hairpins (Cazalla, Xie et al. 2011).

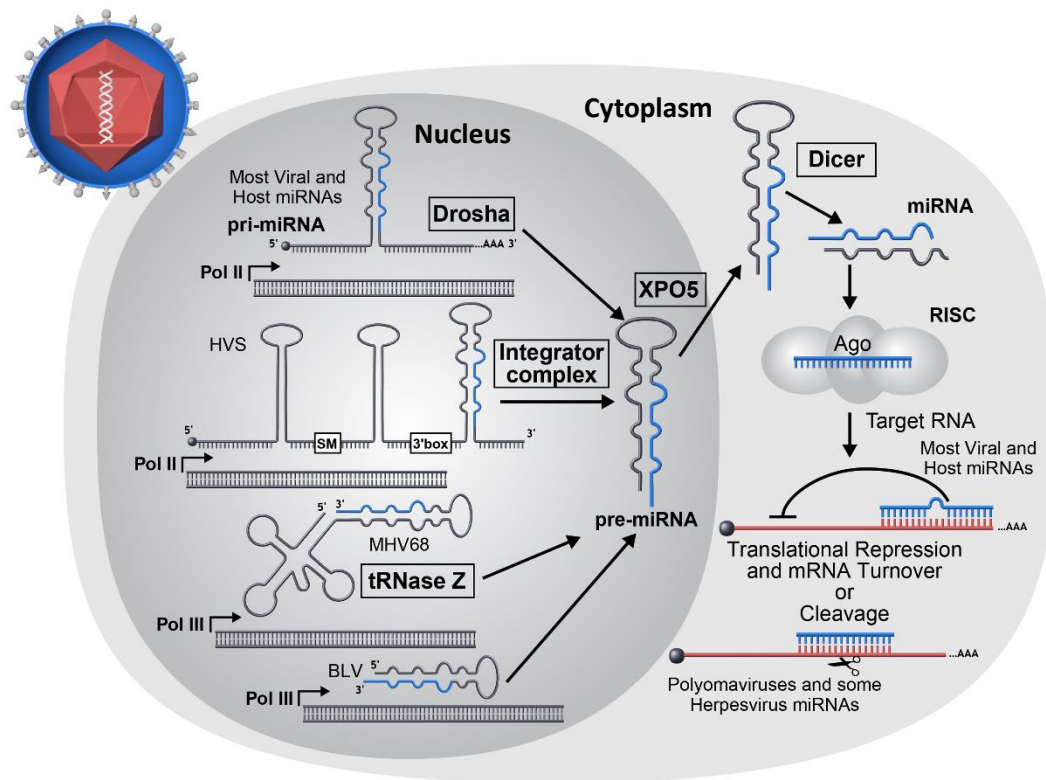


Figure 1.5 miRNAs biogenesis. Most of the host and viral miRNAs are transcribed as pri-miRNAs by host RNA pol II in the nucleus and then processed by the endonuclease Drosha to produce the pre-miRNAs. miRNAs of MHV-68 and BLV are transcribed by the RNA pol III. The MHV-68 pri-miRNAs are then processed by the host tRNase Z, but not Drosha, to generate the pre-miRNAs. BLV miRNAs are transcribed directly as pre-miRNAs and do not require Drosha processing. HVS pre-miRNAs are produced following the integrator complex processing of the HSURs. The pre-miRNAs are then transported to the cytoplasm by Exportin 5 (XPO5) and further processed by the cellular Dicer to form the short RNA duplex. One strand of this RNA duplex (the guide strand) is incorporated into an Argonaute (Ago) protein within the RISC complex and direct the RISC to the target mRNA for degradation or translational inhibition. Figure adapted from (Kincaid and Sullivan 2012).

1.5.3 miRNA-mRNA interaction

miRNAs interact with their target mRNAs by base pairing. In Plants, miRNAs base pair to mRNAs with almost perfect complementarity inducing endonucleolytic cleavage of the mRNA by an RNAi-like mechanism (Jones-Rhoades, Bartel et al. 2006). In contrast, the majority of the metazoan miRNAs base pair with their mRNA targets imperfectly, according to a set of rules determined by bioinformatic and experimental analyses (Bartel 2009, Pasquinelli 2012).

The first rule and a key determinant of the specific miRNA-mRNA base pairing is the perfect and contiguous base pairing of the nucleotide 2 to 8 in the 5' end of the miRNA, representing the 'seed' region, to the seed match site in the target mRNA, which is mostly located in the 3' UTR (Lewis, Burge et al. 2005). However, some miRNAs can specifically target the 5' UTR and/or the coding region of their mRNA targets (Lee, Ajay et al. 2009, Brümmer and Hausser 2014). Mismatches or GU pairs in the seed region can significantly affect the miRNA-mediated repression; nevertheless, an A residue across position 1 of the miRNA and an A or U across position 9 increase efficiency, although they do not need to base pair with the miRNA (reviewed in (Filipowicz, Bhattacharyya et al. 2008)).

Another rule is that mismatches or bulges must be existing in the middle region of the miRNA-mRNA duplex, precluding the Ago-mediated mRNA cleavage by endonucleolytic activity (Filipowicz, Bhattacharyya et al. 2008). The third rule is that there must be some degree of complementarity at the 3' half of the miRNA in order to stabilise the interaction, particularly at nucleotide positions 13-16 of the miRNA (Brennecke, Stark et al. 2005, Lewis, Burge et al. 2005, Grimson, Farh et al. 2007).

1.5.4 Mechanisms of translational repression

The mRNA translation comprises 3 steps: Initiation, elongation, and termination. Translation initiation involves the recognition of the mRNA 5'-terminal 7-methylguanosine (m7G) cap by the eukaryotic translation initiation factor 4E (eIF4E) subunit of the translation initiation factor eIF4F, which also contains eIF4A (an RNA helicase) and eIF4G (a large protein that acts as a scaffold for the assembly of the ribosome initiation complex (Hinnebusch and Lorsch 2012). eIF4G can concurrently interact with the eIF4E and the poly(A) binding protein 1 (PABP1), which is associated with the poly(A) tail, leading to circularisation of the mRNA that induces translation initiation and ribosome recycling (Filipowicz, Bhattacharyya et al. 2008).

eIF4G interacts with another initiation factor, eIF3, enabling the recruitment of the 40S ribosomal subunit, which scans the 5' UTR of the mRNA in search of the initiation codon, and after joining of the 60S ribosomal subunit the elongation stage proceeds (Van Der Kelen, Beyaert et al. 2009, Hinnebusch and Lorsch 2012). When the ribosome finds a termination codon, the ribosomal subunits dissociate from each other and from the mRNA resulting in translation termination. Some cellular and many viral mRNAs initiate translation in an m7G cap and eIF4E-independent manner and the 40S ribosome subunits are recruited to the mRNA via interaction with internal ribosome entry sites (IRES), which are located in the 5' UTR (Jackson 2005).

1.5.4.1 Inhibition of translation initiation

It was reported that the central domain of the human Ago2 protein shows sequence similarity to eIF4E, resulting in competition with eIF4E for m7G cap binding leading to inhibition of translation initiation at the cap recognition step (Kiriakidou, Tan et al. 2007). In the same study, the authors have shown that the two tryptophan residues (W56 and W102) of eIF4E, which bind to the m7G cap, correspond to two conserved phenylalanine residues (F470 and F505) in the Ago proteins and that the substitution of one or both phenylalanine residues of human Ago2 protein with valine resulted in abolition of the translational repression.

A previous published study has shown that the let-7 miRNA and its target mRNAs are associated with the free messenger ribonucleoprotein pool but not with the polysomal fraction in sucrose gradients in the mammalian cells, suggesting an miRNA-mediated inhibition of translation initiation (Pillai, Bhattacharyya et al. 2005). Moreover, the authors have shown that the IRES-mediated translation is resistant to repression by let-7 miRNA. This finding is consistent with other studies that showed that mRNAs translated through cap-independent mechanisms are immune to miRNA-mediated translation repression (Humphreys, Westman et al. 2005, Mathonnet, Fabian et al. 2007, Wakiyama, Takimoto et al. 2007).

In agreement with the notion that miRNAs can inhibit translation initiation, Mathonnet et. al. showed that addition of purified translation initiation complex eIF4F counteracted miRNA-mediated translation repression (Mathonnet, Fabian et al. 2007). Using siRNA screening for eIF4F components, it was recently revealed that the DEAD-box RNA helicase eIF4A2 plays a key role in the miRNA-mediated

silencing and that it favourably binds to the CCR4–NOT complex rather than to eIF4G (Meijer, Kong et al. 2013, Wilczynska and Bushell 2015).

1.5.4.2 Post-initiation mechanisms

1.5.4.2.1 Inhibition of ribosomal subunit association

Chendrimada et. al. showed that the depletion of eIF6, which is a ribosome inhibitory protein that prevents productive assembly of 80s ribosomes (Ceci, Gaviraghi et al. 2003), results in abrogation of miRNAs-mediated translational inhibition, suggesting that miRNAs can suppress translation at an early stage before elongation (Chendrimada, Finn et al. 2007). The authors reported that eIF6 associates with the 60s ribosomal subunit and Ago2 protein in human cells. These findings suggest that Ago2 may recruit eIF6 in order to disrupt the association of large and small ribosomal subunits and thus inhibit an early translation step. eIF6 is required for the biogenesis of the 60s ribosomal subunit (Miluzio, Beugnet et al. 2009, Brina, Miluzio et al. 2015). Therefore, another interpretation of Chendrimada's results is that eIF6 depletion may have secondary effects that may not yet be completely appreciated.

1.5.4.2.2 Premature ribosome dissociation (ribosome drop-off)

Peterson et. al. designed a synthetic luciferase reporter mRNA containing a 3' UTR that carries 6 identical sites partially complementary to a transfected miRNA mimic (Petersen, Bordeleau et al. 2006). In this system, although the transient expression of the reporter mRNA was repressed by the miRNA mimic, the mRNA was associated with polysomes. However, when the translation initiation was inhibited by hippuristanol, which is an eIF4A inhibitor, there was more rapid loss of the repressed

mRNA from the polysome than that detected for the active unrepressed mRNA. These findings suggest that ribosomes are freed from their association with repressed mRNAs more rapidly than with active mRNAs as a result of miRNA-mediated premature ribosome dissociation (Petersen, Bordeleau et al. 2006, Eulalio, Huntzinger et al. 2008, Valinezhad Orang and Safaralizadeh 2014).

1.5.4.2.3 Co-translational protein degradation

miRNAs were suggested to mediate the destruction of the newly formed polypeptide chain co-translationally (Nottrott, Simard et al. 2006, Eulalio, Huntzinger et al. 2008, Valinezhad Orang and Safaralizadeh 2014). This suggestion was based on the evidence that miRNA target is actively translated although the protein product cannot be detected, whereas proteasome inhibitors are not able to restore the protein expression from repressed mRNAs; nevertheless, the putative peptidase remains unknown (Nottrott, Simard et al. 2006).

1.5.4.3 mRNA decay

Studies in *C. elegans*, *D. melanogaster*, zebrafish embryos, and human cells showed that miRNAs induce deadenylation from 3' end and/or decapping from the 5' end resulting in mRNA degradation not by Argonaute endonucleatic cleavage but rather by exonucleases and by enzymes such as DCP1/2 (Valencia-Sanchez, Liu et al. 2006, Garneau, Wilusz et al. 2007, Eulalio, Huntzinger et al. 2008, Fabian, Sonenberg et al. 2010, Valinezhad Orang and Safaralizadeh 2014, Wilczynska and Bushell 2015). Some evidence suggests that translational repression can occur independent of mRNA-mediated mRNA degradation. For example, miR-430 was reported to induce translational repression in zebrafish embryos before complete mRNA deadenylation and decay, where inhibiting mRNA deadenylation did not block translational

repression (Bazzini, Lee et al. 2012). It is possible that miRNAs can combine two mechanisms of action (mRNA decay and translational repression) at the same time in order to maximise their inhibitory effect.

1.5.4.4 Sequestration in P bodies

miRNAs, miRNA targets, and Argonaute proteins colocalise to distinct foci within the cytoplasm known as processing bodies (P bodies). Other P bodies components include GW182, the decapping enzyme DCP2, decapping activators (such as DCP1, EDC3, Ge-1), the CAF1-CCR4-NOT deadenylase complex, and the RNA helicase RCK/p54, all of which were involved in miRNA function (Eulalio, Behm-Ansmant et al. 2007, Parker and Sheth 2007, Filipowicz, Bhattacharyya et al. 2008, Kulkarni, Ozgur et al. 2010, Nishi, Takahashi et al. 2015). The detection of miRNAs, miRNA targets and Argonaute proteins in P bodies led to the notion that miRNA targets get sequestered in P bodies and thus isolated from the translation machinery. It has been debated whether the localisation of miRNA targets in the P bodies is a consequence or cause of miRNA-mediated gene expression silencing. It has been shown that miRNA-mediated silencing is unaffected in cells lacking detectable microscopic P bodies, suggesting that aggregation of miRNA silencing machinery in microscopic P bodies is not essential for miRNA function (Chu and Rana 2006, Eulalio, Behm-Ansmant et al. 2007, Eulalio, Huntzinger et al. 2008, Kulkarni, Ozgur et al. 2010).

1.5.5 miRNA-based therapeutic approaches

Due to their powerful regulatory functions in a wide variety of diseases, miRNAs are considered as an attractive target for therapeutic intervention. Depending on the cause of miRNA disbalance, the therapeutic approach has to be adjusted. miRNA dysregulation can be caused by deficiency in the maturation pathway or altered

regulation of a specific miRNA. If the total miRNA level in the cell is dysregulated, it is highly likely that one or more components of the miRNA processing machinery are altered and an indirect miRNA-targeting therapeutic approach should be used (De Santa, Iosue et al. 2013). If the expression of a specific miRNA is dysregulated, the therapy is based on direct miRNA targeting. The ideal drug should be (1) easily delivered into the target cells; (2) stable in vivo and has long-term effect; (3) specific to its target within the cell; (4) safe with minimal or no off-target effects.

Disease-causing miRNA dysregulation can be due to downregulation of miRNA expression or overexpression of a normally lowly expressed miRNA. In cancer, miRNAs can have oncogenic or tumour suppressive functions. Depending on the function alteration, therapeutic strategy can be developed. Downregulated miRNA can be substituted by miRNA mimic, a synthetic RNA duplex designed to mimic the endogenous function of the miRNA of interest with modifications for cellular uptake and stability. Another way for substitution of the deficient miRNA is by the use of adeno-associated viruses (AAV) as vectors for stable expression of the miRNA of interest (van Rooij and Kauppinen 2014). AAV various serotypes allow for tissue specificity due to the natural tropism of the virus. In addition, the use of tissue-specific promoter for miRNA expression can add another layer of specificity to this system.

Inhibition of highly expressed miRNA can be achieved by miRNA sponges, in which RNA molecules carrying complementary binding sites to the miRNA of interest are transgenically expressed, or by antisense oligonucleotides (ASO), known as anti-miRs (van Rooij and Kauppinen 2014). Anti-miRs are the most widely used approach to regulate miRNA expression in vivo. Anti-miR is an antisense oligonucleotide carrying the full or partial complementary reverse sequence of a

mature miRNA, which can be modified to promote their stability, binding affinity and pharmacokinetic properties. The locked nucleic acid (LNA), which is RNA analogue in which the bicyclic furanose unit is locked in an RNA mimicking sugar conformation, is considered a very promising antisense oligonucleotide with proven therapeutic efficacy (Stenvang, Petri et al. 2012).

1.5.5.1 Examples of miRNAs with therapeutic potential

1.5.5.1.1 Inhibition of miR-122 for treatment of Hepatitis C virus

In 2005, miR-122 was identified as a liver-specific miRNA that can modulate HCV replication and it has been shown to be inhibited by a 2'-OMe-modified anti-miR-122 ASO (Jopling, Yi et al. 2005). Miravirsen, which is a 15-mer LNA- and phosphorothioate-modified anti-miR with a high affinity for miR-122, is currently in clinical trials (Li and Rana 2014). Preclinical trials showed that miravirsen was well-tolerated and effectively inhibited miR-122 expression in healthy monkeys (Krutzfeldt, Rajewsky et al. 2005, Hildebrandt-Eriksen, Aarup et al. 2012). The same drug at a dose of 5 mg per kg decreased HCV infection by two orders of magnitude in chimpanzees with chronic HCV (Lanford, Hildebrandt-Eriksen et al. 2010). In a phase I clinical study, miravirsen was well tolerated and showed no dose-limiting toxicity in single-dose and multiple ascending-dose studies in healthy individuals (Lindow and Kauppinen 2012). The phase II clinical study showed that there is 2–3 log decrease in serum HCV RNA in patients with chronic HCV infection who received five weekly subcutaneous injections of miravirsen. Moreover, HCV RNA was undetectable in 44.4% of patients who received the highest dose tested (7 mg per kg) and no serious side effects were reported (Lindow and Kauppinen 2012). This

interesting data suggests that miravirsen can be the first anti-miR drug to enter the market.

1.5.5.1.2 miR-34 mimic for treatment of liver cancer

The miR-34 family comprises miR-34a, miR-34b and miR-34c whose genes are located in genomic regions involved in cancer (Calin, Sevignani et al. 2004). Several studies showed that miR-34 expression is reduced in cancer cells and that it inhibits cell growth by targeting oncogenes involved in proliferation (histone deacetylase 1 (HDAC1) and MYC), cell cycle control (cyclin-dependent kinase 4 (CDK4), CDK6 and the transcription factor E2F3), metastasis (metastasis-associated protein MTA2), and apoptosis (B cell lymphoma 2 (BCL-2) and sirtuin1 (SIRT1)) (Li and Rana 2014). This data suggested that miR-34 mimic can be a potential therapeutic option for restoring the normal regulation of cell growth and cell death genes in cancer cells. In 2013, Mirna Therapeutics announced the start of a phase 1 clinical study of a liposome-formulated miR-34 mimic, designated MRX34, in patients with liver cancer (Bouchie 2013). However, in 2016, the clinical study halted as a result of multiple immune-related severe adverse events such as severe cytokine release syndrome in patients treated with MRX34.

1.5.5.1.3 Anti-miR-21 for treatment of Alport syndrome

Alport syndrome is genetic kidney disease caused by mutations in the type IV collagen genes (Col4A3, Col4A4 and Col4A5) characterised by chronic glomerulonephritis (Hudson, Tryggvason et al. 2003). It has been shown that miR-21 plays an important role in promoting kidney pathogenic progression in short-term

rodent models of kidney disease (Chau, Xin et al. 2012, Zhong, Chung et al. 2013) and in progression of Alport syndrome (Gomez, MacKenna et al. 2015). Inhibition of miR-21 in Alport kidney mouse model by an anti-miR-21 oligonucleotide, which is chemically modified single-stranded RNA molecule with full sequence complementarity to miR-21, led to a considerably milder kidney disease, where the anti-miR-21 resulted in (i) Enhancement of PPAR α /retinoid X receptor (PPAR α /RXR) activity and downstream signaling pathways in glomerular, tubular, and interstitial cells; (ii) stimulation of mitochondrial function and reduction of mitochondrial ROS production, leading to improved tubular functions; (iii) protection against TGF- β -induced fibrogenesis and inflammation in interstitial and glomerular cells (Gomez, MacKenna et al. 2015). This data suggests that anti-miR21 can be a candidate drug for treatment of Alport syndrome. A chemically modified anti-miR-21, designated RG-012, is currently being developed by Regulus Therapeutics.

1.5.5.1.4 Anti-miR15/195 for post-myocardial infarction remodelling and cardiac regeneration

Overexpression of miR-195 (miR-15 family) in the embryonic heart resulted in ventricular hypoplasia and septal defects (Porrello, Johnson et al. 2011). A mechanistic study has shown that miR-195 controlled cardiomyocyte proliferation by targeting a number of cell cycle genes, including checkpoint kinase 1 (CHEK1) (Porrello, Johnson et al. 2011). Interestingly, inhibiting miR-195 by LNA-modified anti-miRs in pigs and mice resulted in protection of treated animals from myocardial infarction and caused strong effects on cardiac regeneration (Hullinger, Montgomery

et al. 2012, Porrello, Mahmoud et al. 2013). Currently, miRagen Therapeutics and Servier are developing an anti-miR15/195 and it is at the preclinical stage.

1.5.5.2 Future perspectives

The miRNA-based therapeutic field is growing; however, there are some challenges such as the potential drug resistance to anti-miRs and the off-target effects of the miRNA-based therapeutics. The potential resistance to an anti-miR could stem from (1) change in absorption, distribution, metabolism and excretion of the anti-miR of interest; (2) upregulation of targeted miRNAs expression and/or enhancing their processing or biogenesis to counteract the anti-miR effect; (3) upregulation of the expression of other miRNAs that can target the same gene and thus counteract the inhibitory effect of the anti-miR. Further understanding of the miRNA therapeutic resistance can lead to better miRNA-targeting strategies.

Due to the ubiquitous nature of the miRNAs, it is possible that miRNA therapeutics can have off-target adverse effects especially in indications that require chronic treatment. Therefore, the delivery of the miRNA therapeutic to the cell type or tissue of interest is a key factor for a successful drug. One potential approach is to conjugate the miRNA with a bioactive molecule that can enhance the delivery of the miRNA therapeutic to the specific cell types. Another approach can involve encapsulation of the miRNA therapeutic into a lipid-based formulation that enhances specific cell uptake. Despite the challenges, the miRNA-based therapeutics has now become a viable option for addressing the unmet medical needs for serious and life-threatening diseases.

1.6 MHV-68 microRNAs

MHV-68 encodes at least 15 miRNAs that were identified by cloning or deep sequencing (Pfeffer, Sewer et al. 2005, Reese, Xia et al. 2010, Zhu, Strehle et al. 2010). The miRNAs are located immediately downstream of the vtRNAs in the left end of the genome (Figure 1.5). The primary miRNAs are transcribed by RNA pol III as hybrid transcripts with the vtRNAs (Pfeffer, Sewer et al. 2005). It has been shown that several MHV-68 miRNAs are functional and biologically active using luciferase reporter analyses and that this activity varies between different miRNAs (Diebel, Smith et al. 2010).

While this project was underway, two groups published studies that investigated the functions of the MHV-68 miRNAs and vtRNAs using mutant viruses with reduced or abolished expression of 14 MHV-68 miRNAs (Feldman, Kara et al. 2014) or all vtRNAs and their associated miRNAs (Diebel, Oko et al. 2015). Feldman *et. al.* have demonstrated that the MHV-68 miRNAs are not essential for viral lytic replication *in vitro* and *in vivo* (Feldman, Kara et al. 2014). In the same study, although the splenocytes from mice infected with the miRNA mutant virus or the WT virus had identical levels of *ex vivo* reactivation, the miRNAs deletion caused a small reduction (2.3-fold) in the latent genome levels at 16 dpi. However, the *ex vivo* reactivation of virus from the splenocytes from the miRNA mutant virus-infected mice was reduced as compared to that of the WT virus when the mice were infected at a lower dose. The authors utilised a beta lactamase reporter gene fused in frame to the ORF73 within the virus genome in order to isolate the latently infected B cells by flow cytometry.

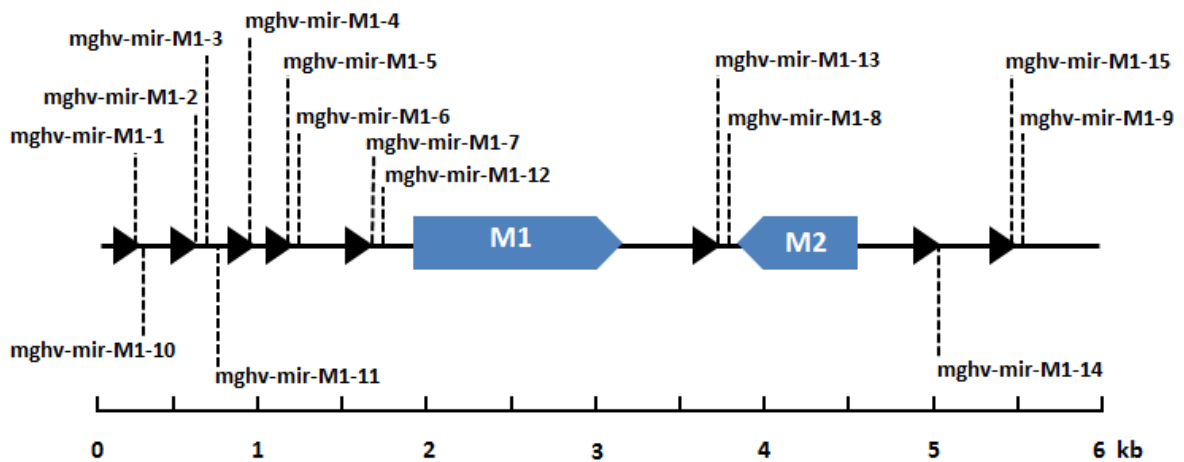


Figure 1.6 Genomic organisation of the MHV-68 encoded vtRNAs and miRNAs in the left end of MHV-68 genome. The vtRNAs are represented as black arrows. The dashed lines indicate the locations of the MHV-68 miRNAs, which are numbered mghv-mir-M1-1 through-15. The miRNAs are named according to the miRBase nomenclature (Kozomara and Griffiths-Jones 2014). The open reading frames for M1 and M1 are shown as blue arrows.

The splenic naïve, germinal centre, and memory B cells were isolated from mice infected with the miRNA mutant virus or the WT virus. It was revealed that the deletion of the 14 miRNAs resulted in approximately 2-fold decrease in the MHV-68 infected memory B cells at 16 dpi. Interestingly, the deletion of the miRNAs resulted in attenuation of viral virulence in acute-lethal pneumonia model (Feldman, Kara et al. 2014).

Diebel *et. al.* have demonstrated that the MHV-68 vtRNAs and their associated miRNAs are not essential for acute replication or establishment of latency *in vivo* (Diebel, Oko et al. 2015). Similar to Feldman *et. al.* observations, the authors demonstrated that the deletion of the vtRNAs and their associated miRNAs led to a reduction in the lethal pneumonia in IFN γ knock-out mice. However, in the same study, a recombinant virus expressing vtRNA1 only in the absence of other vtRNAs and miRNAs was able to partially restore the lethal pneumonia phenotype. Collectively, it is suggested that the vtRNAs may play an active role in MHV-68 virulence and that they may co-operate with the viral miRNAs to achieve this role.

A very recent study has shown that infection of mice with a mutant MHV-68 virus lacking all miRNAs and vtRNAs, resulted in slight reduction of *ex vivo* reactivation during early latency in spleen (17 days post infection) as compared to wild-type and revertant viruses despite similar viral genomic loads (Steer, Strehle et al. 2016). In the same study, the author have shown that during late latency in spleen (42 days post infection), there was significant increase of viral genomic load of the mutant virus as compared to the wild-type and revertant viruses; although, no *ex vivo* reactivation could be detected at this time-point. In contrast to Feldman's and

Diebel's studies, Steer *et al.* found no difference in the lethal pneumonia in the infected IFN γ knock-out mice between wild-type and mutant virus. Possible cause of this discrepancy is that Steer *et al.* sacrificed mice when they lost more than 15% of the initial body weight and animals were not given chance to recover.

1.7 Role of herpesvirus miRNAs during latency

To date, more than 300 virally encoded miRNAs have been identified and found to be encoded by several virus families including adenoviruses, polyomaviruses, and retroviruses; however, the majority of viral miRNAs are encoded by herpesviruses (Kincaid, Burke *et al.* 2012, Kozomara and Griffiths-Jones 2014). There has been an increasing evidence that the miRNAs encoded by herpesviruses play important roles during viral latency (Grey 2015). The best model for studying viral miRNAs is within the natural host; however, obviously this is not possible for human viruses. Animal models can provide a powerful tool for investigating viral miRNAs functions. For example, MHV-68 provides a unique small animal model for studying gammaherpesvirus-encoded miRNAs in the context of natural virus-host interplay.

A common feature among all herpesviruses is their ability to establish latency within the natural host. A single miRNA can suppress the expression of hundreds or even thousands of target mRNAs, and thus an miRNA can inhibit the expression of multiple genes within the same physiological pathway (Feig, Giles *et al.* 2015). Furthermore, miRNAs have a relatively modest effect on their target transcripts and act as fine-tuners of gene expression, which may require time before causing a significant biological effect (Kincaid and Sullivan 2012). This makes viral miRNAs ideal proviral tools during the state of latent infection.

Indeed, several studies demonstrated that the miRNAs encoded by herpesviruses play important roles in promoting latent infection through multiple strategies such as inhibiting lytic replication, evading the immune response, enhancing infected cell survival, and promoting infected cell proliferation. These roles are reviewed in the following sections.

1.7.1 Inhibition of lytic infection

Successful latent infection requires restricting viral lytic gene expression so that the infected cells cannot be detected by the host immune surveillance and be eliminated. However, at the same time the latent virus genome requires a suitable environment for reactivation under the appropriate conditions. This requires intricate homeostatic balance and fine tuning of gene expression, a function that is ideal for miRNAs.

One strategy for restricting viral lytic infection is by inhibiting the viral transcriptional activators which play a key role in stimulating lytic gene expression and in the latent/lytic switch. It has been demonstrated that the KSHV miRNA, miR-K12-9*, targets a sequence in the 3' UTR of the immediate-early transcriptional activator RTA (Bellare and Ganem 2009). The authors demonstrated that the ectopic expression of miR-K12-9* inhibits RTA expression, while its specific knock down in latently infected cells increased lytic reactivation frequency by two to three fold, suggesting that the virally encoded miRNAs can act as fine tuners of the major lytic switch protein, RTA, in order to facilitate latency by preventing any fortuitous reactivation. These findings were reminiscent of the reported inhibition of the expression of the trans-activating protein, IE72, of HCMV by the HCMV miRNA miR-UL112-1 (Grey, Meyers et al. 2007, Murphy, Vanicek et al. 2008).

Similarly, The HSV-1 miRNA, miR-H2-3p, which is transcribed antisense to the transcriptional activator infected cell protein 0 (ICP0), was able to reduce the ICP0 protein levels without affecting ICP0 mRNA levels (Umbach, Kramer et al. 2008). Additionally, co-transfection experiments revealed that HSV-2 miR-H3 and miR-H4 (also referred to as miR-I and miR-III respectively) were able to reduce the levels of ICP34.5 protein (Tang, Patel et al. 2009), which is involved in virus replication, reactivation, and neurovirulence (Whitley, Kern et al. 1993, Bolovan, Sawtell et al. 1994).

Strasheim *et. al.* reported that MDV-1 miRNA, miR-M7-5p, inhibits the expression of the immediate-early genes ICP4 and ICP27 using luciferase reporter assays; in addition, there was an increase in miR-M7-5p expression and decrease of ICP27 expression during virus reactivation, suggesting that this miRNA facilitates latent infection (Strasheim, Stik et al. 2012).

Several other studies revealed the herpesviruses from different families can inhibit their own trans-activating gene expression via their miRNAs (Lu, Li et al. 2010, Lin, Liang et al. 2011, Jung, Choi et al. 2014, Riaz, Dry et al. 2014). Viral miRNA-mediated inhibition of the lytic switch protein expression in different herpesviruses indicates that this is an evolutionary conserved mechanism that allows herpesvirus to restrict lytic infection and thereby establish and maintain latency.

Another strategy for restricting viral lytic gene expression is by regulating cellular targets that are involved in the viral lytic replication. One clear example for this is the inhibition of I κ B α , a suppressor of NF- κ B complexes, by KSHV miR-K12-1

leading to enhanced NF- κ B-dependent viral latency (Lei, Bai et al. 2010). miR-K12-3 can also promote KSHV latent infection via targeting the nuclear factor I/B, an activator of RTA promoter (Lu, Li et al. 2010). Furthermore, the EBV miRNA, BART-miR-18-5p, has been reported to inhibit the expression of the cellular protein MAP3K2 resulting in decreased viral lytic gene expression and virus production (Qiu and Thorley-Lawson 2014).

Pavelin *et. al.* used combined approach of RNA-induced silencing complex immunoprecipitation (RISC-IP) and siRNA screening techniques for identification of HCMV miRNAs targets that have a role in the virus life cycle (Pavelin, Reynolds et al. 2013). The top identified target was the cellular ATP6V0C that is a component of Vacuolar ATPase involved in acidification of endosomal compartments (Forgacs 2007). The authors demonstrated that knock down of ATP6V0C resulted in almost complete inhibition of infectious virus production and that ATP6V0C is targeted by US25-1 miRNA, suggesting that US25-1 can inhibit virus lytic replication through down-regulation of ATP6V0C.

1.7.2 Evading the immune response

The virus use of miRNAs during latency is itself a way of immune evasion because the miRNAs are non-immunogenic and cannot be detected by the conventional immune defence. There has been growing evidence that herpesvirus miRNAs can play a role in evading the host immune response. Dolken *et. al.* provided the first *in vivo* evidence that links viral miRNAs to immune evasion through investigating the phenotype of an MCMV virus lacking 2 miRNAs, miR-M21-1 and miR-M23-2. The authors demonstrated that deletion of the miRNAs resulted in reduction in virus production in the salivary gland, which is a site for virus persistence, a phenotype

seen in C57BL/6 mice but not BALB/c mice and could be rescued by combined depletion of NK and CD4⁺ T cells, suggesting that these miRNAs may help the virus evade the immune response and allow for long-term persistence (Dolken, Krmpotic et al. 2010).

Furthermore, an miRNA encoded by another betaherpesvirus, HCMV UL112-1, inhibits the MHC class I-related chain B (MICB) protein, which is a stress-induced ligand that is targeted by NK cells via the NKG2D ligand, resulting in reduction of the cytotoxic effects of NK cells (Stern-Ginossar, Elefant et al. 2007). Similarly, miRNAs encoded by KSHV and EBV have been shown to target the MICB mRNA and down-regulate its expression (Nachmani, Stern-Ginossar et al. 2009), suggesting that viral miRNAs can have similar function; although, that they share no sequence homology.

Several studies have shown that herpesvirus miRNAs can target genes that inhibit the production of pro-inflammatory cytokines in order to suppress the recruitment of immune cells to the site of infection (Abend, Uldrick et al. 2010, Abend, Ramalingam et al. 2012, Hook, Hancock et al. 2014). For example, the KSHV miRNA, miR-K12-10a, targets TWEAKR (TNF-like weak inducer of apoptosis receptor), resulting in reduction in IL-8 and MCP-1 expression (Abend, Uldrick et al. 2010).

1.7.3 Survival of latently infected cells through anti-apoptotic mechanisms

Survival of the latently infected cells is an absolute requirement for viral long-term latency because cell death means the virus loses its host. EBV miRNAs promote cell survival through anti-apoptotic mechanisms. For instance, EBV-miR-BART5 inhibits the expression of p53-up-regulated modulator of apoptosis (PUMA) (Choy, Siu et al. 2008). Another study demonstrated that BART cluster 1 miRNAs can inhibit the expression of the pro-apoptotic BIM protein as well as PUMA (Marquitz, Mathur et al. 2011). Moreover, EBV miRNAs, BART16 and BART1-3p, target the cellular caspase 3, leading to apoptosis suppression (Vereide, Seto et al. 2014).

A recent study has identified the mRNA targets of BART miRNAs in nasopharyngeal carcinoma cell line using photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP), where 10 pro-apoptotic mRNA targets were validated by indicator assays and ectopic expression of the miRNAs (Kang, Skalsky et al. 2015), suggesting that EBV BART miRNAs enhance survival of the latently infected epithelial cells and thereby promote long-term infection.

It has been demonstrated that a KSHV miRNA, miR-K12-1, hijacks the NF- κ B pathway by inhibiting the expression of I κ B α , an inhibitor of NF- κ B transcription factors, resulting in enhanced NF- κ B-dependent viral latency and cell survival (Lei, Bai et al. 2010). Viral miRNA-mediated anti-apoptotic activity is not only restricted to gammaherpesviruses, an alphaherpesvirus miRNA, MDV-1 miR-M3, has been reported to promote cell survival through suppression of cisplatin-induced apoptosis by targeting SMAD2 (Xu, Xue et al. 2011).

1.7.4 Cellular proliferation and oncogenesis

Remarkably, both KSHV miR-K12-11 and MDV-1 miR-M4 share sequence homology with the cellular oncomiR miR-155, which plays crucial roles in physiological lymphocyte development and activation but whose overexpression is associated with lymphoid cancer development (Eis, Tam et al. 2005, Gottwein, Mukherjee et al. 2007, Rodriguez, Vigorito et al. 2007, Skalsky, Samols et al. 2007). Indeed, it has been demonstrated that miR-K12-11 and miR-155 target common sets of mRNAs and that miR-K12-11 plays a role in B cell transformation in humanised mice and can complement the deficiency of miR-155 *in vivo* (Gottwein, Mukherjee et al. 2007, Skalsky, Samols et al. 2007, Boss, Nadeau et al. 2011, Sin, Kim et al. 2013). An MDV-1 virus lacking miR-M4 expression was unable to transform infected T-cells, suggesting a major role played by miR-M4 in MDV tumourigenesis (Zhao, Xu et al. 2011).

A KSHV mutant virus lacking a cluster of 10 primary miRNAs revealed that the KSHV miRNAs are crucial for cellular transformation and oncogenesis as well as for cell survival and apoptosis inhibition in primary rat mesenchymal cells (Moody, Zhu et al. 2013). EBV miRNAs have also been reported to contribute to the virus-induced oncogenesis and to B cell proliferation (Seto, Moosmann et al. 2010, Babu, Ponia et al. 2011, Vereide, Seto et al. 2014).

Recently, it has been shown that EBV-miR-BART1 targets the tumour suppressor, phosphatase and tensin homologue (PTEN), resulting in activation of the PTEN-dependent pathways and induction of epithelial–mesenchymal transition and metastasis in nasopharyngeal carcinoma (Cai, Ye et al. 2015).

1.8 miRNAs encoded by viruses other than herpesviruses

1.8.1 Polyomavirus miRNAs

Polyomaviruses is a family of non-enveloped double stranded DNA tumour viruses with circular genome of approximately 5,100 bp that can infect humans and animals (DeCaprio, Imperiale et al. 2013). The closely related polyomaviruses, Simian virus 40 (SV40), JC polyomavirus (JCPyV), BK polyomavirus (BKPyV), and Baboon polyomavirus 1 (SA12), encode at least one primary miRNA from a late strand of the viral genome downstream of the late polyadenylation site (Cantalupo, Doering et al. 2005, Seo, Fink et al. 2008, Bauman, Nachmani et al. 2011). Murine polyomavirus (MuPyV) and Merkel Cell Virus (MCPyV) encode a single primary miRNA located at the 5' end of the second LTA_g exon and it is complementary to the early mRNA transcript (Seo, Chen et al. 2009, Sullivan, Sung et al. 2009).

The miRNAs of SV40, the first polyomavirus found to encode miRNAs, down-regulate the expression of viral early proteins and thus reduce the viral T antigens presentation (Sullivan, Grundhoff et al. 2005). Furthermore, Zhang *et al.* have demonstrated that a SV40 mutant virus lacking the miRNAs expression showed higher levels of viral DNA in liver and kidney tissues of infected syrian golden hamsters as compared to those infected with the WT virus (Zhang, Sroller et al. 2014). The higher levels of viral T-antigens in the mutant SV40-infected animals are likely to support the increased viral DNA replication. However, a mutant SV40 virus lacking the primary miRNA exhibited levels of virus production in cell culture similar to that of the WT virus (Sullivan, Grundhoff et al. 2005). This highlights the importance of *in vivo* models for analysis of viral miRNAs functions.

The miRNA mutant SV40-infected cells were more sensitive to lysis by cytotoxic T cells and triggered more cytokine production than the WT SV40-infected cells (Sullivan, Grundhoff et al. 2005), suggesting that SV40 miRNAs auto-regulate early viral protein expression and play a role in evading the cytotoxic immune response. The miRNAs of the other polyomaviruses, JCPyV, BKPyV, MuPyV, MCPyV, and SA12, have been shown to down-regulate early gene expression (Cantalupo, Doering et al. 2005, Seo, Fink et al. 2008, Seo, Chen et al. 2009, Sullivan, Sung et al. 2009, Bauman, Nachmani et al. 2011).

1.8.2 Adenovirus miRNAs

Adenoviruses encode several non-coding virus associated (VA) RNAs that can use the RNA interference processing pathway such as Exportin 5 and Dicer (Carnero, Sutherland et al. 2011). A number of these VA RNAs are cleaved by Dicer into viral miRNAs (mivaRNAs). Suppression of mivaRNAs with 2-o-methyl (2ome)-modified oligonucleotides, which prevents miRNA interaction with the target, resulted in deficient virus production (Aparicio, Razquin et al. 2006). There are no predicted mivaRNAs targets in the adenoviral genome by bioinformatic analysis (Aparicio, Razquin et al. 2006), excluding potential auto-regulatory roles of these miRNAs. Potential cellular targets have been identified using genomic and bioinformatics analyses (Aparicio, Carnero et al. 2010). These targets include proteins involved in gene expression, cell cycle, and DNA repair but further work is required for validation.

1.8.3 Retrovirus miRNAs

1.8.3.1 Human immunodeficiency virus 1 (HIV-1) miRNAs

Multiple HIV-1 miRNAs are derived from the TAR motif, which is a stable hairpin structure found at the 5' end of all HIV-1 transcripts that facilitates the binding of the trans-activator protein Tat in order to enhance transcription (Berkhout, Silverman et al. 1989, Berkhout 1992, Bennasser, Le et al. 2004, Ouellet, Plante et al. 2008, Harwig, Das et al. 2014). The TAR miRNAs can be detected in both latently and lytically infected human cells and they play a role in transcriptional silencing at the long-terminal repeat (LTR) promoter by chromatic remodelling (Bennasser, Le et al. 2004, Klase, Kale et al. 2007, Ouellet, Plante et al. 2008).

Microarray analysis has shown that TAR miRNAs target apoptotic genes for down-regulation and therefore it can promote cell survival and chronic infection (Klase, Winograd et al. 2009, Ouellet, Vigneault-Edwards et al. 2013). This is consistent with the detection of TAR miRNAs in chronically infected CD4⁺ T cells where virus production is very low (Klase, Kale et al. 2007). On the other hand, a HIV-1-encoded miRNA (miR-H3) located in the reverse transcriptase gene can target the 5' LTR promoter and induce viral transcription, thereby facilitate exit from latency (Zhang, Fan et al. 2014). This indicates that HIV-1 miRNAs can have contrasting effects.

Narayanan *et al.* have shown that TAR miRNAs are also found in exosomes from HIV-infected cells in cell culture and in the serum of HIV-infected patients, suggesting that these miRNAs may have trans-cellular effects on naïve cells (Narayanan, Iordanskiy et al. 2013).

1.8.3.2 Bovine leukemia virus (BLV) miRNAs

BLV is the causative agent of enzootic bovine leukosis, an economically important disease in cattle. BLV encodes at least 5 primary miRNAs that are transcribed by RNA pol III (Rosewick, Momont et al. 2013). These miRNAs are abundantly expressed in pre-leukemic and malignant cells where viral gene expression is restricted, raising the possibility that these miRNAs may play roles in latency and tumorigenesis (Rosewick, Momont et al. 2013). Indeed, the seed sequence of BLV-miR-B4 is homologous to that of the cellular pro-oncogenic miR-29 and both miRNAs share common target mRNAs, suggesting a role of BLV-miR-B4 in BLV oncogenesis (Kincaid, Burke et al. 2012). This mimicry is reminiscent of the KSHV miR-K12-11 and MDV-1 miR-4 homology with the cellular oncogenic miRNA, miR-155 (Gottwein, Mukherjee et al. 2007, Skalsky, Samols et al. 2007, Zhao, Yao et al. 2009).

1.9 Project outline and aims

The human gammaherpesviruses such as KSHV and EBV are associated with lymphoproliferative disorders and some types of cancer during their latent infection within the host. The exact molecular mechanisms behind this latent infection are poorly understood. The discovery of gammaherpesvirus encoded miRNAs opened doors for better understanding of latent infection through investigating their functions during latency, providing potential novel therapeutic approaches.

Due to species specificity of gammaherpesviruses, dissecting the functions of the human gammaherpesvirus-encoded miRNAs *in vivo* is impossible. MHV-68 is

genetically related to and shares pathological features with KSHV and EBV. Hence, it provides a small animal model for studying gammaherpesvirus life cycle and pathogenesis. In this project, the MHV-68 was used as a model for characterising the expression of MHV-68 miRNAs and investigating their potential functions *in vivo*.

The first aim of this study was to establish qRT-PCR assays for quantification of MHV-68 miRNAs *in vivo*. In order to provide a platform for characterising the miRNAs expression *in vivo*, the miRNAs expression was first examined in lytically and latently infected cells *in vitro*. The expression kinetics of the MHV-68 miRNAs were then evaluated at different time-points during the different stages of latent infection *in vivo* (latency establishment and latency maintenance). The second aim was to construct an miRNA mutant MHV-68 virus lacking the expression of 9 miRNAs in order to investigate the miRNAs functions *in vivo*. The final objective was to carry out a comprehensive phenotypic analysis of this mutant virus *in vitro* and *in vivo* in order to examine viral lytic replication, viral latency, *ex vivo* reactivation, viral gene expression, virus-induced B cell activation and proliferation.

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2 Chapter 2: Materials and Methods

2.1 Cell culture

Baby Hamster Kidney fibroblast 21 (BHK-21) cells were cultured in Glasgow's Modified Eagle's Medium (GMEM) (Life Technologies, UK) supplemented with 10% (v/v) fetal calf serum (FCS) (Life Technologies, UK), 10% (v/v) tryptose phosphate broth (Life Technologies, UK), 2 mM L-glutamine (Life Technologies, UK), and 100 U/ml penicillin and streptomycin (Life Technologies, UK). NIH 3T3 cells (mouse embryo fibroblasts) were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Life Technologies, UK) supplemented with 10% (v/v) FCS (Life Technologies, UK), 2 mM L-glutamine (Life Technologies, UK), and 100 U/ml penicillin and streptomycin (Life Technologies, UK).

NS0 cells (murine myeloma) were grown in Rosewell Park Memorial Institute 1640 (RPMI-1640) medium (Life Technologies, UK) containing 10% (v/v) FCS (Life Technologies, UK), 2 mM L-glutamine (Life Technologies, UK), and 100 U/ml penicillin and streptomycin (Life Technologies, UK). Cell master stocks (density of 5×10^6) were stored as 1 ml FCS with 10% dimethyl sulfoxide (DMSO) in liquid nitrogen. In order to make working stocks, the cells were rapidly thawed in water bath at 37°C. Pre-warmed media was then added drop by drop to the cells followed by centrifugation at 470 x g for 5 min at room temperature. The cell pellet was then re-suspended in 10 ml media and cultured in T25 sterile flask (Nunc, UK) and incubated at 37°C in the presence of 5% CO₂. BHK-21 and NIH 3T3 were split when they reached 80% confluent growth using 0.25% trypsin (Life Technologies, UK). The trypsin was inactivated by addition of an equal amount of medium and the cells were pelleted by centrifugation at 470 x g for 5 min at room temperature. The cell

pellet was re-suspended in medium and a cell aliquot was diluted 1:10 with 0.1% trypan blue (Sigma, UK) so that the unstained viable cells could be counted in a haemocytometer. The cells were seeded at density of 3×10^6 or 5×10^6 in T80 or T175 flasks (Nunc, UK) respectively.

NS0 cells were grown as suspension and flasks were tilted in the incubator (37°C and 5% CO₂) to allow the cells to stick together. These cells were split at ratio of 1:3 every 3 or 4 days depending on the cell density.

2.2 *In vivo* infection

Female BALB/c mice (4-5 weeks old) purchased from Harlan, UK were housed in a pathogen-free facility either at the Roslin Institute or the Hugh Robson Building (The University of Edinburgh) in accordance with all Home Office and university guidelines. Mice were infected with MHV-68 virus intranasally at dose of 4×10^5 pfu in 40 µl Phosphate Buffer Saline (PBS) (Life Technologies, UK) under brief isofluorane anaesthesia. The infection was carried out by dropping the solution containing the virus onto the mouse's nostrils. Mice were sacrificed by CO₂ asphyxiation. Spleens were harvested and placed in sterile plastic universals containing 5 ml RPMI-1640 media. Lungs were kept in cryovials on dry ice immediately after harvesting.

2.3 Virological methods

2.3.1 Preparation of virus stocks

Working stocks of MHV-68 virus were prepared by infection of BHK-21 cells at a multiplicity of infection (MOI) of 0.001 pfu/cell. The virus was added to the cells

and incubated for one hour at 27°C with shaking to allow virus adsorption. The cells were plated out into T175 flasks at 3×10^6 cells/flask and incubated for 6 days. The cells were subsequently scraped and centrifuged at 1000xg for 20 min at 4°C. Supernatant was decanted and the cell pellet was re-suspended in PBS, in a volume of about third of the number of the flasks used in mls (e.g. 3 ml for 10 flasks). The cells were homogenised in a glass dounce homogeniser 20-30 times, sonicated in an ice bath for 15 min, and then centrifuged at 1000xg for 20 min at 4°C and the supernatant (contains the virus) was collected. The pellet was re-suspended in 1 ml PBS and homogenised again, spun down at 1000xg for 20 min at 4°C and the supernatant was pooled with previous one. The virus suspension was aliquoted into cryovials (Nunc) and stored at -80°C.

2.3.2 Plaque assay for virus titration

The virus was serially diluted in bijoux tubes at a range of 10^{-2} to 10^{-10} in GMEM medium. Two million BHK-21 cells were added to each bijoux tube and incubated for one hour at 27°C with shaking. Each dilution was divided between two 60 mm plates resulting in 1×10^6 cells/plate and 3 mls of GMEM was added to each plate. Two plates containing uninfected cells were used as a negative control. The plates were incubated at 37°C in CO₂ incubator for 4 days, then the medium was discarded and the cells were fixed with 10% (v/v) Neutral Buffered Formaldehyde (Surgipath Europe Ltd) for at least 1 hour followed by staining with 0.1% Toluidine blue (BDH Laboratory Supplies) for 30 min. Plates were washed in water, left to dry and plaques formed by the virus were counted under light microscope. The virus titre was calculated as: (average number of plaques) / (lowest dilution x final dilution volume added to the plate (2 ml)).

2.3.3 Infection of NIH 3T3 and NS0 cells

NIH 3T3 cells were seeded in a 6-well plates at density of 10^6 cells per well. Next day the wells were infected with MHV-68 virus at MOI of 5 pfu/cell. Uninfected wells were used as a control. The 6-well plates were then incubated in CO₂ incubator for 1 hour at 37°C to allow virus attachment then the media was discarded and 2 ml of fresh DMEM was added to each well. The plates were incubated for 2, 8 or 24 hours at 37°C followed by cell harvesting and total RNA and DNA extraction. NS0 cells were counted and 2×10^6 cells were infected with MHV-68 virus at MOI of 5 and incubated in bijou tubes at 37°C for 1 hour while shaking. The cell-virus mixture (1 ml) was transferred from the bijou to T25 flasks containing 9 ml of pre-warmed RPMI-1640 media. Uninfected cells were used as a control. The flasks were incubated for 5 days at 37°C followed by cell harvesting and total RNA and DNA extraction.

2.3.4 MHV-68 reactivation induction *in vitro*

NS0 cells were infected with MHV-68 virus as described above. On day 3 post infection, the cells were treated with 20 ng/ml Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). Cells were harvested for RNA and DNA extraction at 2, 4, 8, 24, and 48 hours post treatment. Untreated cells were used as a control and were harvested at 5 days post infection.

2.3.5 Inhibition of MHV-68 replication *in vitro*

Two mg/ml solution of the antiviral thionucleoside analogue 2'-deoxy-5-ethyl-beta-4'-thiouridine (4'-S-EtdU) was prepared in sterile PBS and heated at 60°C until dissolved. The solution was filtered through a 0.2 µM filter using a syringe. NS0

cells were infected with MHV-68 virus as described above. On day 3 post infection, the cells were treated with 4'-S-EtdU at concentration of 1µg/ml in medium. Cells were harvested for RNA and DNA extraction at 0 and 48 hours after treatment. Untreated cells were used as a control.

2.4 Flow cytometry for isolation of mLANA expressing cells

2.4.1 Harvesting of spleen cells

A small portion of the spleen was kept in -80°C for DNA extraction and determination of viral genomic load. The rest of the spleen was teased out of the capsule with a scalpel blade into RPMI-1640 medium (with 10% FCS, 2 mM L-glutamine, and 100 U/ml Pen-Strept). Red blood cells were lysed using RBC Lysis Buffer (Biolegend) according to the manufacturer's instructions. The cells were pelleted by centrifugation at 470 x g/ 5 min/ 4°C. The cell pellet was re-suspended in 10 ml fresh medium and left on ice for 5 min to allow settling of any intact portions of the tissue out of the fluid. The supernatant was transferred to a new tube and the splenocytes were counted and cells were adjusted to a concentration of 5x10⁷ cells/ml in PBS supplemented with 2% FCS. All of the harvested cells were subjected to the flow cytometry.

2.4.2 β-lactamase detection and isolation of mLANA positive splenocytes

The splenocytes were adjusted to a concentration of 5x10⁷ cells/ml in PBS supplemented with 2% FCS. The CCF2/AM β-lactamase substrate loading kit (Life Technologies) was used according to the manufacturer's instructions and adapted as

needed. Freshly prepared CCF2/Am substrate was added to the splenocytes (2 μ l substrate/100 μ l cell suspension) for 5 min at room temperature and light exposure was minimised. Cells were then washed in PBS supplemented with 2% FCS in PBS twice before being subjected to flow cytometry. Controls including unstained splenocytes and splenocytes infected with the wt virus were performed in each experiment. The flow cytometry was carried out using a FACS Aria III sorter (BD Biosciences) and the results were analysed using FACS Diva software. The mLANA positive as well as the mLANA negative populations were collected in RPMI-1640 medium supplemented with 20% FCS or collected directly in 0.5 ml TRI Reagent[®] (Ambion). Splenocytes collected in medium were subjected to centrifugation at 7000xg / 10 min / 4°C. The supernatant was discarded and 0.5 ml TRI Reagent[®] (Ambion) was added and samples were kept at 80°C for RNA extraction.

2.5 DNA isolation

DNA extraction was carried out using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's protocol.

2.6 RNA isolation

Total RNA was extracted using the TRI Reagent[®] (Ambion). One or 0.5 ml of TRI Reagent[®] was added to cells in 1.5 ml screw capped tubes for 5 min at room temperature allowing complete dissociation of the nucleoprotein complex in cells, followed by addition of 0.2 volume Chloroform (Sigma-Aldrich, UK). Tubes were shaken vigorously for 15 sec and incubated at room temperature for 3 min. Samples were then centrifuged at 12,000 xg for 15 min at 4°C. The aqueous layer (contained the RNA) was then transferred to a new 1.5 ml screw capped tube. Fifteen

micrograms of GlycoBlue (Ambion) were added to the aqueous phase in order to visualise the RNA pellet. Half volume of 100% RNase-free Isopropanol (ACROS organics) was added to the aqueous layer and incubated at RT for 10 min. Samples were then centrifuged at 12,000 $\times g$ /10 min/4°C. The supernatant was discarded and RNA pellet was washed twice with RNase-free 75% Ethanol (Sigma-Aldrich, UK). Samples were vortexed briefly and centrifuged at 7500 $\times g$ /5 min/4°C. Ethanol was discarded and RNA pellets were air-dried. RNA pellets were then re-suspended in appropriate volumes of RNase-free water. Samples were then incubated in a heat block set at 55-60°C for 10-15 min. RNA samples were stored at -80°C.

2.7 RNA quality control

The quality of the total RNA extracted from splenocytes was determined using an RNA 6000 Pico kit on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) or using the more recent High Sensitivity RNA ScreenTape in combination with the 2200 TapeStation system (Agilent Technologies, Santa Clara, CA). RNA integrity was judged by RNA Integrity Number (RIN), which was calculated with 2100 Expert Software (Agilent, version B.02.07, eukaryote total RNA pico mode). The software algorithm categorises total RNA quality on a scale from 1 to 10, in which 10 corresponds to intact RNA and 1 corresponds to highly degraded RNA.

2.8 DNase treatment of RNA

Contaminating genomic DNA was removed from the samples using the TURBO DNA-free™ Kit (Ambion, UK). One μ l TURBO DNase and 0.1 volume 10x TURBO DNase buffer were added to the RNA sample for 30 min at 37°C. Following

the DNase treatment, the reaction was stopped by the addition of 0.1 volume (at least 2 μ l) inactivation reagent and the samples were incubated at room temperature for 5 min. The inactivation reagent was pelleted by 1.5 min centrifugation at 10,000 xg at room temperature and the supernatant was transferred to a fresh RNase free tube.

2.9 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

2.9.1 miRNA qRT-PCR

2.9.1.1 Reverse transcription

RNA was reverse transcribed into cDNA using miScript II RT Kit (Qiagen, Crawley, UK) following the manufacturer's protocol. The mature miRNAs were polyadenylated by poly (A) polymerase and reverse transcribed into cDNA using oligo-dT primers, which have a universal tag sequence on the 5' end and a 3' degenerate anchor, allowing amplification of mature miRNA in the real-time PCR step. The combination of polyadenylation and the universal tag addition ensures that the assays do not detect genomic DNA.

2.9.1.2 Real-time PCR

RT-PCR was performed using miScript SYBR green PCR kit (Qiagen, UK) following the manufacturer's protocol. Specific forward primers identical to the full length of the mature miRNAs (200 nM final concentration per reaction) were used with the supplied universal reverse primer. The forward miRNA-specific oligonucleotides were synthesised by Eurofins MWG Operon (Ebersberg, Germany). The primers sequences and the relevant annealing temperatures are shown in table 2.1.

2.9.1.3 Relative quantification ($\Delta\Delta\text{CT}$ method)

Cycle Threshold (CT) is the value where the PCR curve crosses the threshold in the linear part of the curve. The qPCR was performed using both the gene of interest and the reference gene. A calibrator was also used. A calibrator is the sample which all other samples are compared to (e.g. untreated sample). The relative quantification of the calibrator is 1 because it does not vary compared to itself.

$$\Delta\text{CT}_{\text{gene of interest}} = \text{CT}_{\text{gene of interest}} - \text{CT}_{\text{reference gene}}$$

$$\Delta\text{CT}_{\text{calibrator}} = \text{CT}_{\text{calibrator}} - \text{CT}_{\text{reference gene}}$$

$$\Delta\Delta\text{CT} = \Delta\text{CT}_{\text{gene of interest}} - \Delta\text{CT}_{\text{calibrator}}$$

$$\text{Relative quantification (fold increase or decrease)} = 2^{-\Delta\Delta\text{CT}}$$

| miRNA | Primer sequence (5'-->3') | Annealing temperature (°C) |
|-------------------|---------------------------|----------------------------|
| mghv-miR-M1-1-3p | TAGAAATGGCCGTA CTTTCCTTT | 58 |
| mghv-miR-M1-2-3p | CAGACCCCTCTCCCCCTCTTT | As indicated |
| mghv-miR-M1-3-3p | GAGGTGAGCAGGAGTTGCGCTT | 62 |
| mghv-miR-M1-4-5p | TCGAGGAGCACGTGTTATTCTA | 62 |
| mghv-miR-M1-5-5p | AGAGTTGAGATCGGGTCGTCTC | 62 |
| mghv-miR-M1-6-3p | TGAAACTGTGTGAGGTGGTTTT | 62 |
| mghv-miR-M1-7-3p | GATATCGCGCCACCTTTATT | 58 |
| mghv-miR-M1-8-5p | AGCACTCACTGGGGGTTTGGTC | 58 |
| mghv-miR-M1-9-3p | TCACATTTGCCTGGACCTTTTT | 58 |
| mghv-miR-M1-10-3p | TGATTACACGGAAGGTTCTTTT | 58 |
| mghv-miR-M1-12-5p | AAGGGTACTCTCATCACCAATGT | 62 |
| mghv-miR-M1-13-3p | TATCTCATGTGAGCTCTTCTTT | 58 |
| mghv-miR-M1-14-3p | TGCTACAGCGTGCAGAACGTTT | 62 |
| mghv-miR-M1-15-5p | AGCTACCCGCGTGGCCGGAGTGTTT | 62 |
| mmu-miR-191-5p | CAACGGAATCCCAAAGCAGCTG | 58 |

Table 2-1 Sequence of the forward miRNA-specific primers used in the miRNA qRT-PCR assays. The primers are DNA oligos that are identical to the entire mature miRNA sequences according to miRBase database (<http://www.mirbase.org/>).

2.9.2 mRNA qRT-PCR

qRT-PCRs for quantification of viral mRNAs were carried out using FastStart Universal SYBR Green Master (Roche) following the two-step programme in the manufacturer's protocol. qPCR for viral DNA polymerase, RTA, ORF73, M3 and the housekeeping gene SDHA were set up by Ian Bennet in the Dutia/Nash lab (The primers sequences and the relevant annealing temperatures are shown in Table 2.2). qPCR for ORF65 was set up for this project. The primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany). The first step in the ORF65 qRT-PCR assay set up was to generate a standard template for the qPCR absolute quantification. For this reason an ORF65 DNA fragment was amplified by conventional PCR using the MHV-68 BAC as a template. Primer sequences are shown in Table 2.3. The thermal conditions of the PCR were as follow:

| | | |
|------|--------|---------------|
| 94°C | 3 min | |
| 94°C | 45 sec | } X 35 cycles |
| 55°C | 45 sec | |
| 72°C | 1 min | |
| 72°C | 7 min | |

The PCR product was analysed on 1% agarose gel. The band was excised and DNA was extracted using QIAquick Gel Extraction Kit (Qiagen). Then the DNA fragment was cloned using TOPO TA Cloning Kit (Invitrogen) for sequencing according to the manufacturer's protocol. After cloning, miniprep plasmid DNA was obtained using PureLink® Quick Plasmid Miniprep Kit (Invitrogen). Diagnostic restriction endonuclease digestion using EcoRI enzyme was performed to detect positive colonies. The plasmid was then sent for sequencing. The sequencing was carried out by GenePool (The University of Edinburgh School of Biological Sciences). The

sequence was analysed and compared to the known sequence using DNASTAR Lasergene 10 Core Suite programme. After the correct sequence had been confirmed, the plasmid DNA was used as a standard material for the qPCR for making the standard curve. The concentration of the DNA plasmid was determined by Nanodrop spectrophotometry. The number of copies of the plasmid was calculated using the following equation:

$$\text{Copy number per } \mu\text{l} = 6.032 \times 10^{23} \text{ (copies/mol)} \times \text{plasmid concentration (g/}\mu\text{l)} / \text{molecular weight (MW) of the whole plasmid (g/mol)}.$$

Plasmid MW (g/mol) = (number of insert base pairs + number of vector base pairs) x 660 dalton. According to the calculated copy number, the standard plasmid was diluted to 10^9 copies/ μl and stored at -20°C .

ORF65 qRT-PCR was carried out using FastStart Universal SYBR Green Master (Roche) following the two-step programme in the manufacturer's protocol. The optimum conditions for 100% reaction efficiency and detection of the specific product were determined. The annealing temperature of 62°C was used. ORF65 transcript qPCR primers were synthesised by Eurofins MWG Operon (Ebersberg, Germany). The primer sequences are shown in Table 2.3.

| mRNA | Primer sequence (5'-->3') | Annealing temperature (°C) |
|----------------|---|-----------------------------------|
| DNA polymerase | Fwd AGAGTGTTTGGGTGAATGTGG Rev GCTTGGAGATGGAGTTGGTG | 63 |
| ORF50 (RTA) | Fwd CAAAGTCCATAACAGGCATCC Rev GCCAGAGGTTGAGGTAGC | 62 |
| ORF73 | Fwd CGTCTGTCTCTCCTACATCTAAACC Rev CACCAACACTTCCCTCATCC | 62 |
| M3 | Fwd TAACAGGCAGATTGCCATTCCC Rev TGGCACTCAAACCTTGGTTGTGG | 65 |
| SDHA | Fwd GTCCTACTGATGAAACCTG Rev AACTCAATCCCTTACAGCAA | 62 |

Table 2-2 Sequence of the primers used for viral mRNAs and cellular SDHA qRT-PCR assays.

| Primer | Sequence (5'-->3') |
|-----------------------|--|
| ORF65 cloning primers | Fwd GATAACACATTGCCTACATTG Rev CTTCCATTTCCACTCCTT |
| ORF65 qPCR primers | Fwd TGTCTTGGGTATCTATCACTTGG Rev CTCCCTCCGTCAAATGCTC |

Table 2-3 Sequence of the primers used for ORF65 cloning and qRT-PCR.

2.10 Agarose gel electrophoresis

DNA fragments were analysed using 1% agarose gel electrophoresis. The gels were prepared by dissolving 1 gram SeaKem[®] LE agarose (Lonza, USA) in 100 ml TAE buffer. To visualize the DNA, 10µl of SYBR[®] Safe DNA gel stain (Invitrogen, UK) was added to 100 ml 1% agarose solution. The DNA was mixed with 6x gel loading buffer and electrophoresis was performed at 90 V. The DNA was detected using UV light and the size of the DNA was determined using the relevant DNA ladder.

1L (50X) TAE Buffer

242 g Tris-base

57 ml (glacial) Acetic acid

100 ml (0.5 M) EDTA, pH 8.0

2.11 Construction of MHV-68.ΔmiRNAs virus and its WT parental counterpart

2.11.1 Cloning of the miRNAs mutated sequence into pORI plasmid

A pUCIDT Ampicilin resistance plasmid carrying the synthetic miRNAs mutated sequence flanked by EcoRI sites (GAATTC) was purchased (IDT, UK). Ten nanograms of the pUCIDT.ΔmiRNAs plasmid were transformed into One Shot[®] TOP10 competent cells (Life technologies, UK). A pUCIDT.ΔmiRNAs plasmid midi-prep was carried out using Plasmid Midi Kit (Qiagen) according to the manufacturer's instructions. Two µg of the plasmid midi-prep were digested with 20 units EcoRI restriction enzyme (NEB). The digest was analysed on 1% agarose gel and the released DNA fragment was extracted from the gel using QIAquick Gel Extraction Kit (Qiagen). The DNA fragment was then ligated to pORI plasmid (kindly provided by Prof Jay Nelson, Oregon Health and Science University, USA)

that carries Kanamycin cassette and FRT sites using T4 DNA ligase kit (Thermo Scientific). The ligation mixture was then transformed into chemically competent cells. The cells were plated on LB agar supplemented with Kanamycin (50 ug /ml). Six colonies were picked and plasmid DNA was isolated using PureLink® Quick Plasmid Miniprep Kits (Invitrogen). Positive colonies were determined by restriction enzyme (EcoRI) diagnostic digestion. The orientation of the inserted miRNA knock out (KO) sequence was determined using restriction enzyme diagnostic digestion. Double digestion with XhoI/XbaI (NEB) or SacI/XhoI (NEB) was carried out on the pORI.ΔmiRNAs plasmids extracted from 6 different colonies. The sizes of the released fragments determined the orientation of the miRNA KO sequence in the plasmid.

2.11.2 PCR amplification of the DNA fragment that carries the miRNAs KO sequence, Kanamycin cassette, and FRT sites

Expand long template PCR system (Roche) was used to amplify a DNA fragment carrying the miRNAs KO sequence, kanamycin resistance sequence flanked by FRT sites, and MHV-68 homologous sequence for homologous recombination into EL250 cells. pORI.ΔmiRNAs plasmid mini-prep was used as a template (0.5 μl). The PCR reaction was carried out in 100 μl final volume. The primers' sequences were:

Forward primer 5'

GGAGCTAGGCCACGCCCATTGGAGTTTTCCCCCTCTCCCCTCGCCTGGCT
CTCAGCCCCGAAAAGTGCCACCTGCAGAT -3'

Reverse primer 5'-GCCTGTGGAGCAAAAGGGG -3'.

Primers concentration used was 500 nM per reaction. Thermal conditions were as follow: Hold for 7 min at 95°C followed by 45x cycles of 95 °C for 30 sec, 55 °C for

30 sec, and 68 °C for 4 min followed by 10 min at 72 °C and 4 °C indefinitely. The PCR products were analysed on 1% agarose gel, the band was excised and extracted from the gel as previously mentioned. The extracted DNA was subjected to ethanol precipitation by adding 1/10 volume of 3 M sodium acetate, pH 5.2, mixed well and 2.5 volume of cold 100% ethanol were added and mixed well again before placing in -20 °C for 30 min. The mixture was centrifuged at 18000 xg/15 min/4°C. Supernatant was carefully decanted and DNA pellet was washed twice in 1 ml 70% ethanol. The supernatant was decanted after the washes and DNA pellet was air dried. The DNA pellet was re-suspended in 25 µl DNase free water.

2.11.3 Transformation of the WT-MHV-68 BAC into E.coli strain EL250

2.11.3.1 Preparation of WT-MHV-68 BAC midi-prep

DH10B cells containing WT-MHV-68 BAC were plated out on LB agar containing Chloramphenicol (12.5µg/ml) and incubated o/n at 37°C. A single colony was picked and dropped in 3 ml LB broth containing 12.5µg/ml Chloramphenicol and incubated all day (8 hours) at 37°C with vigorous shaking to form the starter culture. This culture was added to 500 ml LB broth plus Chloramphenicol (12.5µg/ml) and incubated o/n at 37°C with vigorous shaking. Midi-prep BAC DNA was then isolated using QIAGEN® Plasmid Purification kit (Qiagen) following the very low-copy plasmid/cosmid purification protocol. The BAC DNA pellet was re-suspended in 50µl TE buffer.

2.11.3.2 Preparation of electrocompetent EL250 cells

A sterile tip was dipped in the frozen EL250 cells and immersed in 3 ml LB broth and incubated o/n at 30°C with shaking. Next morning, 1 ml of the culture was added to 50 ml of LB broth in 250-ml flask and incubated at 30°C with shaking until the OD₆₀₀ reached 0.4. The culture was centrifuged at 6000 xg for 10 min at 4°C in pre-chilled autoclaved tubes. The cell pellet was re-suspended in 10 ml of cold autoclaved 10% glycerol (made with Milli-Q water). It was then divided between 2 cold 250 ml centrifuge bottles. The bottles were filled with the 10% glycerol and were centrifuged at 6000 xg for 10 min at 4°C. After centrifugation, the bottles were kept on ice and supernatant was decanted and bottles were inverted briefly to drain. The bugs were then re-suspended in the remaining liquid (1-2 ml) and were transferred to sterile Eppendorf tubes. The cells were centrifuged at 6000 xg for 10 min at 4°C. The cell pellets were re-suspended in 200µl 10% glycerol and kept as 100µl volume in -80°C.

2.11.3.3 Electroporation of WT-MHV-68 BAC DNA into electrocompetent EL250 cells

One µl of purified WT-MHV-68 BAC DNA was mixed with 50µl electrocompetent E-coli strain EL250 (Dy380 derived) that harbours λ prophage, which provides heat-inducible recombinase enzyme and arabinose-inducible FLP. The mixture was left on ice for 5 min and then transferred to 0.1 cm gap cuvette. The Gene Pulser was set at 2.5k V and the BAC DNA was electroporated into electrocompetent EL250 cells and 1 ml LB broth was immediately added to the cells. The cells were incubated at 30°C for 1 hour with shaking (200 rpm). It was then centrifuged for 5 min at 450x g and re-suspended in 200µl LB broth, plated on LB agar plate containing Chloramphenicol (12.5µg/ml), and incubated at 30°C for 2 days.

2.11.4 Minipreping BAC cultures

Single colonies (EL250 cells containing transformed BAC or DH10B cells containing original BAC) were picked and were inoculated into 10 ml LB broth + Chloramphenicol (12.5µg/ml) and incubated o/n at 30°C with shaking (200 rpm). Next morning, the cultures were spun down in 15 ml falcon tubes for 15 min at 3000 rpm in table top centrifuge. The cell pellet was re-suspended in 300µl of cold P1 buffer (Qiagen) by dragging along tube rack. It was then transferred to 2 ml Eppendorf tubes and 300µl of P2 buffer were added and mixed gently by inverting. This was followed by addition of 300µl of P3 buffer and mixed gently again by inverting. One ml of Phenol/Chloroform/Isoamyl alcohol, pH6.7 (25:24:1) was added to the mixture and rotated in cold room (4°C) for 15 min. The mixture was then centrifuged at 12000 xg for 10 min at 4°C. The upper phase was transferred to a new 1.5 ml screw-capped tube using a snipped tip in order not to shear the DNA. One tenth volume of 3M sodium acetate (pH 5.2) and 1 ml isopropanol were added and incubated at -80°C for at least 30 min. The mixture was then centrifuged at 12000 xg for 10 min at 4°C. The supernatant was discarded and BAC DNA pellet was washed in 500µl of 70% ethanol followed by centrifugation at 12000 xg for 10 min at 4°C. The ethanol was discarded and the BAC DNA pellet was re-suspended in 100µl of EB buffer. Miniprep BAC DNA was kept at 4°C until used.

2.11.5 Preparation of electrocompetent, recombination-induced EL250 cells

Single colony (EL250 cells carrying MHV-68 BAC) was picked and grown in 3 ml o/n culture at 30°C with shaking (200 rpm). Next morning, 1 ml of the culture was added to 50 ml of LB broth. The culture was incubated in 250-ml flask at 30°C with

shaking until the OD₆₀₀ reached 0.4. The culture was then induced in water bath at 42°C for 17 min with swirling every couple of minutes for expression of recombination proteins. The culture was placed on ice for 5 min, centrifuged at 6000x g for 10 min at 4°C in pre-chilled autoclaved tube. The pellet was re-suspended in 10 ml of cold, autoclaved 10% glycerol v/v (made with milli-Q water). The pellet suspension was divided between 2 cold 250 ml centrifuge bottles. The bottles were filled with the 10% glycerol v/v, centrifuged at 6000x g for 10 min at 4°C in pre-chilled autoclaved tubes. The supernatant was discarded and the bottles were inverted briefly to drain. The bacterial pellets were re-suspended in the remaining fluid (1-2 ml) and were transferred to sterile Eppendorf tubes. The cells were centrifuged at 6000 xg for 10 min at 4°C. The cell pellets were re-suspended in 200µl 10% glycerol v/v. The cells were then kept in volumes of 100µl in sterile Eppendorf tubes and kept at -80°C or used immediately for electroporation.

2.11.6 Transformation to obtain BAC clones by homologous recombination

Approximately 300 ng of the DNA fragment carrying the miRNAs KO sequence, Kanamycin cassette, FRT sites, and flanking MHV-68 homology arms designed from the target sequence (see section 1.12.2) was electroporated into electrocompetent heat-induced EL250 cells carrying the wt-MHV-68 BAC. The Gene Pulser was set at 2.5k V. One ml LB broth was added to the cells immediately after electroporation. The cells were incubated at 30°C for 1 hour with shaking (200 rpm). It was then centrifuged for 5 min at 450x g and re-suspended in 200µl LB broth then plated on LB agar plate containing Chloramphenicol (12.5µg/ml) and Kanamycin (50µg/ml) and incubated at 30°C for 48 hours.

2.11.7 FLP recombination

A single colony from the previous transformation was picked and 2 ml o/n culture was grown with Chloramphenicol (12.5µg/ml) and Kanamycin (50µg/ml) and incubated at 30°C while shaking. Next day, the miniculture was added to 98 ml of LB with Chloramphenicol (12.5µg/ml) only and was grown at 30°C with shaking until the OD₆₀₀ reached 0.5. EL250 cells contain an endogenous L-arabinose-inducible FLP gene which can excise the kanamycin resistance gene that is flanked by FRT sites via FLP-mediated recombination. Therefore, 1 ml of 10% L-arabinose (1 g L-arabinose (Sigma Aldrich, Germany) in 10 ml LB broth and sterilised with 0.2µM filter) was added to the culture and returned to 30°C for one hour with shaking. Then, the culture was diluted 1/10 in LB and was grown for an additional hour at 30°C with shaking.

The culture was centrifuged at 1000xg for 3 min at RT. The pellet was re-suspended in 1 ml LB broth and 10-fold serial dilution was carried out. Fifty µl of 10⁻⁴, 10⁻⁵, and 10⁻⁶ dilutions were plated on LB agar with Chloramphenicol (12.5µg/ml) and incubated at 30°C for 2 days. Antibiotic selection was used to determine the removal of the kanamycin cassette. Thirty colonies were picked and each colony was plated on LB agar with Chloramphenicol (12.5µg/ml) only and with Kanamycin only (50µg/ml). The colonies which grow in the presence of Chloramphenicol, but not in the presence of Kanamycin were selected.

2.11.8 Screening PCR for detection of clones that carry all the expected mutations

The following components (Life technologies) were added to a sterile PCR tube placed on ice: 0.1 volume of 10x PCR buffer, 0.2 mM dNTP mixture, 1.5 nM MgCl₂,

0.5 μ M of each primer, 10 ng BAC DNA template, 1-2 units Taq DNA polymerase enzyme, and PCR grade water to final volume. Contents were collected to the bottom of the tube by brief centrifugation. The primer sequences were:

Fwd primer: 5' GCGCGCCCTGGAGATCCGGG 3'

Rev primer: 5' GCATAGAAACTGAGAGCAAGGC 3'

The thermal conditions were as follow: hold at at 94°C for 3 minutes followed by 25 cycles of denaturing at 94°C for 45 s, annealing at 55°C for 30 s, and extension 72°C for 1.5 min; followed by additional incubation at 72°C for 10 min and the reactions were maintained at 4°C. PCR products were analysed by agarose gel electrophoresis as described previously (section 2.10) and visualised by SYBR[®] Safe staining (Invitrogen, UK) using UV light.

The PCR product was excised from gel and the DNA was extracted using QIAquick Gel Extraction Kit (Qiagen). The DNA fragment was cloned into TOPO vector using TOPO TA Cloning Kit (Invitrogen) for sequencing according to the manufacturer's protocol. After cloning, miniprep plasmid DNA was obtained using PureLink[®] Quick Plasmid Miniprep Kit (Invitrogen). Diagnostic restriction endonuclease digestion using EcoRI enzyme was performed to detect positive colonies. The plasmid was then sent for sequencing. The sequencing was carried out by GenePool (The University of Edinburgh School of Biological Sciences). The sequence was analysed and compared to the known sequence using DNASTAR Lasergene 9 Core Suite programme.

The same protocol was used after the generation of the miRNA mutant virus and its WT counterpart except that 100 ng total genomic DNA was extracted from NIH 3T3 cells infected with the mutant or WT virus at MOI of 5 for 24 hours and used as a template. The primer sequences were:

Fwd primer: 5' GCACCCCACTATCTCTGGTTCTGC 3'

Rev primer: 5' CCTGTGGAGCAAAAGGGGTA 3'

2.11.9 Determination of the BAC genomic arrangement by restriction endonuclease digestion

Fifty μ l of the miniprep BAC DNA from EL250 cells (transformed BAC) or DH10B cells (original BAC) were digested with 2 μ l XhoI enzyme (NEB, 20,000 units/ml) using buffer 4 (NEB) in a total reaction volume of 60 μ l for 1 hour at 37°C followed by addition of another 2 μ l of XhoI enzyme and incubated at 37°C o/n. The next day, 6x loading buffer was added to the digested BAC DNA and was analysed on a 0.8% agarose gel. The gel electrophoresis was performed at 30V o/n. The next day, the gel was immersed in TAE buffer containing SYBR[®] Safe DNA gel stain (Invitrogen, UK) at dilution of 1/1000 and was gently shaken on a rocker for 20 min. The DNA fragments were visualised using UV light.

2.11.10 BAC transfection, removal of BAC cassette, and generation of the mutant virus and its WT parental counterpart

Parental WT MHV-68 BAC or MHV-68. Δ miRNAs BAC (200 ng) were transfected into 3T3 cells expressing Cre recombinase that can excise the BAC vector flanked by *loxP* sites. The day before transfection, Cre 3T3 cells were seeded in 24 well plates at

density of 5×10^4 cells/ well. The next day, cells were approximately 50% confluent and the transfections were carried out using Effectene[®] Transfection Reagent (Qiagen) following the manufacturer's protocol. Seven days later, widespread of CPE was observed and a mix of green (GFP expression) and colourless (BAC vector removed) plaques were seen under the fluorescence microscope. The viruses were harvested by pipetting up and down. Cre 3T3 cells were seeded in 96 well plates at density of 2×10^4 cells / well. The following day, cells were approximately 50% confluent. The viruses were frozen and thawed 3 times and diluted 1/100, 1/200, 1/500, 1/1000, 1/5000, and 1/50000 for 12 wells of each. The viruses were added to the cells and plaque formation was monitored. Single colourless (no GFP expression) plaques were harvested. Cre 3T3 cells were set up in 24 well plates and the virus was grown in them by adding 1/4 harvest from the 96 well plate after freezing and thawing 3 times. The viruses were harvested when there was widespread of CPE.

MHV-68 miRNAs were detected in the mutant virus, indicating that there is a mixture of mutant and wt-viruses. Therefore, the virus was diluted 1/100, 1/200, 1/500, 1/1000, 1/5000, and 1/50000 and was added to Cre 3T3 cells in 96 well plate (12 wells of each dilution). Single plaques were harvested and screened for the presence of viral miRNAs by qRT-PCR. A plaque in which the deleted miRNAs were absent was used for infecting Cre 3T3 cells in 24 well plate (1/4 harvest from the 96 well plate). The mutant and the WT viruses from the 24 well plates were titrated and grown at MOI of 0.001 in BHK cells as previously described.

2.12 One-step growth curve

The day before experiment, BHK-21 cells were seeded in 24 well plates at density of 10^5 cells per well in 2 ml GMEM medium. The next day, cells were infected with

WT parental MHV-68 virus or MHV-68.ΔmiRNAs virus at MOI of 5. Uninfected cells served as a control. Virus was allowed to attach to cells for 1 hour at 37°C in CO₂ incubator. The medium was then removed and cells were washed 3 times with medium. One ml of fresh medium was added to each well and zero hour samples were taken immediately after the washes. Plates were incubated at 37°C in CO₂ incubator and samples (in duplicates) were kept at -80°C at appropriate time points (6, 12, 18, 24, 30, 36, 48, 60, and 72 hours after infection). Virus was released from the cells by freezing and thawing at -80°C for 3 times. Virus titration was carried out as previously described.

2.13 Measuring viral titre in the lung

Lung tissues were chopped into small pieces and homogenised in 1 ml of complete GMEM medium using TissueLyser (Qiagen) operating for 2 min at 28 Hz (2x). The lung homogenate was centrifuged at 2000xg for 5 min at 4°C and supernatant was kept at -80°C until used. Tenfold serial dilution of the homogenate was carried out and each dilution was incubated with 2 x 10⁶ BHK-21 cells in bijoux tubes at 27°C for 1 hour with continuous shaking (200 rpm). The virus-cells mixture in each bijoux tube was divided between two 60mm culture plates and 3 ml of GMEM was added to each plate and incubated at 37°C in CO₂ incubator for 4 days. The plaques were counted after being fixed and stained as described previously.

2.14 Infective centre assay

Spleens were harvested and single-cell suspensions were obtained by pressing through a 40-µm cell strainer in 5ml of complete RPMI-1640 medium. The cell suspensions were transferred to 20ml universal tubes and centrifuged at 450xg for 5

min at 4°C. The supernatant was removed and the pellet was re-suspended in 1 ml of sterile water for disruption of red blood cells and followed immediately by adding 9 ml of sterile PBS. Cells were centrifuged at 450xg for 5 min at 4°C and the pellets were re-suspended in 10 ml RPMI-1640 medium. Cell debris was allowed to settle and the cells were transferred to fresh universal tubes. The cells were diluted in 0.1% Trypan Blue for counting viable cells using a haemocytometer. The volume equal to 10^7 , 10^6 or 10^5 splenocytes were co-cultivated with 10^6 BHK-21 cells in 60mm culture plates (duplicates) in 5 ml RPMI-1640 medium supplemented with 10% FCS, 2mM L-Glutamin, 100U pen/strept, 50µM 2-mercaptoethanol, 100µM HEPES, and 0.25µg/ml Fungizone. The cells were incubated at 37°C in CO₂ incubator for 5 days. The infective centres were counted after being fixed and stained as described previously. BHK cells only without addition of splenocytes were plated to serve as a negative control. Excess splenocytes were kept at -80°C to test for the presence of free infectious virus.

2.15 Measuring the produced infectious virus titre in splenocytes

Live splenocytes were kept at -80°C. These cells were frozen and thawed 3 times to disrupt the cells and prevent reactivation of latent virus. Volumes equivalent to 10^7 splenocytes were added to 10^6 BHK-21 cells (in duplicates) and incubated at 27°C for 1 hour with continuous shaking. The cells were then transferred to 60mm culture plates and incubated at 37°C in CO₂ incubator for 4 days. The plaques were counted after being fixed and stained as described previously. Plates containing BHK cell only served as a negative control.

2.16 Flow cytometry for determination of B cell activation and proliferation

Spleen cell suspensions were prepared as described above (section 2.14). Splenocytes were counted to make 5×10^6 cells / ml. Fifty μl cells (2.5×10^5) were added to each Polystyrene FACS test tube. The cells were pre-incubated with 25 μl purified anti-CD16/CD32 antibody (BD Pharmingen Biosciences, USA) for 30 min at 4°C at final concentration of 1:200 in FACS buffer (PBS containing 1% BSA w/v and 0.1% sodium azide w/v). The splenocytes were then labelled with 25 μl of anti-CD19-PE antibody alone (Invitrogen, UK) (1:200 in FACS buffer), anti-CD69-FITC antibody alone (AbD Serotec, UK) (1:50 in FACS buffer), or a combination of both and incubated for 15 min at 4°C in the dark. FACS buffer (700 μl) was added to the labelled cells, centrifuged for 5 min at 430xg, supernatant was discarded, and cells were washed 2 times with 700 μl FACS buffer. If no further staining was required 200 μl FACS buffer were added followed by addition of 2% formaldehyde (formal buffered saline diluted 1:1), mixed, and stored in the dark at 4°C until ready to analyse.

For Ki-67 intracellular staining, cells were fixed with 50 μl of 1% paraformaldehyde for 15 min at RT in the dark and washed 2 times with 700 μl FACS buffer. Cells were permeabilised with 50 μl of 0.5% Tween-20 (diluted in sterile PBS (v/v) for 10 min at RT in the dark and then washed as above. Rabbit poly-clonal anti-ki-67 antibody (100 μl) (Abcam, UK) at a final concentration of 1:200 in FACS buffer were added to the cells for 15 min in the dark at 4°C. Then the cells were washed as above and 100 μl biotinylated goat anti-rabbit IgG (H+L) (Vector Laboratories, USA) antibody (1:300 in FACS buffer) were added and cells were incubated for 15 min at 4°C in the dark. Cells were washed as above and 100 μl Streptavidin

AlexaFlour[®]647(Invitrogen, UK) at a final concentration of 1:500 in FACS buffer were added and incubated for 15 minutes in the dark at 4°C. Cells were then washed as above and fixed by addition of 200µl FACS buffer followed by addition of 2% formaldehyde v/v. Samples were stored in the dark at 4°C until ready to analyse.

PE rat IgG2a (BD Pharmingen Biosciences, USA), FITC hamster IgG₁ (BD Pharmingen Biosciences, USA), and rabbit IgG (GeneTex) isotype negative controls were used at the same concentration of each antibody. Unstained splenocytes before and after cell permeabilisation were also used as negative controls. Flow cytometry was performed on BD FACSCalibur (BD Bioscience) and analysed with FlowJo software. For each sample 10,000 events were counted.

2.17 Statistical analysis and graphs production

Experimental statistical analyses were performed using Minitab 16 statistical software. Graphs were produced using GraphPad Prism 6 software

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Chapter 3: Expression profiles of MHV-68 miRNAs *in vitro* and *in vivo*

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3 Chapter 3: Expression profiles of MHV-68 miRNAs *in vitro* and *in vivo*

3.1 Introduction

One of the aims of this project was to carry out a systematic analysis of the expression pattern of the MHV-68 miRNAs during latent infection *in vivo*. In order to do this, it was necessary to establish an assay for quantification of the miRNAs expression levels. There are different ways for miRNAs expression profiling such as qRT-PCR, microarray, and next-generation sequencing. qRT-PCR technology is highly specific, highly sensitive, and has a wide dynamic range. In contrast, miRNA microarrays are less sensitive and have lower dynamic range. Next-generation sequencing is mainly used for discovery of novel miRNAs. Therefore, qRT-PCR is the method of choice for quantification of the expression of previously known miRNAs.

Previous published studies identified at least 15 MHV-68 primary miRNAs by deep sequencing (Reese, Xia et al. 2010, Zhu, Strehle et al. 2010). These primary miRNAs are expected to be processed into at least 28 mature miRNAs. The mature miRNAs are the functional molecules; it is therefore of interest to characterise the expression pattern of this class of miRNAs. There are two common priming strategies in the commercially available qPCR-based platforms for amplification of mature miRNAs. One strategy involves the use of specific stem-loop RT primers for the cDNA synthesis (Chen, Ridzon et al. 2005, Kramer 2011). The other strategy depends on the use of universal RT primers, where the miRNAs are polyadenylated by a poly (A) polymerase enzyme and reverse transcribed into cDNA using oligo-dT primers,

which have a universal tag sequence on the 5' end and a 3' degenerate anchor (Shi and Chiang 2005). Both approaches have advantages and disadvantages. The stem-loop RT primers are long and bind to the stem-loop region of the miRNA, whereas the universal RT primers are short and utilise almost the entire mature miRNA sequence; therefore, the stem-loop priming method is more specific than the universal RT priming. However, the poly (A) tailing and universal RT reaction produce a stable pool of cDNA that can be used to detect different miRNAs and/or mRNAs from the same cDNA; thus, this method is convenient when using limiting amount of sample. In contrast, the stem-looping requires different RT primers for detection of individual miRNAs and only allows for detection of the miRNA of interest, but not the mRNA from the same cDNA preparation. This makes stem-loop sequence specific RT priming more time-consuming and more expensive than universal tailing RT priming.

Stem-loop qPCR requires the use of an miRNA-specific forward primer, a reverse primer, and a probe. This assay does not allow the user to control the reaction specificity by melting curve analysis. The qPCR performed using cDNA from the universal tailing RT reaction requires a miRNA-specific forward primer and a universal reverse primer; therefore, there is little degree in freedom of designing the miRNA-specific primer and the assay requires more stringent optimisation measures in order to ensure the reaction specificity. This specificity can be observed by melting curve analysis.

In this project, the quantification of the mature MHV-68 miRNAs expression levels was performed using the universal tailing RT priming for the cDNA synthesis followed by qPCR using miRNA-specific forward primer and a universal reverse primer. Optimisation of each miRNA qRT-PCR assay was carried out in order to

ensure high specificity and high efficiency of the assay. This chapter demonstrates the establishment and optimisation of the miRNA qRT-PCR assays and the use of these assays to characterise the expression profiles of the mature MHV-68 miRNAs *in vitro* and *in vivo*.

3.2 Establishment and optimisation of qRT-PCR assays for detection and quantification of the MHV-68 mature miRNAs

qRT-PCR assays were used to detect and quantify the mature MHV-68 miRNAs. Figure 3.1 shows a workflow diagram of the qRT-PCR reaction. Initially, the total RNA was polyadenylated with a poly (A) polymerase enzyme. The polyadenylation is a random process and results in addition of Adenine residues to the 3' end of the miRNAs. Subsequently, cDNA molecules were synthesised using reverse transcriptase enzyme and oligo-dT primers with universal tag sequence on the 5' end and a 3' degenerate anchor. This allows the amplification of the mature miRNAs without detection of genomic DNA. A miRNA-specific forward primer and a qPCR universal reverse primer were used for the subsequent qRT-PCR reaction. The miRNA-specific primer consists of the full-length mature miRNA sequence based on the miRBase database (<http://www.mirbase.org>) (Kozomara and Griffiths-Jones 2014). The final miRNAs quantifications were carried out by qRT-PCR assays using the SYBR Green fluorescence (see Materials and Methods section 2.9.1)..

Each miRNA has a different sequence and different GC content which can significantly affect the specificity and efficiency of the qRT-PCR assays. Therefore, optimisation of the individual qRT-PCR assays for each miRNA was carried out,

aiming at determining the optimum miRNA-specific primer concentration, assay annealing temperature, efficiency, and specificity of the reaction.

The qRT-PCR assays were established using total RNA extracted from BHK-21 cells infected with MHV-68 virus for 24 hours at MOI of 5. Analysis and optimisation of each miRNA qRT-PCR assay was carried out. As an example of the qRT-PCR assay optimisation, figure 3.2 shows the melting curve analyses of the mghv-miR-M1-4-5p using different annealing temperatures and primer concentrations in order to maximise the specificity of the reaction. A non-specific qPCR product appeared when an annealing temperature of 60°C and a primer concentration of 250 nM or 200 nM were used (Figure 3.2A and B). However, this non-specific product disappeared when the annealing temperature was increased to 62°C (Figure 3.2C and D). Increasing the annealing temperature to 63°C with the aim of minimising any presence of non-specific products led to the decrease in the peak of the melting curve and increase in the background noise (Figure 3.2E). Therefore, the annealing temperature of 62°C and primer concentration of 200 nM were selected as the optimum conditions for this assay. Optimisation of some miRNA qRT-PCR assays failed, for example, mghv-miR-M1-2-3p showed a non-specific qRT-PCR product represented by melting curve at 84°C, whereas the qRT-PCR product of the target melts at 77.5°C (Figure 3.3A). Increasing the annealing temperature failed to eliminate the non-specific product in the mghv-miR-M1-2-3p qRT-PCR assay (Figure 3.3B-E). Another example is mghv-miR-M1-6-3p qRT-PCR, which consistently showed amplification signals in the uninfected negative control (Figure 3.4). Therefore, these miRNAs were excluded from further analysis. qRT-PCR melting curves of different MHV-68 miRNAs are shown in figure 8.1 in the appendix.

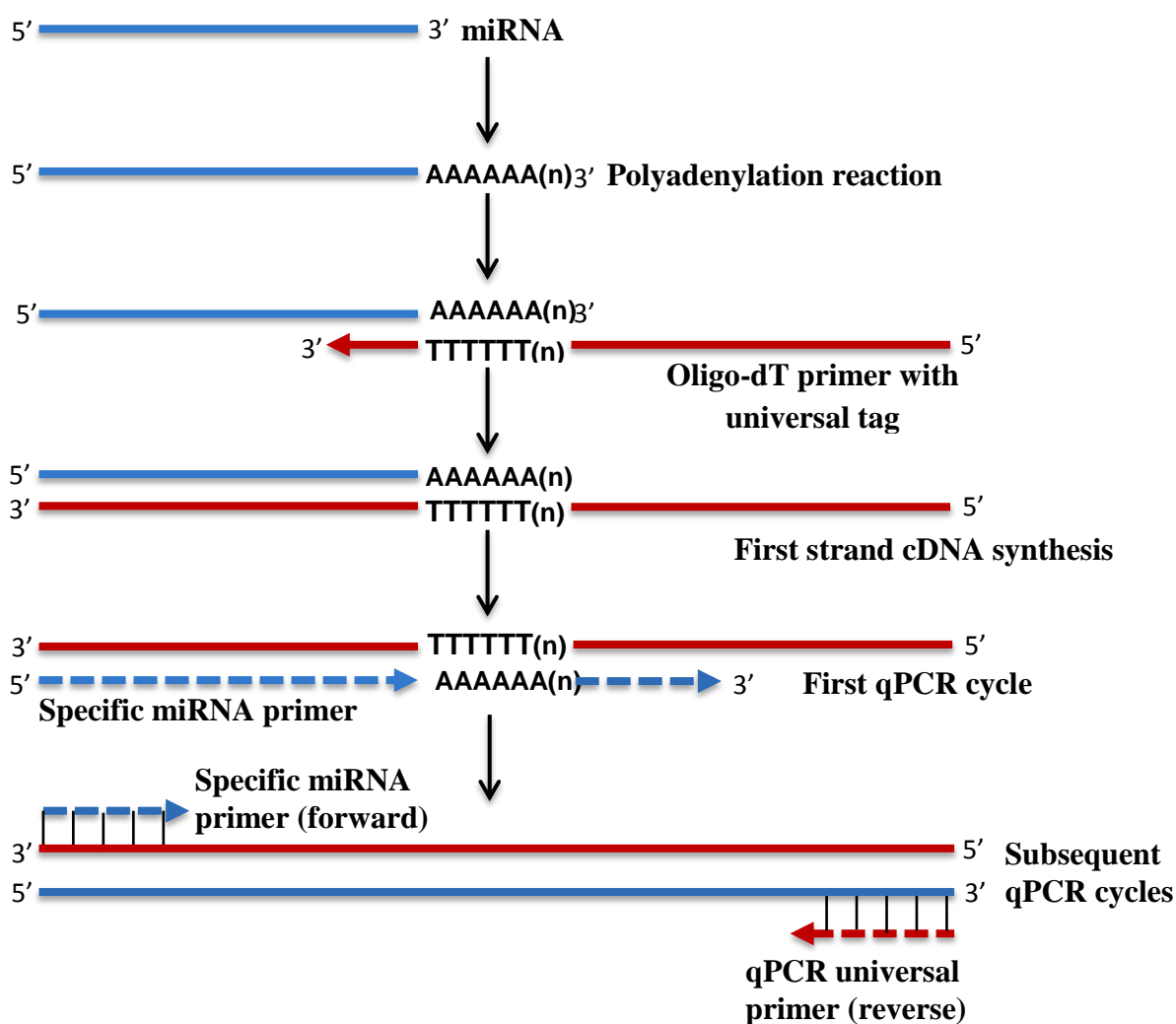


Figure 3.1 Schematic diagram of the qRT-PCR amplification of the MHV-68 mature miRNAs. qRT-PCR includes 3 steps: polyadenylation, reverse transcription and cDNA synthesis, and real-time qPCR. Poly (A) polymerase enzyme adds poly (A) tail to the 3' end of the miRNA. cDNA synthesis is performed by reverse transcription using oligo-dT primer carrying a 5' universal tag and 3' degenerate anchor. A miRNA-specific primer and a universal qPCR primer complementary to the cDNA molecules are used in the qPCR reactions to amplify the target miRNA.

Figure 3.5 shows an example of examining the qRT-PCR assay efficiency and specificity using mghv-miR-M1-7-3p specific primer. The efficiency of the amplification reaction for each miRNA set of primers was determined using 2-fold serial dilution of the cDNAs (Figure 3.5A). The amplification efficiency was determined based on the slope of a standard curve with arbitrary values. The standard curve is graphically represented as regression line plot of CT values versus log of input cDNA. Reaction with 100% efficiency means that the product is doubled each cycle with standard curve slope of -3.32 i.e. there is 10-fold increase in the qRT-PCR amplicon every 3.32 cycles ($\log_2 10 = 3.3219$). Slopes more negative than -3.32 indicate reaction efficiency less than 100%, whereas slopes more positive than -3.32 indicate efficiency greater than 100% that can be due to presence of primer dimer, non-specific amplicon, or pipetting error. Generally, qRT-PCR amplification efficiency between 90-110% is considered acceptable (Life Technologies).

Table 3.1 shows the efficiency of the qRT-PCR assays of the tested miRNAs. All the miRNAs tested showed reaction efficiency in the range of 92% - 105% except mghv-miR-M1-13-3p that had low reaction efficiency and therefore it was excluded from further analysis. The narrow reaction efficiency range between different miRNA qRT-PCR assays confirms that any differences in the quantification are real and not an artefact. The reaction specificity was determined by melting curve analysis where a single peak represents one specific product (Figure 3.5B).

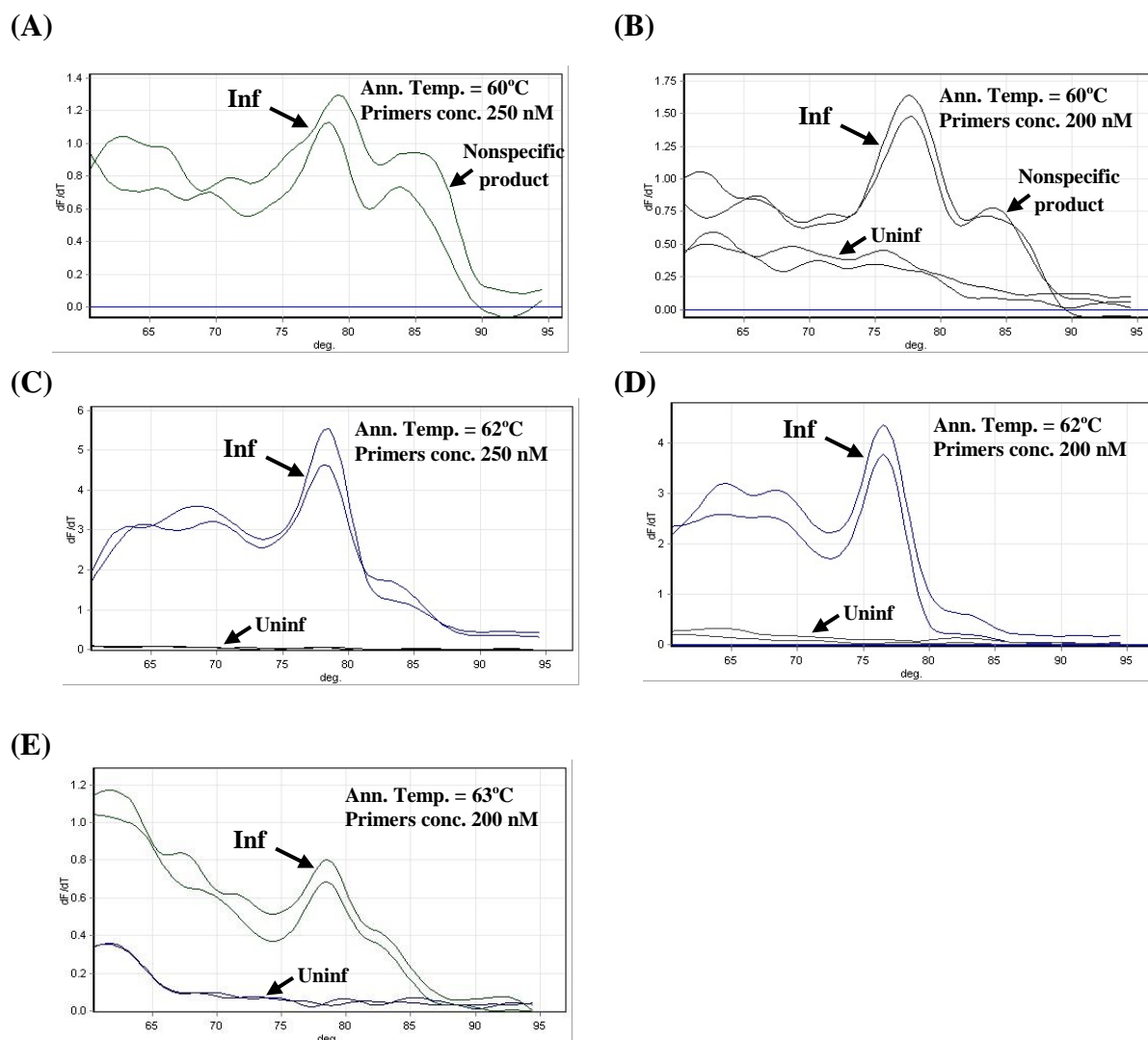


Figure 3.2 Optimisation of the qRT-PCR assay for detection of mghv-miR-M1-4-5p.

The graphs show the melting curve analyses of the mghv-miR-M1-4-5p qRT-PCR assay using different annealing temperatures and primers concentrations (indicated). The vertical values represent the negative first derivative of the fluorescence and the horizontal values represent the melting temperature of the qPCR product(s). The two peaks indicate the presence of two qPCR products (A and B), whereas a single peak represents one product (C and D). When the annealing temperature increased to 63°C, the amount of the specific qRT-PCR product decreased and the non-specific products increased (E). The cDNAs were synthesised from the total RNA samples harvested from infected (Inf) or uninfected (Uninf) BHK-21 cells (MOI of 5, 24 hours after infection).

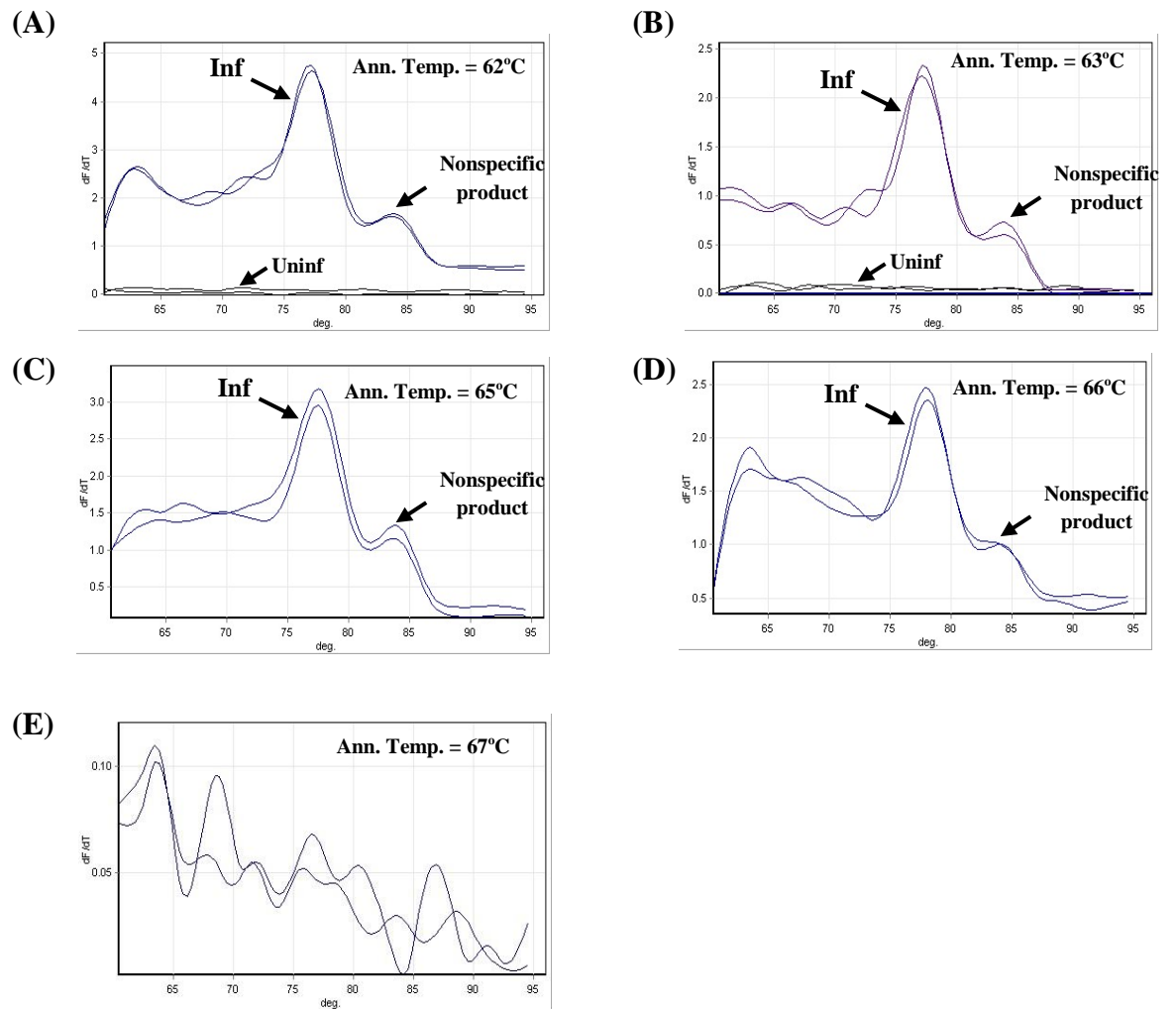


Figure 3.3 Melting curve analyses of mghv-miR-M1-2-3p qRT-PCR assay.

The graphs show the mghv-miR-M1-2-3p qRT-PCR melting curve analyses using different annealing temperatures (indicated). The primers concentrations used were 200 nM. The mghv-miR-M1-2-3p qRT-PCR product melts at 77.5°C while a non-specific product melts at 84°C (A). Increasing the annealing temperature failed to eliminate the non-specific product (B-D). Further increase of the annealing temperature at 67°C showed no qRT-PCR product (E). The cDNAs were synthesised from the total RNA harvested from infected (Inf) or uninfected (Uninf) BHK- 21 cells (MOI of 5, 24 hours post-infection).

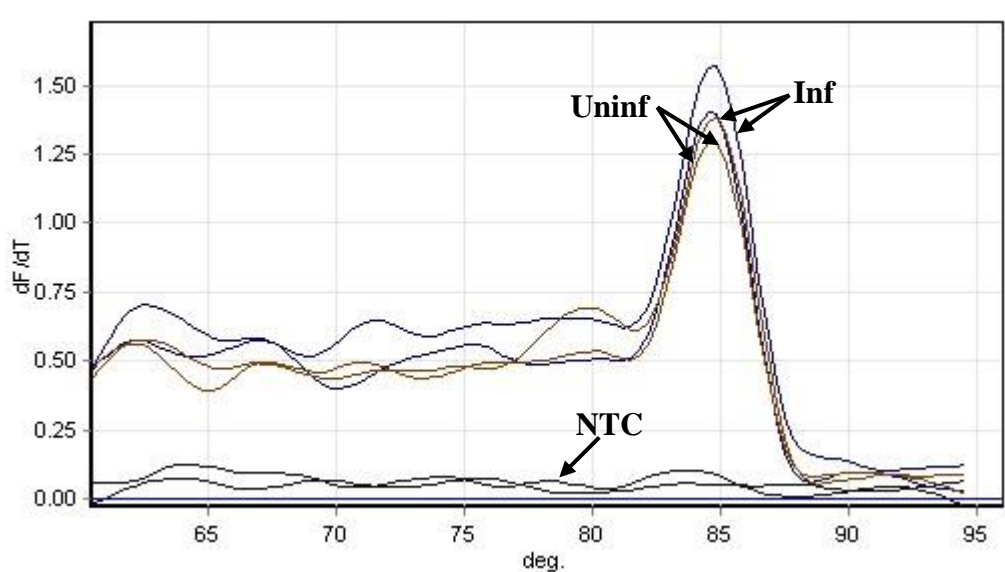


Figure 3.4 Melting curve analysis of mghv-miR-M1-6-3p qRT-PCR.

The graph shows the melting curve of the mghv-miR-M1-6-3p qRT-PCR assay. The total RNA harvested from infected (Inf) or uninfected (Uninf) BHK- 21 cells (MOI of 5, 24 hours after infection) was reverse transcribed into cDNA that was subjected to qPCR. NTC = No template control.

| miRNA | qRT-PCR efficiency (%) |
|-------------------|------------------------|
| mghv-miR-M1-1-3p | 96 |
| mghv-miR-M1-3-3p | 95 |
| mghv-miR-M1-4-5p | 97 |
| mghv-miR-M1-5-5p | 92 |
| mghv-miR-M1-7-3p | 105 |
| mghv-miR-M1-8-5p | 94 |
| mghv-miR-M1-9-3p | 100 |
| mghv-miR-M1-10-3p | 94 |
| mghv-miR-M1-13-3p | 77 |
| mghv-miR-M1-14-3p | 102 |
| mghv-miR-M1-15-5p | 95 |
| mmu-miR-191-5p | 99 |

Table 3-1 Reaction efficiency of the MHV-68 miRNA qRT-PCR assays.

The total RNA harvested from MHV-68-infected BHK- 21 cells (MOI of 5, 24 hours after infection) was reverse transcribed into cDNA. Two-fold serial dilution of the cDNA was carried out and subjected to qPCR in order to make a standard curve with arbitrary values. The reaction efficiency was calculated via the Rotor-Gene Q series software based on the slope of the standard curve.

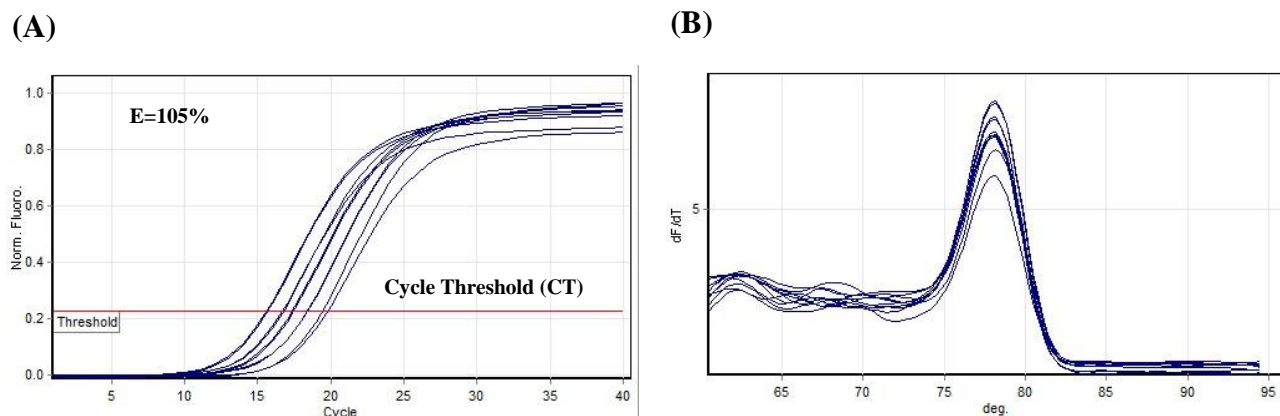


Figure 3.5 Determination of the efficiency and specificity of the miRNAs qRT-PCR assays.

BHK-21 cells were infected with MHV-68 at MOI 5 for 24 hours and the miRNAs were detected by qRT-PCR. (A) Two fold serial dilution of the cDNA was carried out and subjected to qPCR using mghv-miR-M1-7-3p specific primer for determination of reaction efficiency (E). (B) Melting curve analysis of mghv-miR-M1-7-3p qRT-PCR for determination of reaction specificity. The single peak indicates the presence of a single qPCR product.

3.3 The MHV-68 miRNAs increase in abundance over time during the lytic infection *in vitro*

The expression kinetics of MHV-68 miRNAs during productive infection were determined by infecting NIH 3T3 cells with MHV-68 at MOI 5. The total RNA was extracted from uninfected cells or infected cells that were harvested at 2, 8, and 24 hours post infection (hpi). The total RNA was converted into cDNA that was subsequently subjected to qRT-PCR analysis. A reference miRNA was required as a normaliser in order to correct for any experimental variability. miR-191 has been shown to be the most consistently expressed miRNA among various healthy human solid tissues (Peltier and Latham 2008) and it has been found not to be regulated following MHV-68 infection (Zhu et al., 2010 and figure 8.2 in the appendix). Therefore, mmu-miR-191-5p was used as an endogenous reference control to normalise the data in the present study. The viral miRNAs expression levels are presented as values relative to the cellular mmu-miR-191-5p expression levels. Ten miRNAs were examined and all of them were readily detected at variable levels as early as 2 hpi except mghv-miR-M1-4-5p whose expression was below level of detection at 2 or 8 hpi, which can be due to either late expression kinetics of this miRNA or the limitation of the assay (Figure 3.6). The amplification signals in the uninfected controls were below the cycle threshold (data not shown). It was found that mghv-miR-M1-7-3p, mghv-miR-M1-1-3p, mghv-miR-M1-3-3p, mghv-miR-M1-8-5p, mghv-miR-M1-5-5p, mghv-miR-M1-15-5p, and mghv-miR-M1-9-3p are the most abundant miRNAs at 24 hpi (Figure 3.6 A). In contrast, mghv-miR-M1-14-3p, mghv-miR-M1-10-3p, and mghv-miR-M1-4-5p are the least abundant miRNAs (Figure 3.6 B).

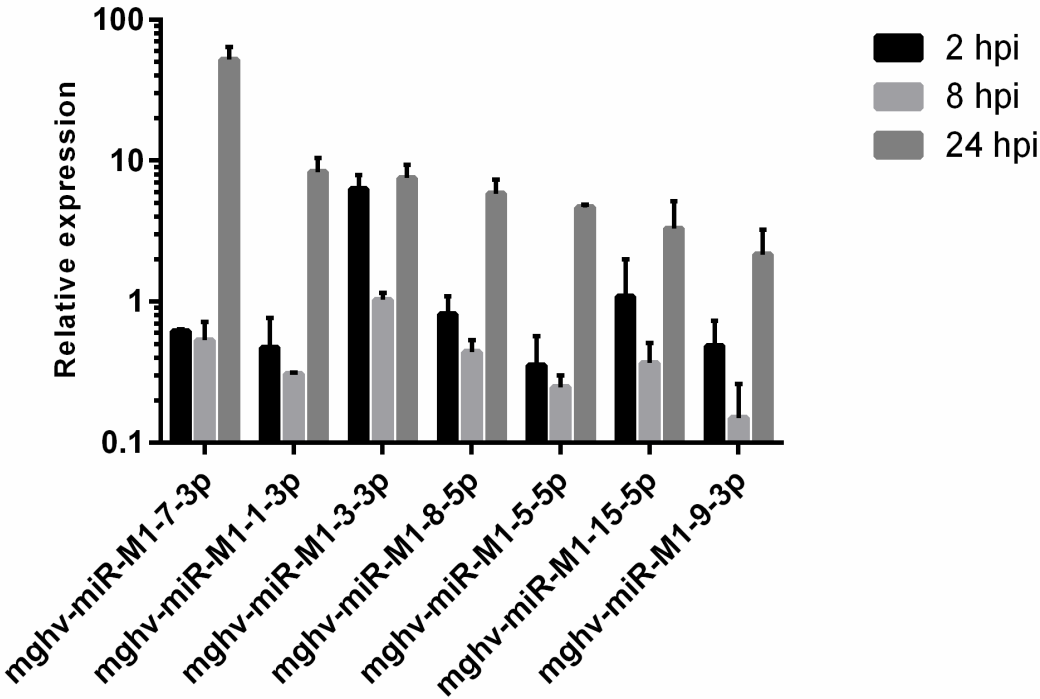
The tested miRNAs showed a slight decrease in expression at 8 hpi and then accumulated over the course of infection. The abundance of mghv-miR-M1-7-3p, mghv-miR-M1-1-3p, mghv-miR-M1-5-5p, mghv-miR-M1-8-5p, mghv-miR-M1-9-3p, mghv-miR-M1-14-3p, mghv-miR-M1-15-5p, and mghv-miR-M1-10-3p at 24 hpi was increased by approximately 84.2, 17.5, 13.2, 7.2, 4.4, 4.2, 3, and 2.6-fold, respectively, as compared to their abundance at 2 hpi. Interestingly, mghv-miR-M1-3-3p expression level at 2 hpi and 24 hpi was almost the same and its abundance at 2 hpi was higher than the other miRNAs at the same time point, indicating that this miRNA may play a role during the early stage of lytic infection.

3.4 Expression of MHV-68 miRNAs during latent infection

3.4.1 Profiling of MHV-68 miRNAs expression during latency *in vitro*

In order to investigate the miRNAs expression profiles during latent infection *in vitro* and compare it to that of the lytic infection, NS0 cells were infected with MHV-68 virus at an MOI five for five days. The NS0 cell line is derived from a mouse myeloma and it supports latent infection; however, approximately 5% of the infected cells undergo reactivation and express lytic proteins (Nash, Dutia et al. 2001). The infected or uninfected NS0 cells were harvested and total RNA was extracted and analysed by qRT-PCR for determination of the expression levels of the MHV-68 miRNAs (Figure 3.7A). The amplification curves of the uninfected controls were below the threshold (data not shown).

(A)



(B)

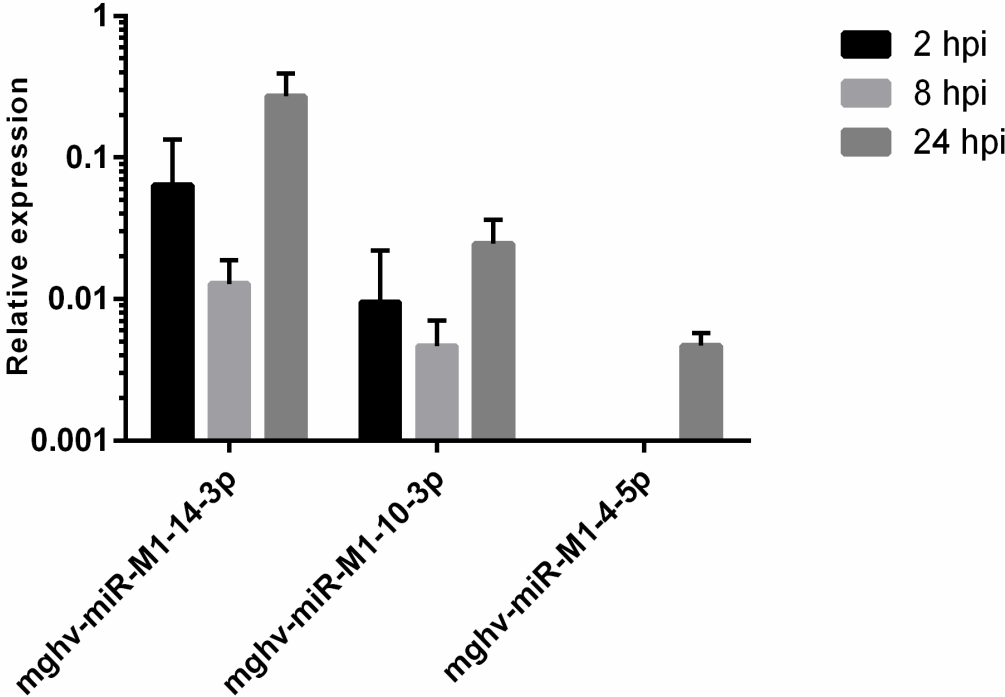


Figure 3.6 Expression kinetics of the MHV-68 miRNAs during lytic infection.

NIH 3T3 cells were infected with MHV-68 virus at MOI of 5 and total RNA was harvested from infected cells at 2, 8, and 24 hours post-infection (hpi). qRT-PCR was carried out using specific forward primers (see Materials and Methods) for quantification of the mature miRNAs. (A) Expression levels of the miRNAs with relatively high abundance. The relative expression was calculated as $2^{-\Delta CT} \times 10^2$ where $\Delta CT = CT_{\text{miRNA of interest}} - CT_{\text{reference miRNA}}$. The mmu-miR-191-5p miRNA was used as a cellular reference miRNA in order to correct for any experimental variability. (B) Expression levels of the miRNAs with relatively low abundance. Error bars represent standard deviation from the mean values of 3 experiments.

In general, the miRNAs were detected during both lytic and latent infection *in vitro* (Figure 3.6 and 3.7A). It was found that the expression pattern of the miRNAs in NS0 cells was similar but not identical to that in the NIH 3T3 cells (Figure 3.6 and figure 3.7A). For example, mghv-miR-M1-9-3p was ranked the 7th in terms of abundance among the tested miRNAs in NIH 3T3 cells at 24 hpi, whereas it is the 3rd most abundant miRNA in NS0 cells (Figure 3.6A and figure 3.7A). The expression level of mghv-miR-M1-7-3p was approximately 6.3-fold higher than mghv-miR-M1-1-3p in 3T3 cells at 24 hpi, but it was approximately 2-fold less than mghv-miR-M1-1-3p in NS0 cells (Figure 3.6A and figure 3.7A). mghv-miR-M1-14-3p, mghv-miR-M1-10-3p, and mghv-miR-M1-4-5p were the least abundant miRNAs in NS0 and NIH 3T3 cell lines, whereas mghv-miR-M1-1-3p and mghv-miR-M1-7-3p were the most abundant miRNAs in both cell lines. Collectively, these findings suggest that the regulation of the expression of some MHV-68 miRNAs can differ depending on the phase of infection.

3.4.2 MHV-68 miRNAs expression during the peak of viral latency *in vivo*

After detecting the MHV-68 miRNAs during latent infection *in vitro*, the expression levels of the miRNAs during latency were examined *in vivo* at 14 dpi, a time point that coincides with the peak latent viral load in the spleen (Cardin, Brooks et al. 1996, Weck, Kim et al. 1999, Marques, Efstathiou et al. 2003). BALB/c mice were infected intranasally with 4×10^5 PFU of MHV-68 virus. Fourteen days post infection, mice were sacrificed and spleens were harvested. The total RNA was extracted from splenocytes and cDNAs were synthesised for qRT-PCR analysis and quantification of the MHV-68 miRNAs. In general terms, the miRNAs expression levels were higher in cell lines than in splenocytes (Figure 3.6 and 3.7). This is expected because

of the higher percentage of infected cells in the cell culture (approximately 100%) as compared to the percentage of infected splenocytes during the peak expansion of latently infected cells, which is approximately 0.5 – 1% (Nealy, Coleman et al. 2010).

It was found that mghv-miR-M1-1-3p and mghv-miR-M1-9-3p were expressed at relatively high levels as compared to other viral miRNAs during latency *in vitro* and *in vivo* (Figure 3.7A and B). Surprisingly, mghv-miR-M1-10-3p and mghv-miR-M1-14-3p, which are expressed at low levels in NS0 cells, were expressed at relatively higher levels *in vivo* when compared to the expression levels of the other miRNAs (Figure 3.7A and B). Although the percentage of infected splenocytes *in vivo* is much lower than the infected cells *in vitro* as mentioned above, the mghv-miR-M1-10-3p abundance in splenocytes was 3.5-fold higher than NS0 cells and 16.9-fold higher than NIH 3T3 fibroblasts at 24 hpi (Figure 3.6B and

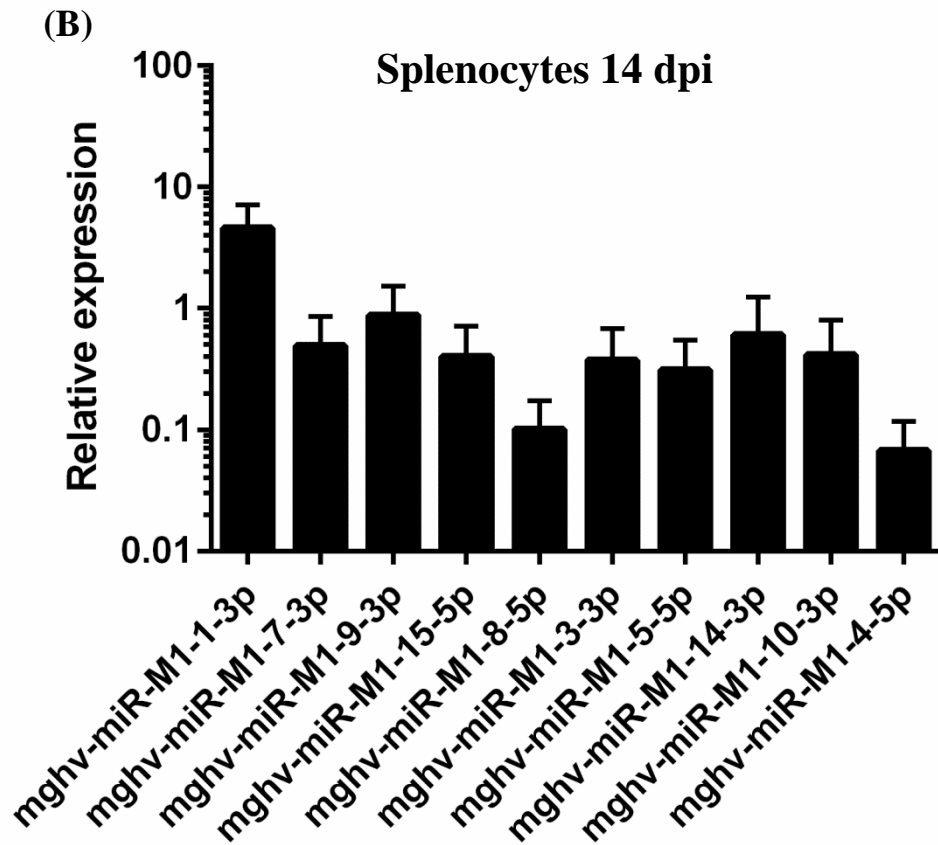
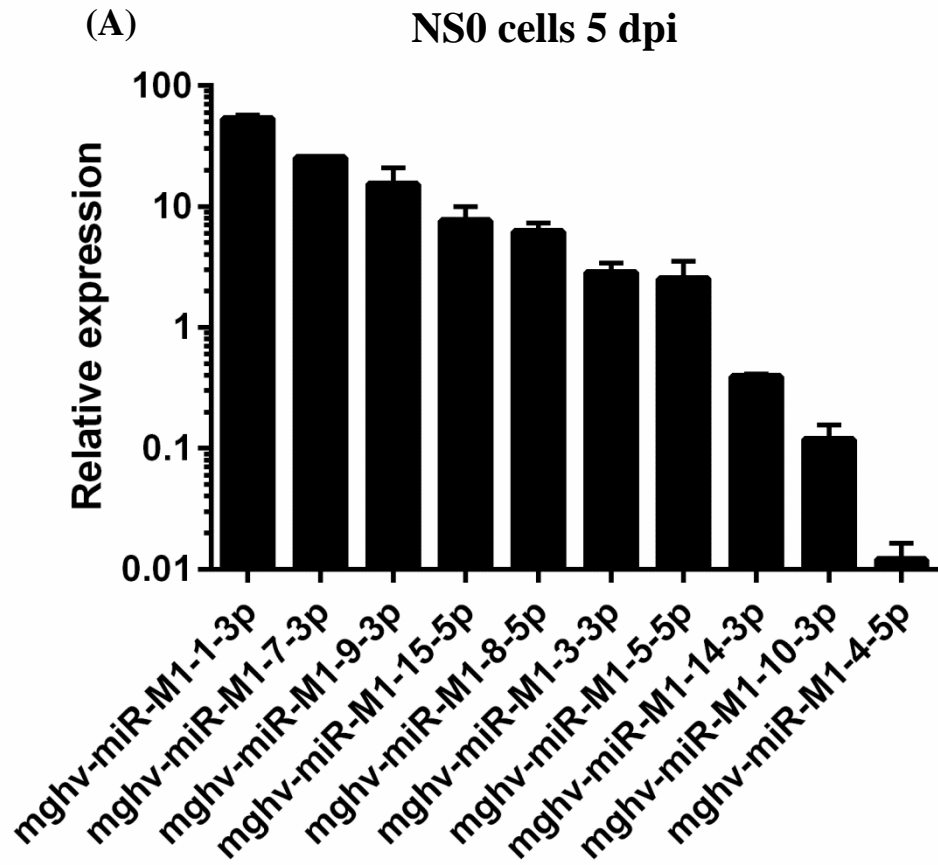


Figure 3.7 MHV-68 miRNAs expression during latent infection.

qRT-PCR was carried out to quantify the mature miRNAs expression levels. Viral miRNA expression levels are presented as values relative to the endogenous cellular miRNA mmu-miR-191-5p. Relative expression is $2^{-\Delta CT} \times 10^2$. (A) NS0 cells were infected with MHV-68 virus at MOI of 5 for 5 days. Total RNA was extracted and qRT-PCR was performed. Error bars represent standard deviation from the mean of 2 independent experiments. (B) BALB/c mice were infected intranasally with 4×10^5 PFU WT MHV-68 virus. At day 14 post-infection, mice were sacrificed and total RNA from splenocytes was extracted for determination of mature miRNAs expression levels by qRT-PCR. Error bars represent means \pm SD from 5 individual mice.

figure 3.7A and B). In addition, mghv-miR-M1-14-3p expression level in splenocytes was 2.2-fold more abundant than NIH 3T3 cells at 24 hpi (Figure 3.6B and figure 3.7B). Although mghv-miR-M1-4-5p is the least abundant miRNA in NIH 3T3 cells, NS0 cells, and splenocytes, its abundance during latency in splenocytes was 5.6-fold higher than NS0 cells and 14.4-fold higher than 3T3 cells. mghv-miR-M1-8-5p was detected in NIH 3T3 cells and NS0 cells at relatively high levels; however it was one of the least abundant miRNAs during latency *in vivo*. Taken together, these results demonstrate that the expression of MHV-68 encoded miRNAs depends on the phase of virus infection and it highlights the importance of the *in vivo* model for studying gammaherpesviruses miRNAs.

3.5 Expression kinetics of MHV-68 miRNAs during virus reactivation *in vitro*

3.5.1 The stimulation of NS0 cells with PMA results in a robust induction of virus reactivation

In order to examine the kinetics of MHV-68 miRNAs during *in vitro* reactivation, NS0 cells were infected with MHV-68 virus at MOI of 5. Three days after infection, the cells were treated with Phorbol 12-myristate 13-acetate (PMA) at concentration of 20ng/ml. The cells were harvested at 2, 4, 8, 24, and 48 hours post treatment (hpt). Total RNA and genomic DNA were extracted in order to look at viral gene expression and viral genomic load. Before investigating the viral miRNAs expression kinetics following reactivation, viral transcripts and viral genome load were analysed by qRT-PCR and qPCR. Immediate early ORF50 (RTA), early DNA polymerase (DNA pol), and late ORF65 (M9) as well as the latency associated ORF73 expression kinetics were analysed after the PMA-induced reactivation. As expected,

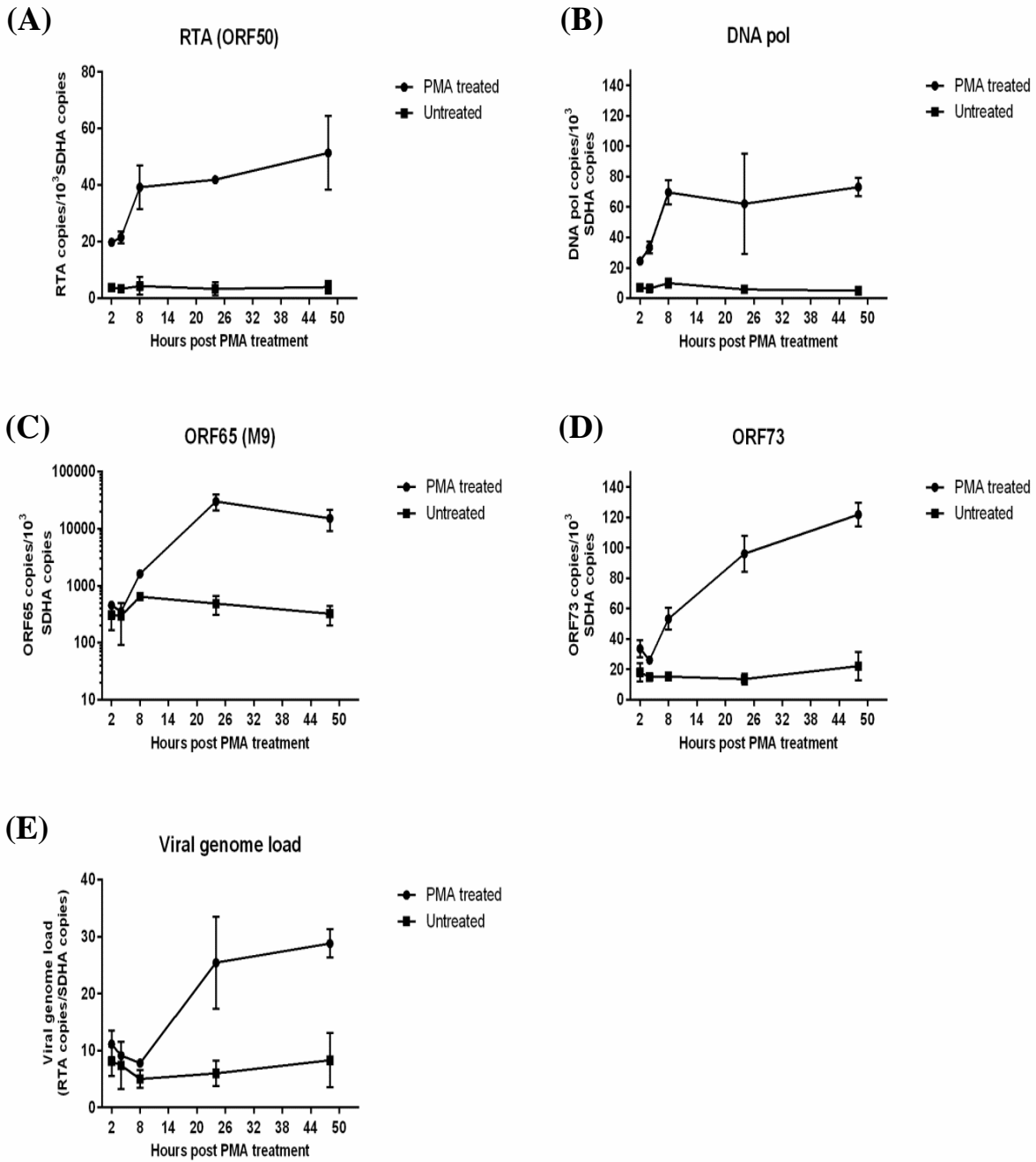


Figure 3.8 MHV-68 mRNAs expression kinetics during *in vitro* reactivation.

Murine myeloma NS0 cells were infected with MHV-68 virus at MOI 5. At 3 days post-infection, the cells were treated with PMA at concentration of 20 ng/ml in medium. The cells were harvested at 2, 4, 8, 24, or 48 hours post-treatment (hpt). Untreated cells were used as a control. Total RNA and genomic DNA were extracted and the viral mRNA expression and viral genomic load were examined by qRT-PCR and qPCR, respectively. The mRNAs copy numbers as well as the genome copy numbers were determined using serial dilution of a Plasmid DNA standard curve. The expression kinetics of the immediate-early ORF50 (RTA) (A), the early viral DNA polymerase (B), the late ORF65 (C), and the latency associated ORF73 (D) transcripts were analysed by qRT-PCR over time after PMA treatment. The viral mRNAs copy numbers were normalised against the endogenous cellular SDHA copy numbers. The viral genomic load was determined following PMA treatment by qPCR using 100 ng genomic DNA per reaction (E). The viral genome copy numbers were normalised against the cellular genome copy numbers. RTA and SDHA primers were used for determination of the viral and cellular genome copy numbers, respectively. The data represent 2 independent experiments. Error bars represent mean \pm SD.

the viral mRNAs expression was strongly induced after reactivation (Figure 3.8). RTA, DNA pol, and ORF73 mRNAs reached peak levels at 48 hpt with approximately 51.4, 73.2, and 121.9 copies per 10^3 SDHA copies, respectively, and with fold increases of approximately 13.2, 14.9, and 5.5-fold, respectively, as compared to the untreated control (Figure 3.8A, B, and D). ORF65 mRNA expression reached peak level at 24 hpt with approximately 30,078 copies/ 10^3 SDHA copies and a fold increase of 61.8-fold as compared to the untreated control (Figure 3.8C). The viral genomic load reached peak levels at 48 hpt with approximately 27.8 copies/cellular genome copies and a fold increase of approximately 4-fold higher than the untreated control (Figure 3.8E). These results indicate that PMA-induced reactivation of MHV-68-infected NS0 cells is efficient.

3.6 MHV-68 miRNAs are differentially expressed following PMA-induced virus reactivation *in vitro*

After confirming the efficient PMA-induced virus reactivation in NS0 cells, the next step was to evaluate the expression kinetics of MHV-68 miRNAs. Seven out of 10 tested miRNAs were induced following PMA treatment, however, at variable levels (Figure 3.9). The expression levels of these miRNAs reached peak levels at 48 hpt except mghv-miR-M1-8-5p that reached peak levels at 24 hpt. mghv-miR-M1-3-3p, mghv-miR-M1-14-3p, and mghv-miR-M1-15-5p showed the highest expression induction levels with approximately 9.4, 9.5, and 14.4-fold increase over the untreated control at 48 hpt, respectively. Interestingly the expression of 3 miRNAs (mghv-miR-M1-4-5p, mghv-miR-M1-9-3p, and mghv-miR-M1-10-3p) was not induced after PMA treatment to a level that is substantially higher than the untreated control (Figure 3.9). These results indicate that the MHV-68 miRNAs are differentially expressed following PMA-induced reactivation *in vitro*.

3.6.1 Expression of MHV-68 mRNAs and miRNAs following *in vitro* PMA-induced reactivation after standardisation against the viral genomic DNA levels

In order to determine the rate of viral mRNAs and miRNAs expression during the lytic reactivation, the normalised mRNAs or miRNAs expression levels were standardised against the normalised levels of viral genomic DNA levels and presented as fold change of PMA-treated versus untreated (Figures 3.10 and 3.11). The rate of RTA transcript expression was approximately 3.6-fold higher than in the untreated sample as early as 2 hpt and reached peak levels at 8 hpt with an expression rate that is 6-fold higher than the untreated control (Figure 3.10A). The DNA pol transcript was also induced as early as 2 hpt with an approximately 2.4-fold higher than the untreated control with peak of expression at 48 hpt that was 4.4-fold higher than the untreated control (Figure 3.10B). ORF65 transcript showed increase in the expression rate only after 8 hpt, which is consistent with its late expression kinetics (Ebrahimi, Dutia et al. 2003), with expression level that is approximately 14-fold higher than the untreated control at 24 hpt (Figure 3.10C). The latency-associated ORF73 transcript showed a modest increase in the expression rate with approximately 1.8-fold higher than untreated control at 8 hpt and almost stayed the same after this time-point (Figure 3.10E).

In terms of the expression kinetics of the miRNAs after standardisation against the viral genomic loads, there was differential expression of the miRNAs (Figure 3.11). mghv-miR-M1-3-3p, mghv-miR-M1-14-3p, and mghv-miR-M1-15-5p were up-regulated at 48 hpt with approximately 2.5-, 2.7-, and 4-fold higher than the untreated control. In contrast, the expression rate of the other miRNAs was either down-regulated or stayed unchanged. mghv-miR-M1-1-3p, mghv-miR-M1-4-5p,

mgHV-miR-M1-9-3p, and mgHV-miR-M1-10-3p were down-regulated following PMA treatment with peak of down-regulation at 24 hpt, showing expression rate of approximately 3.3-, 5-, 14.3-, and 5-fold less than untreated control. mgHV-miR-M1-8-5p was down-regulated as early as 2 hpt with approximately 2-fold decrease in the expression rate as compared to untreated control and this down-regulation continued at same level over the reactivation time-course. The expression rate of mgHV-miR-M1-5-5p did not change after PMA treatment at any time-point when compared to the untreated control. Taken together, the differential expression of different miRNAs during reactivation suggest a differential regulation of the expression of these miRNAs, which may play different roles in modulating reactivation. It is important to note that during lytic reactivation not all of the viral genomic DNA is used for gene transcription because a proportion of this DNA is packaged within the newly formed virions. However, the expression pattern of the well-characterised MHV-68 mRNAs was determined and it showed the expected expression kinetics following PMA-induced reactivation, which validates this system.

3.7 MHV-68 DNA replication is not a requirement for the miRNAs expression

In order to determine whether the inhibition of MHV-68 DNA replication may affect the expression profiles of the miRNAs, NS0 cells were infected with MHV-68 of MOI 5. Three days after infection, the cells were treated with the antiviral thionucleoside analogue 2'-deoxy-5-ethyl-beta-4'-thiouridine (4'-S-EtdU) at concentration of 1µg/ml in the medium. 4'-S-EtdU can inhibit MHV-68 replication due to its nucleoside analogue activity; however it does not inhibit latency establishment (Barnes, Dyson et al. 1999). The cells were harvested at 0 or 48 hpt. Viral transcript expression and viral miRNA expression levels were analysed by

qRT-PCR. There were no significant changes in RTA and ORF73 expression levels (Figure 3.12A and D), however, DNA pol showed a decrease in expression by 4-fold as compared to the untreated control at 48 hpt (Figure 3.12 B).

Notably, the expression level of ORF65, which is a late transcript proposed to express a capsid protein (Ebrahimi, Dutia et al. 2003, Martinez-Guzman, Rickabaugh et al. 2003), was decreased by 17.9-fold at 48 hpt as compared to the untreated control (Figure 3.12C). The viral genomic load was decreased by approximately 3.5-fold at 48 post 4'-S-EtdU treatment (Figure 3.12E). This indicates that the inhibition of virus replication was efficient. Next, the expression profiles of the viral miRNAs were determined. None of the miRNA tested showed significant decrease in expression level after 4'-S-EtdU treatment at 48 hpt (Figure 3.13). These results demonstrate that virus replication is not required for efficient expression of MHV-68 miRNAs.

3.8 Discussion

MHV-68 provides a unique tractable model for investigating the potential roles of a gammaherpesvirus-encoded miRNAs in the virus life cycle and pathogenesis within the natural host. In order to investigate the potential functions of MHV-68 encoded miRNAs, it is important to characterise their expression pattern during lytic and latent infection. qRT-PCR assays were established and optimised for quantification of MHV-68 mature miRNAs expression levels.

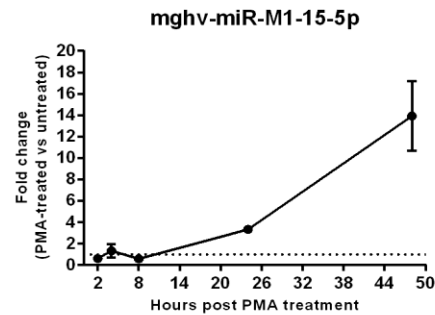
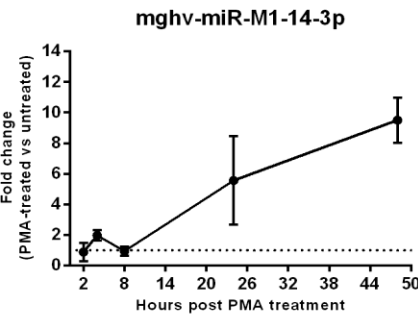
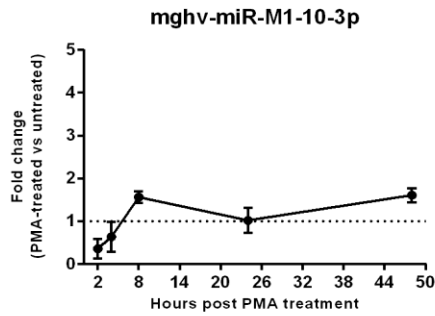
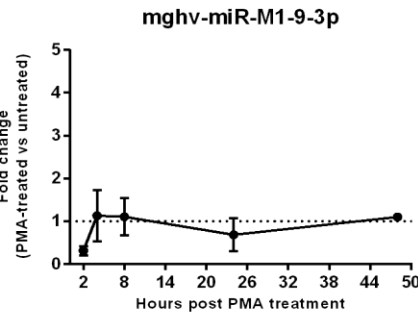
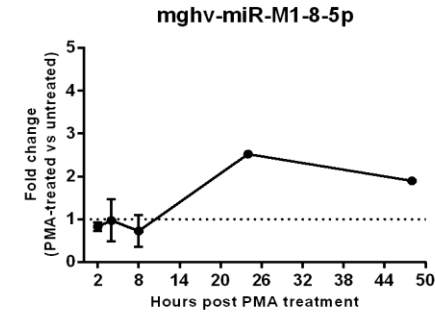
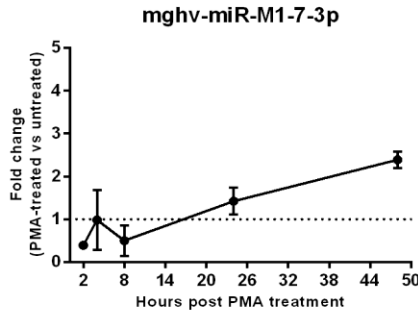
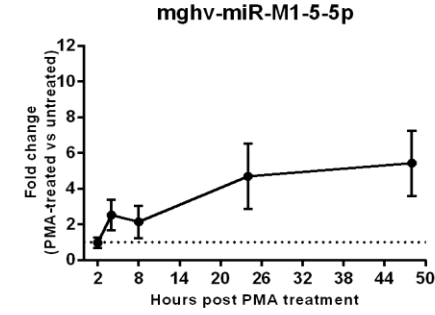
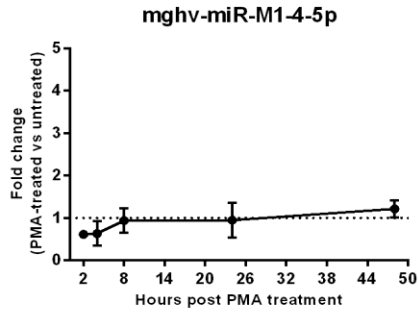
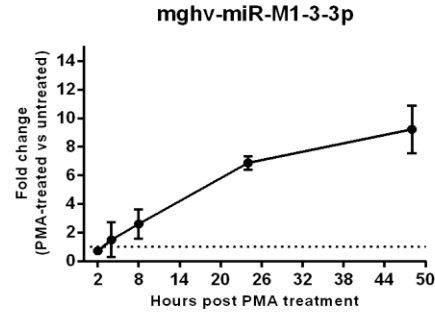
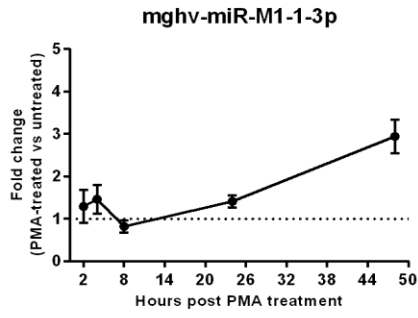


Figure 3.9 MHV-68 miRNAs expression kinetics following *in vitro* reactivation.

Murine myeloma NS0 cells were infected with MHV-68 virus at MOI of 5. Three days after infection, the cells were treated with PMA at concentration of 20 ng/ml. The cells were harvested at 2, 4, 8, 24, or 48 hpt. Untreated cells were used as a control. The total RNA was extracted from treated or untreated infected cells and the viral miRNA expression kinetics were analysed by qRT-PCR. The miRNAs expression levels were normalised against the reference cellular miRNA mmu-miR-191-5p. Data are presented as fold expression change in the treated over the untreated samples based on the comparative $2^{-\Delta\Delta CT}$ method (see Materials and Methods section 2.9.1.3). Error bars represent mean \pm SD. The data were compiled from 2 independent experiments.

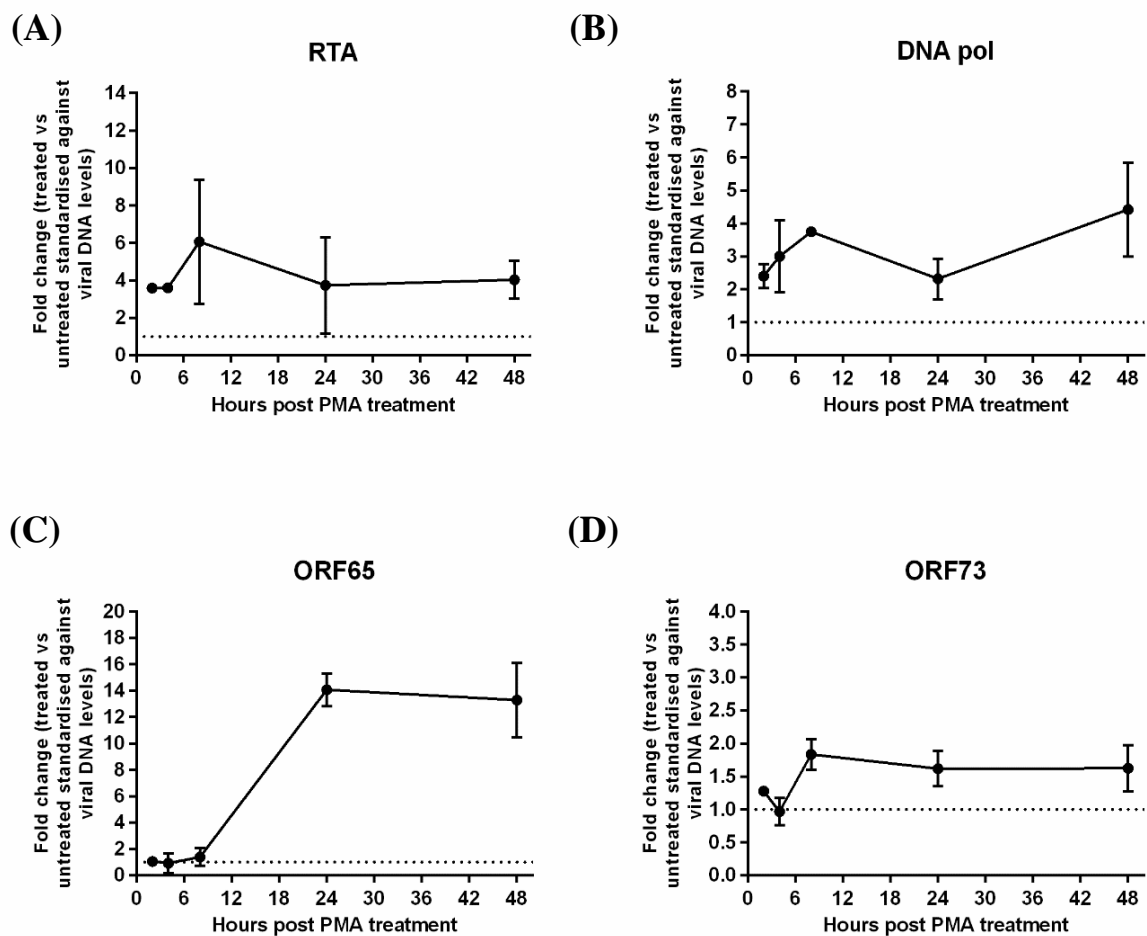


Figure 3.10 Expression kinetics of MHV-68 transcripts standardised against viral genomic loads following *in vitro* PMA-induced reactivation.

This is the same experiment described in figure 3.8, but here the normalised MHV-68 mRNA expression levels in the PMA-treated samples are presented as fold change from that of the untreated samples after standardisation against the viral genomic DNA loads. The data represent 2 independent experiments. Error bars represent mean \pm SD.

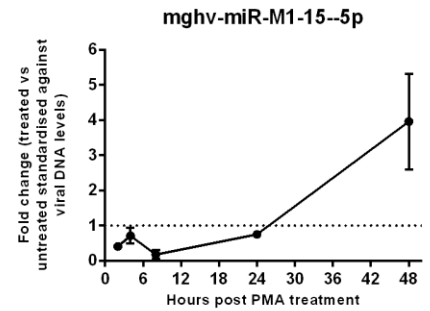
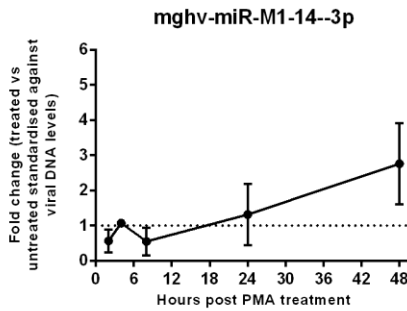
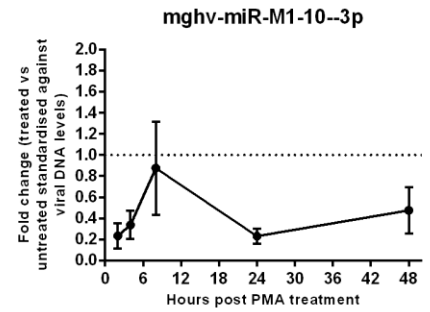
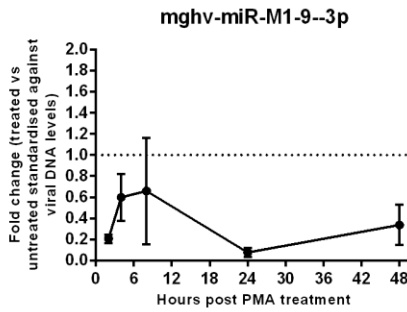
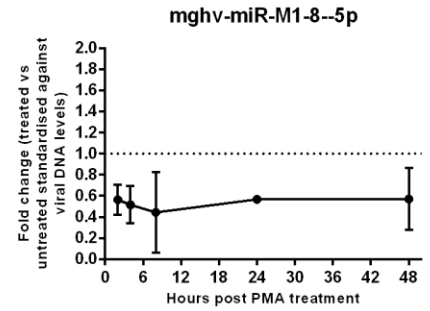
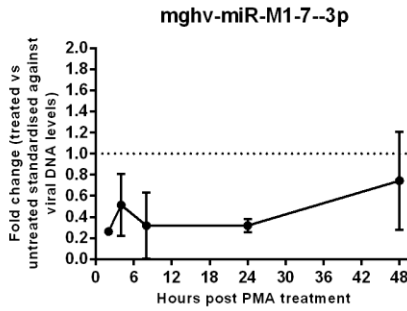
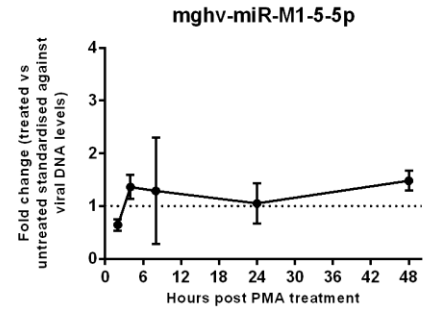
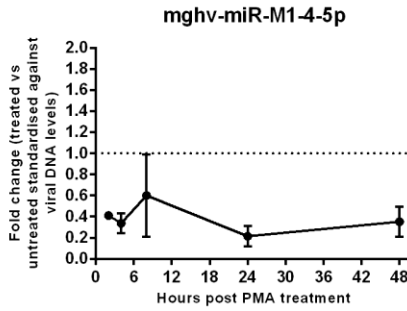
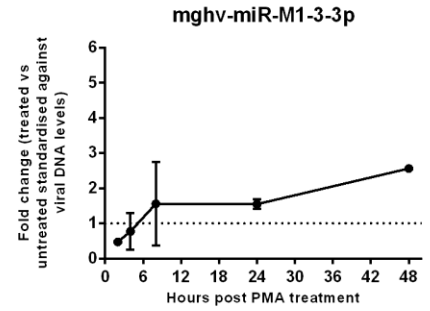
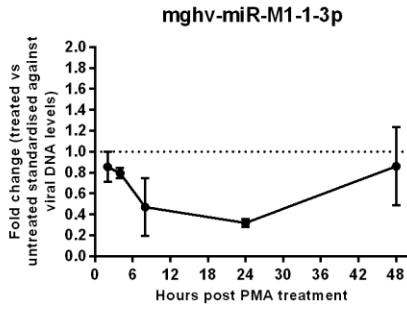


Figure 3.11 miRNAs expression kinetics following *in vitro* reactivation after standardisation against viral genomic DNA levels.

This is the same experiment described in figure 3.9, but the normalised MHV-68 miRNA expression levels in the PMA-treated samples are presented as fold change from that of the untreated samples after standardisation against the viral genomic DNA loads. The data represent 2 independent experiments. Error bars represent mean \pm SD.

The current study has shown that the MHV-68 miRNAs expression is dependent on the stage of virus life cycle and the infection model employed. This is consistent with a recent published study that investigated the MHV-68 miRNAs expression *in vitro* and *in vivo* using stem-loop qRT-PCR (Feldman, Kara et al. 2014), suggesting that the regulation of the MHV-68 miRNAs expression is dependent on the stage of infection. In the present study it has been shown that the abundance of the MHV-68 mature miRNAs increases over time in the lytically infected NIH 3T3 cells. mghv-miR-M1-14-3p, mghv-miR-M1-10-3p, and mghv-miR-M1-4-5p were the least abundant miRNAs among the tested miRNAs during this lytic infection. It has also been shown that mghv-miR-M1-8-5p expression level is very low during latent infection *in vivo*, whereas it is relatively high during latent infection *in vitro*. These results are consistent with Feldman's study. However, although mghv-miR-M1-1-3p and mghv-miR-M1-7-3p were among the most abundant miRNAs during lytic and latent infection in the present study and showed high read frequency in NIH 3T3 and S11 cell lines by deep sequencing (Zhu, Strehle et al. 2010), they were of low abundance in Feldman's study. These differences are likely due to the different approaches used for the miRNAs quantifications. Stem-loop primers and TaqMan assays, which are very sensitive to miRNA sequence specificity, were used in the Feldman's study and thus the alternate miRNA isoforms cannot be detected using these assays. On the other hand, SYBR Green qRT-PCR was used in the current study and it allows for detection of different miRNA isoforms.

While mghv-miR-M1-9-3p is one of the most highly expressed miRNAs during latent infection *in vitro* and *in vivo*, it is one of the least abundant miRNAs during lytic infection in NIH 3T3 cells. Interestingly, the expression of this miRNA was not induced following PMA-induced virus reactivation in NS0 cells. This suggests that

mghv-miR-M1-9-3p may play a role in promoting latent infection. The difference in miRNAs expression may reflect differential regulation and functions of these miRNAs. It is plausible that some miRNAs may have more fundamental roles during latency by facilitating inhibition of productive infection and lytic reactivation as well as increasing the longevity of infected cells (reviewed in section 1.5), whereas, other miRNAs may play roles in supporting lytic replication. Indeed, the observation that the MHV-68 miRNAs, mghv-miR-M1-3-3p, mghv-miR-M1-14-3p, and mghv-miR-M1-15-5p, are upregulated during the PMA-induced lytic reactivation in latently infected NS0 cells suggests that these miRNAs may play a role in MHV-68 lytic reactivation. This finding is consistent with a previous published study showing the upregulation of kshv-miR-K12-10 and kshv-miR-k12-12 during PMA-induced lytic reactivation of KSHV (Umbach and Cullen 2010). MHV-68 miRNAs are located adjacent to the unique latency-associated M genes within the genome, suggesting that MHV-68 miRNAs predominately promote latent infection.

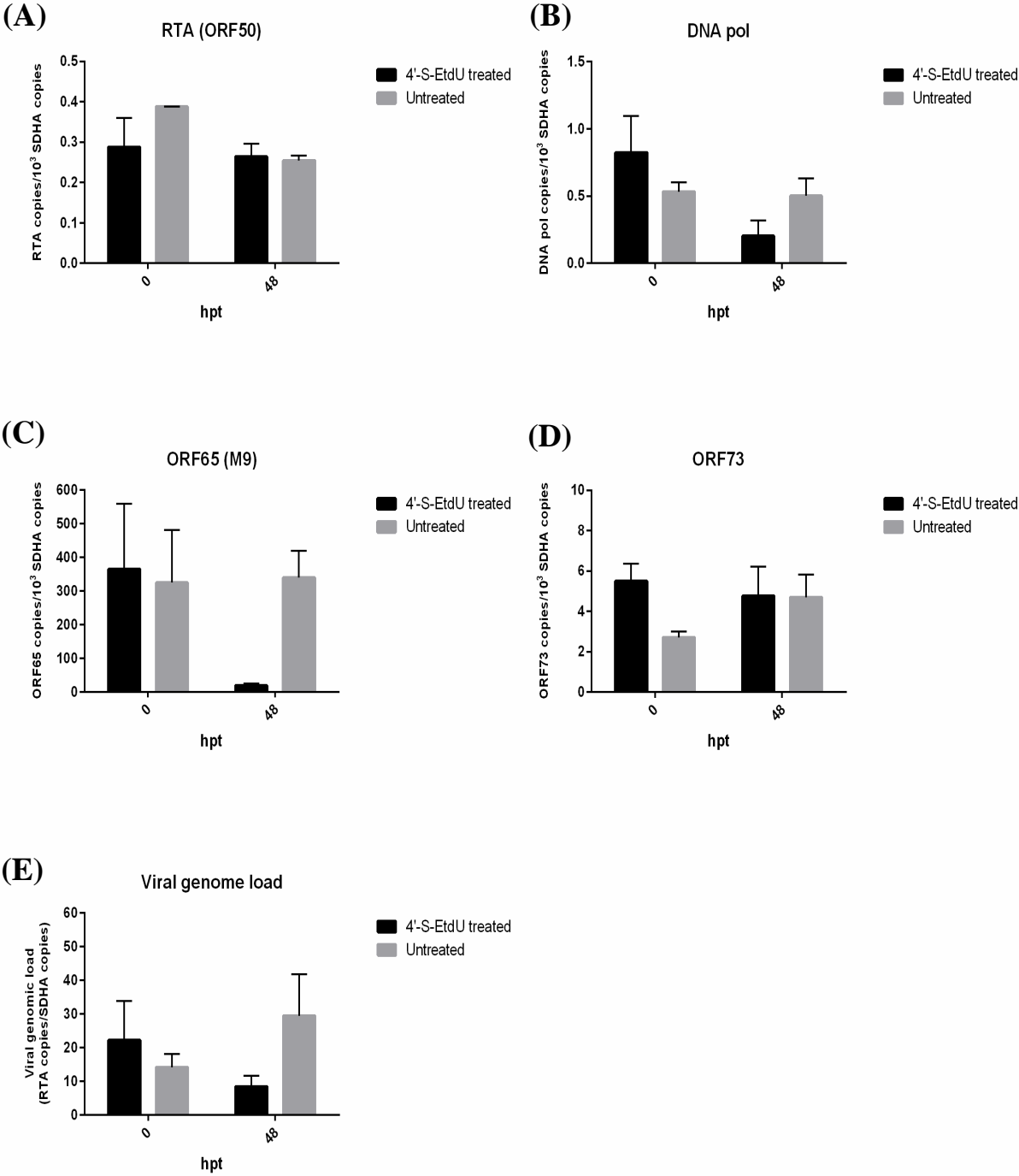


Figure 3.12 Expression of MHV-68 mRNAs after inhibition of lytic virus replication in NS0 cells.

NS0 cells were infected with MHV-68 virus at MOI of 5. Three days after infection, the cells were treated with 4'-S-EtdU (concentration 1 µg/ml in medium). The cells were harvested at 0 and 48 hours post treatment (hpt). Untreated cells were used as a control. Total RNA and genomic DNA were extracted and viral mRNA expression and viral genomic load were examined by qRT-PCR and qPCR, respectively. The mRNAs copy numbers were determined using serial dilution of Plasmid DNA standard curve. The expression levels of RTA (A), DNA pol (B), ORF65 (C), and ORF73 (D) were determined as copy numbers normalised against reference cellular SDHA copy numbers. The viral genomic load is presented as viral genome copy numbers normalised against the cellular genome (SDHA) copy numbers (E). The data represent 2 independent experiments. Error bars represent mean ± SD.

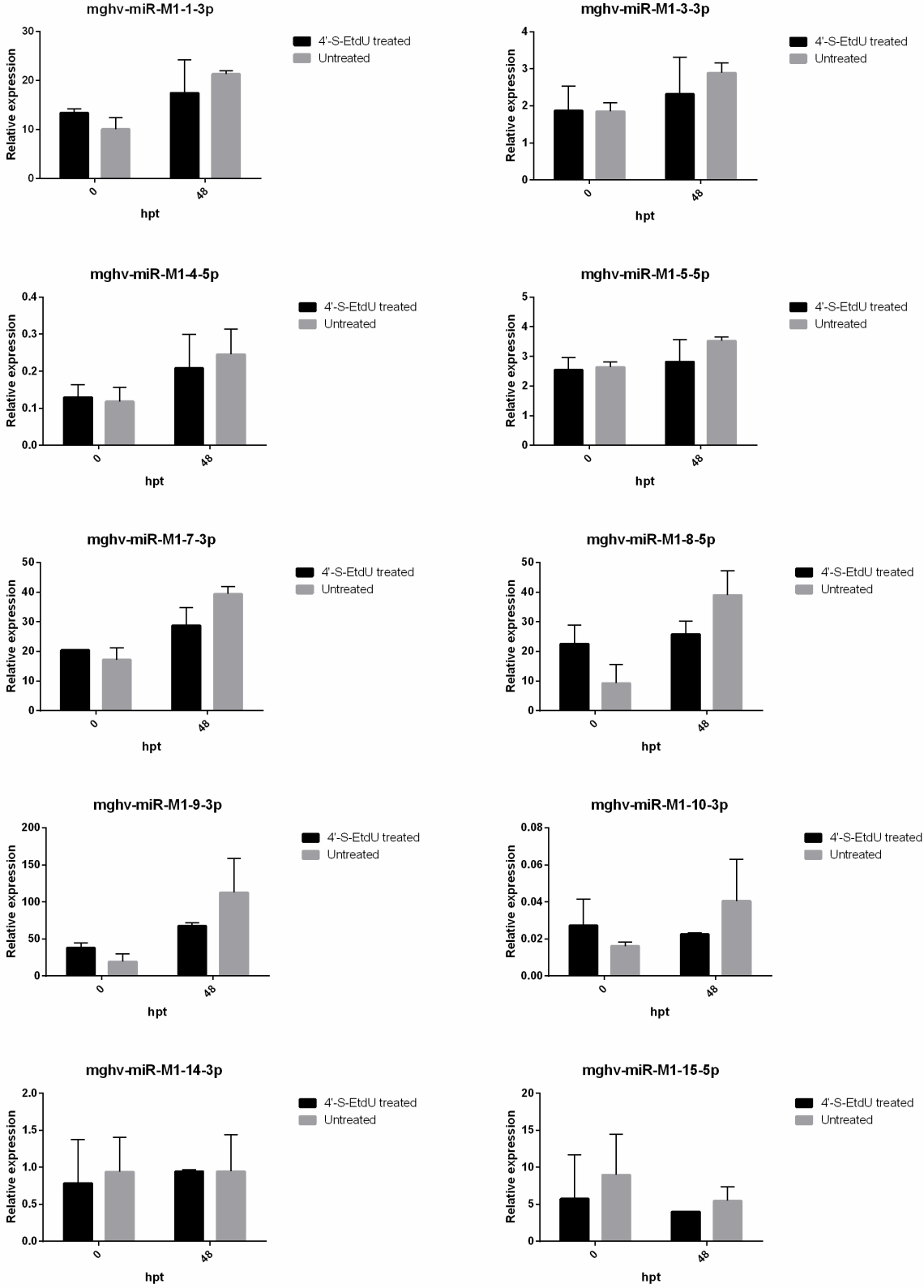


Figure 3.13 Expression profiles of MHV-68 miRNAs following inhibition of virus lytic replication in NS0 cells.

Murine myeloma NS0 cells were infected with MHV-68 virus at MOI of 5. Three days after infection, the cells were treated with 4'-S-EtdU at concentration of 1 µg/ml in medium. The cells were harvested at 0 or 48 hours post-treatment (hpt). Untreated cells were used as control. Total RNA was extracted and viral miRNA expression kinetics were analysed by qRT-PCR. The miRNAs expression levels are presented as values relative to the reference cellular miRNA mmu-miR-191-5p. Relative expression is $2^{-\Delta\text{CT}} \times 10^2$. Error bars represent standard deviation from the mean of 2 independent experiments.

In general, the expression levels of the miRNAs were higher in cell lines than in splenocytes which is likely due to higher number of infected cells *in vitro* than *in vivo*.

Interestingly, mghv-miR-M1-1-3p and mghv-miR-M1-10-3p whose primary miRNAs are transcribed from the same vtRNA promoter sequence (vtRNA1) have different expression profiles. For instance, the expression level of mghv-miR-M1-1-3p is higher than the expression level of mghv-miR-M1-10-3p by approximately 331-, 450-, and 11-fold in NIH 3T3 cells (24 hpi), NS0 cells (5 dpi), and splenocytes (14 dpi), respectively. This suggests a post-transcriptional regulation of these miRNAs such as miRNA processing and/or stability.

The kinetics of MHV-68 miRNAs expression were evaluated after stimulation of NS0 cells for virus reactivation. There was strong reactivation induction evidenced by the increase of viral mRNAs expression levels and the increase of viral genomic load over time. The expression level of RTA, which is a key regulator of lytic infection and reactivation (Liu, Pavlova et al. 2000, Pavlova, Virgin et al. 2003), was increased by 13.2-fold at 48 hpt. The viral DNA pol was also increase by 14.9-fold at 48 hpt. Interestingly, the expression level of ORF73, which is a latency-associated transcript, was increased by 5.5-fold at 48 hpt, suggesting that ORF73 may play an important role in facilitating MHV-68 reactivation in a manner similar to that described during viral lytic replication in the fibroblasts (Forrest, Paden et al. 2007).

The kinetics of MHV-68 miRNAs expression were investigated following PMA-induced virus reactivation in NS0 cells. It was found that the MHV-68 miRNAs were differentially expressed during reactivation, suggesting different roles played by these miRNAs during lytic reactivation. Interestingly, the mghv-miR-M1-9-3p expression was down-regulated, whereas the expression of mghv-miR-M1-15-5p was

up-regulated following the PMA-induced reactivation; although, their primary miRNAs are transcribed from the same promoter in association with vtRNA8, suggesting that the differential regulation of the MHV-68 miRNAs expression during reactivation can occur at the post-transcriptional level such as miRNA processing or stability.

When the MHV-68 infected NS0 cells were treated with 4'-S-EtdU, which inhibits MHV-68 DNA replication, there were no substantial changes in expression levels of the tested miRNAs, indicating that the MHV-68 DNA replication is not essential for the miRNAs expression, a feature that is required during latent infection when the viral DNA replication is restricted.

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Chapter 4: Expression kinetics of MHV-68 miRNAs *in vivo* during latent infection in the spleen

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4 Chapter 4: Expression kinetics of MHV-68 miRNAs *in vivo* during latent infection in the spleen

4.1 Introduction

The hallmark of herpesviruses infection is their ability to establish and maintain latency within the natural host (Speck and Ganem 2010). Following the primary lytic infection in lung epithelial cells, MHV-68 establishes latent infection mainly in B cells where it exploits B cell biology to gain access to the long-lived memory B cells through germinal centre reactions (Flano, Kim et al. 2002, Collins and Speck 2012). During latency, the viral gene expression is restricted and the production of infectious virus is absent; however, the virus possesses the ability to reactivate and produce infectious virus under certain conditions in order to disseminate the virus and infect new host. The molecular mechanisms behind the establishment, maintenance, and reactivation from latency are not fully understood.

Since their discovery, there has been growing evidence that miRNAs encoded by herpesviruses can play important roles during virus latency (reviewed in Grey F, 2015). The gamma-herpesvirus miRNAs are readily expressed and detected during the latent infection and their genes are clustered within regions of the genome characterised by latent gene expression, suggesting that these miRNAs might be involved in regulating the viral latent state (Cai, Lu et al. 2005, Pfeffer, Sewer et al. 2005, Umbach, Kramer et al. 2008, Cosmopoulos, Pegtel et al. 2009, Grundhoff and Sullivan 2011). Furthermore, KSHV miRNAs were found to play roles in inhibiting lytic viral gene expression during latency in order to inhibit virus productive

replication and to help the virus lay low (Bellare and Ganem 2009, Lei, Bai et al. 2010, Lu, Stedman et al. 2010).

The human gamma-herpesviruses, EBV and KSHV, are oncogenic viruses that establish and maintain latency in immunocompetent individuals; however, the viruses can reactivate and develop malignant tumours in immunocompromised individuals. KSHV causes Kaposi's sarcoma, primary effusion lymphoma, and some types of multicentric Castleman's disease (Cesarman, Chang et al. 1995, Boshoff and Weiss 2002). EBV is involved in Hodgkin's lymphoma, Burkitt's lymphoma, and nasopharyngeal carcinoma (Niedobitek, Meru et al. 2001). It is therefore important to investigate the potential roles of the viral miRNAs during latent gamma-herpesvirus infection in the immunocompetent host.

Due to strict species specificity of herpesviruses, the roles played by the miRNAs encoded by human gamma-herpesviruses during latency cannot be directly assessed *in vivo*. MHV-68 is genetically related to the human gamma-herpesviruses (Virgin, Latreille et al. 1997, McGeoch, Gatherer et al. 2005) and provides a tractable small animal model to investigate the establishment and maintenance of gamma-herpesvirus latency and other pathologic features that are difficult to assess in humans. MHV-68 causes a productive infection of the lung following intranasal infection, followed by establishment of splenic latent infection mainly in B cells, which peaks 14 days post-infection and is characterised by prominent splenomegaly and activation and clonal expansion of B cells (Sunil-Chandra, Efstathiou et al. 1992, Sunil-Chandra, Efstathiou et al. 1993, Weck, Barkon et al. 1996, Flano, Husain et al. 2000). Three weeks after infection, there is reduction in the CD4⁺ T-cell mediated B cell proliferation and expansion of CD8⁺ T-cells (Tripp, Hamilton-Easton et al.

1997, Flano, Hardy et al. 2004, Evans, Moser et al. 2008). These pathological features are reminiscent of the infectious mononucleosis induced by EBV virus (Nash and Sunil-Chandra 1994, Doherty, Tripp et al. 1997). MHV-68 virus then maintains lifelong latent infection within the host.

Quantification of the MHV-68 miRNAs expression and determination of their expression kinetics during the latency *in vivo* can allow us to better define the possible biological roles of these miRNAs. This chapter shows evaluation of the expression kinetics of MHV-68 miRNAs during different stages of MHV-68 splenic latency.

Investigation of viral miRNAs expression pattern in latently infected cells requires detection and isolation of those cells during the course of infection because they exist in small numbers. There have been a number of attempts to construct MHV-68 marked viruses. Bennett *et. al.* constructed an MHV-68 recombinant virus expressing enhanced green fluorescent protein (eGFP), which was co-expressed with mLANA by insertion of an internal ribosome entry site downstream of the ORF73 coding region (Bennett, May et al. 2005, Smith, Rosa et al. 2006). This virus showed similar lytic replication and establishment of latency to that of the WT virus, but it was attenuated during long-term latency in BALB/c mice. Collins and colleagues constructed a transgenic virus that expresses enhanced yellow fluorescent protein (MHV68-YFP) (Collins, Boss et al. 2009), where the human cytomegalovirus immediate-early promoter was used for the expression. This virus replicated and established latency to comparable levels to that of the WT virus. However, the expression of the transgene was significantly reduced during late latency, suggesting

that a latency associated promoter may be required for marking infected B cells during late latency.

Another approach to isolate latently infected cells involved the use of a recombinant MHV-68 virus expressing Cre-recombinase under the control of an RSV promoter (Dutia, Reid et al. 2009). However, this virus was attenuated *in vivo* and *in vitro* as compared to the WT virus. (mLANA), a protein that has sequence homology to KSHV LANA and plays a central role in maintaining the viral genome as an extra chromosomal episome in dividing cells . Published data indicated that the MHV68.ORF73 β la virus replicated lytically and established latency at similar levels to that of the wild-type virus.

In the current study, for the purpose of isolating latently infected splenocytes in a mouse model by flow cytometry, with the aim of determining the kinetics of MHV-68 miRNAs expression in these cells, an initial attempt involved the use of a recombinant MHV-68 virus carrying a β -lactamase gene inserted in-frame downstream of ORF73 (designated MHV68.ORF73 β la), which was constructed by Nealy and co-workers (Nealy, Coleman et al. 2010). This recombinant virus allowed for quantification of MHV-68 miRNAs expression in the isolated latently infected splenocyte population at 14 and 21 dpi, despite the unpredicted attenuation of this virus in establishing latency, which was evidenced in this study by significantly lower MHV68.ORF73 β la splenic genomic loads as compared to that of the wild-type virus, in disagreement with Nealy's study. Therefore, this virus was not used for further profiling of MHV-68 miRNAs expression at later time-points. Alternatively, the determination of the latent miRNAs expression kinetics in total splenocytes

throughout latency was performed using wild-type MHV-68. This involved quantification of the miRNAs expression levels during the course of latent infection and normalisation of these levels to the respective levels of the viral DNA genomic loads, aiming to eliminate the possibility that any changes in the miRNAs expression levels are not merely due to changes in the viral genomic load.

It was found that the standardised miRNAs expression levels are high during the early stage of latency (10 or 14 dpi) and then decrease over time until day 21 post-infection. The miRNAs expression increases again after 21 dpi as determined at 32 dpi.

4.2 Detection and isolation of latently infected splenocytes using the MHV68.ORF73 β la virus

One challenge in studying MHV-68 infection is the very low frequency of latently infected cells *in vivo*. Therefore, evaluation of the MHV-68 encoded miRNAs expression kinetics during different stages of virus latency *in vivo* requires detection and isolation of individual latently infected cells, aiming at enriching these cells. MHV68.ORF73 β la virus was used for this purpose. This virus carries β -lactamase gene that is fused in-frame downstream of ORF73 (Figure 4.1A). The Fluorescence Resonance Energy Transfer (FRET)-based substrate, CCF2, allows the use of β -lactamase as a reporter for ORF73 (mLANA) expression in latently infected splenocytes. The lipophilic esterified form of this substrate (CCF2-AM) enters the cells via passive diffusion. The endogenous cytoplasmic esterases cleave the AM ester from the CCF2-AM, converting it into its negatively charged form (CCF2) that is retained in the cytoplasm (Figure 4.1B). In the absence of β -lactamase, excitation

of the coumarin in the intact CCF2 at 405 nM by violet laser causes FRET to the fluorescein that emits a green light at 520 nM. When the β -lactamase is expressed in the cell, it cleaves the CCF2 and disrupts FRET; therefore, excitation of the coumarin at 405 nM now produces a blue fluorescence signal at 447 nM (Zlokarnik, Negulescu et al. 1998) (Figure 4.1B).

A previous published study has shown that the MHV68.ORF73 β la virus allowed for detection and isolation of rarely infected mouse splenocytes expressing β -lactamase/mLANA by flow cytometry. For example, the frequency of splenocytes from MHV68.ORF73 β la-infected mice was 1 in 1,120, 1 in 18,600, and 1 in 30,800 at 16, 42, and 90 days post intranasal infection, respectively (Nealy, Coleman et al. 2010).

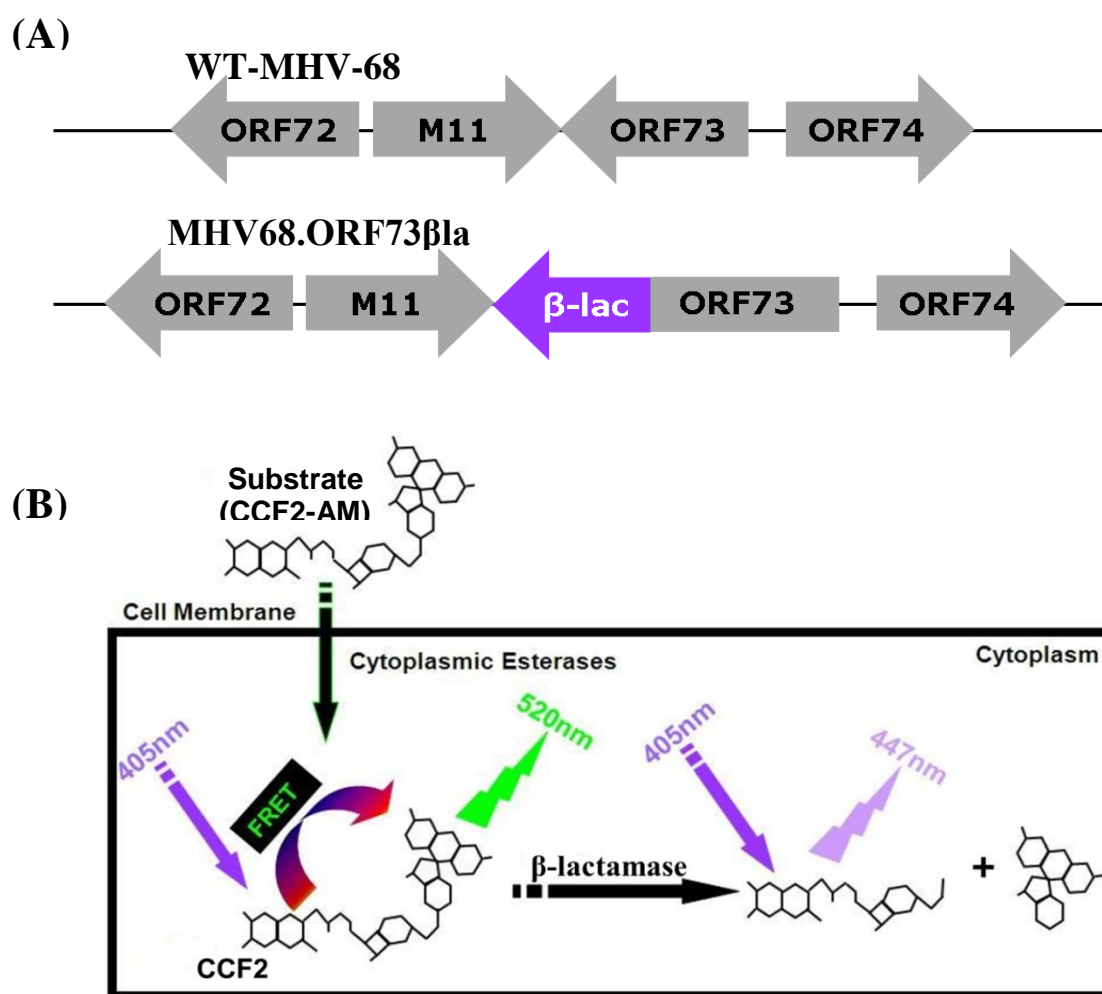


Figure 4.1 The β -lactamase reporter system.

(A) Schematic diagram showing the insertion of the β -lactamase gene in-frame downstream of ORF73 in the MHV68.ORF73 β la virus. The recombinant virus was constructed from the parental MHV-68 bacterial artificial chromosome (BAC) using allelic exchange (Nealy, Coleman et al. 2010). (B) A diagram showing the β -lactamase reporter system. The FRET based substrate, CCF-AM, is loaded into splenocytes by incubation at 37°C for 5 min in dark place. The AM ester is cleaved from the CCF2 by the cytoplasmic esterases, producing a fluorescence dye that emits a green signal when excited at 405 nM. The β -lactamase cleaves the CCF2 causing a change in emission to 447 nM when excited at 405 nM.

In order to isolate latently MHV-68 infected splenocytes by flow cytometry-based cell sorting, female BALB/c mice were infected intranasally with 4×10^5 PFU of MHV68. ORF73 β la virus or WT-MHV-68 virus. The spleens were harvested at 14 and 21 dpi and single cell suspensions were prepared and loaded with CCF2-AM β -lactamase substrate. The splenocytes were subsequently subjected to flow cytometry that allowed for distinguishing the cells expressing mLANA from the cells not expressing mLANA (see Materials and Methods section 2.4). Splenocytes from WT MHV-68 infected mice and unloaded splenocytes (untreated with CCF2-AM) were used as negative controls for adjusting the flow cytometry gates.

At 14 dpi, approximately 0.2% of the splenocytes from MHV68. ORF73 β la infected mice were β -lactamase/mLANA positive, whereas 0.0% of splenocytes from WT MHV-68 infected mice were mLANA positive (Figure 4.2 upper panels). At 21 dpi, approximately 0.06% of the splenocytes were β -lactamase/mLANA positive (Figure 4.2 lower panels). The decrease in number in mLANA positive cells is due to the drop of the number of MHV-68 infected splenocytes over time (Nealy, Coleman et al. 2010). The mLANA positive cells were isolated for RNA extraction and viral miRNAs expression profiling by qRT-PCR. At least 10^5 mLANA negative cells were also isolated to be used as a negative control in the qRT-PCR. The mLANA negative splenocytes are present in a very high frequency; therefore, a small portion of this cell population was gated as a representative of the mLANA negative cells.

4.3 Quality control of the RNA extracted from sorted splenocytes

Effective extraction and processing of the RNA from mLANA positive cell populations is crucial for the subsequent qRT-PCR assays. RNA is very sensitive to degradation by the ubiquitous RNases. This problem is further enhanced during the process of RNA extraction from the sorted mLANA positive splenocytes because 1) these cells are rare and exist in very low numbers (a range of 3,000 to 20,000 cells at 14 dpi and less than 1,000 to 5,000 cells at 21 dpi) and 2) the passage of the cells through the nozzle of the flow cytometer can stress the cells by shearing forces.

At the beginning of this study, several attempts for isolating intact RNA from the mLANA positive cells were not successful when the routine flow cytometry and RNA extraction procedures were used. This was evidenced by the highly degraded RNA when analysed by the Agilent bioanalyzer. Collecting the sorted cells in RNAlater[®] did not overcome this problem. Although RNAlater[®] can stabilise and protect the RNA from degradation, loss of the cells during the centrifugation step before the RNA extraction dramatically decreased the RNA yield from the small number of the sorted cells. Therefore, more stringent measures were taken in order to recover RNA of acceptable quantity and quality for the qRT-PCR assays. These measures included cleaning the FACS machine thoroughly with RNase decontamination solution prior to the cell sorting and sorting the cells directly into Trizol or foetal calf serum-rich medium rather than the FACS buffer or RNAlater[®]. This significantly improved the RNA yield and quality following RNA extraction.

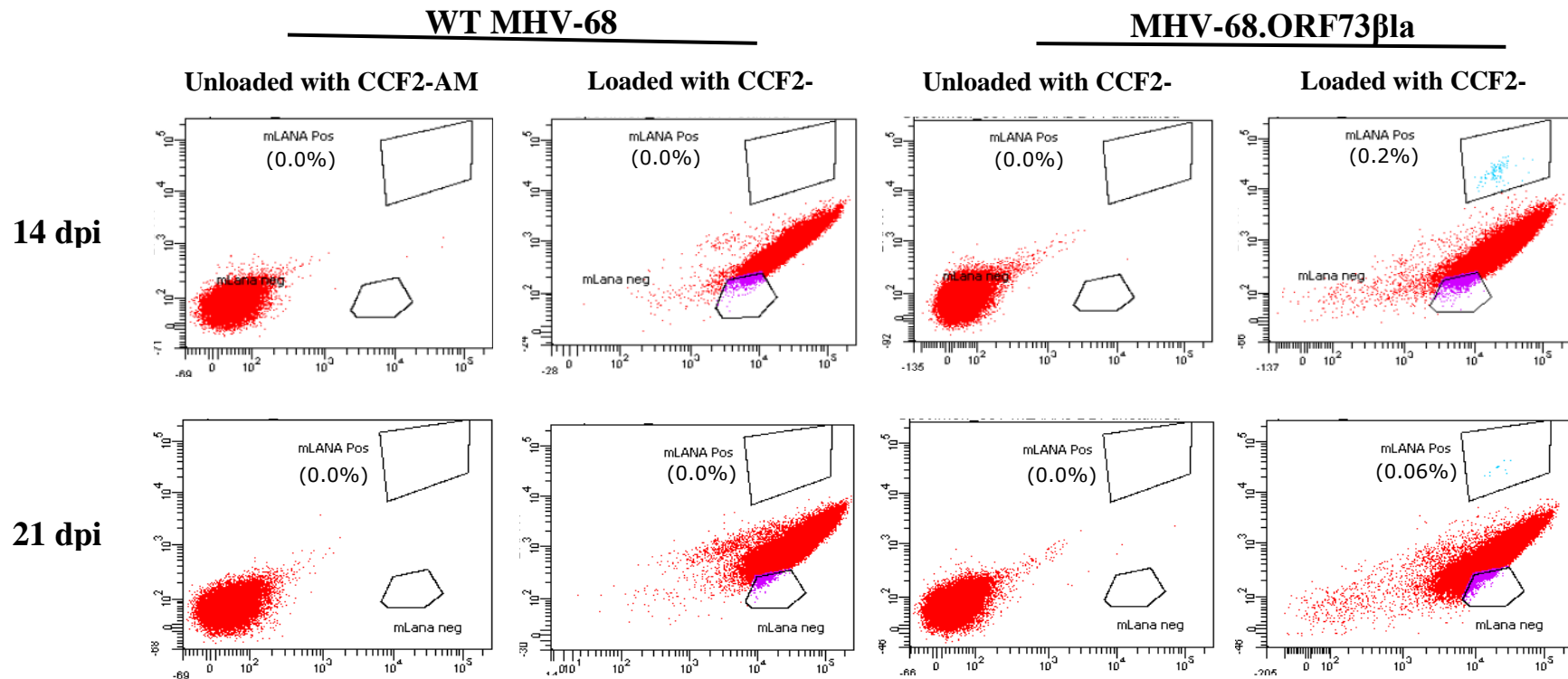


Figure 4.2 Detection of the mLANA expressing splenocytes by flow cytometry.

Spleens were harvested at 14 dpi (upper panels) or 21 dpi (lower panels). WT MHV-68 (left) or MHV68.ORF73 β la (right) virus was used for the infections. Single-cell suspensions were loaded or unloaded with CCF2-AM substrate followed by flow cytometric analysis. β -lactamase/mLANA positive cells (blue) and a representative cell population of the β -lactamase/mLANA negative cells (purple) are indicated by the boxed gates. The data are representative of 2 independent experiments, using 5 mice per time-point per group per experiment.

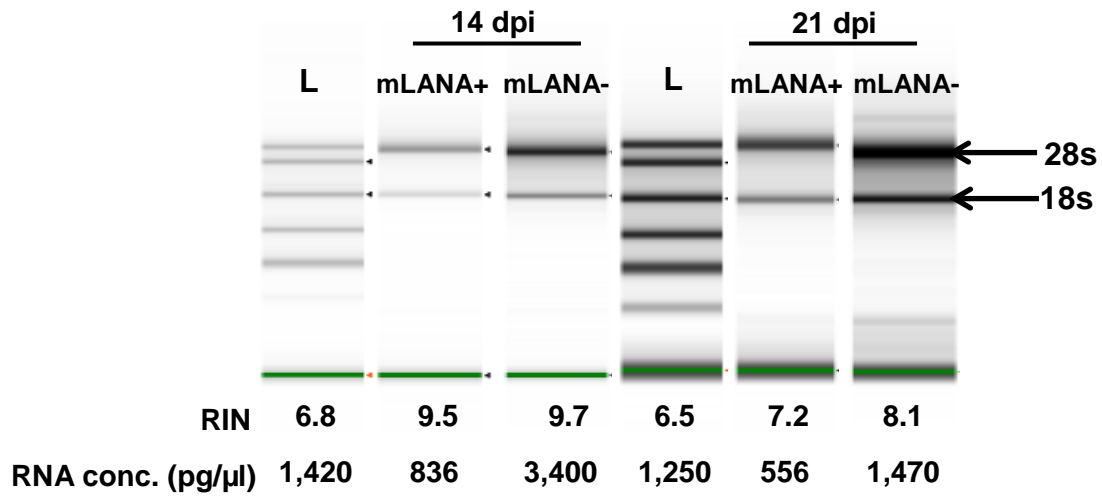


Figure 4.3 Quality control of the RNA extracted from the sorted splenocytes.

Agilent High Sensitivity RNA ScreenTape[®] gel image showing RNA extracted from mLANA positive (mLANA+) and mLANA negative (mLANA-) splenocytes at 14 or 21 dpi. RNA integrity numbers (RIN) and RNA concentrations (RNA conc.) are given at the bottom. The 28 and 18s rRNA bands are indicated by arrows. L, Ladder.

The quality of the RNA samples from sorted cells was determined using the Agilent bioanalyzer or the Agilent High Sensitivity ScreenTape[®] in combination with the 2200 TapeStation system. The RNA quality in these systems is based on the integrity of the 18s and 28s subunits of the ribosomal RNA (rRNA). This integrity was judged by RNA Integrity Number (RIN). The scale of RIN ranges from 1 to 10 where 10 corresponds to intact RNA and 1 corresponds to highly degraded RNA. Only the samples with intact RNA were included in the qRT-PCR analyses. Figure 4.3 shows a representation of the samples subjected to Agilent RNA analyses, which were selected for the qRT-PCR analyses. The 28s and 18s rRNA bands in the RNA from the sorted cells were intact with no signs of degradation and absence of low molecular weight RNA bands (Figure 4.3).

The RNA concentrations from mLANA positive cells were very low; therefore, the maximum volume of RNA was required for cDNA synthesis and qRT-PCR reactions. However, increasing the volume of the RNA also increases the amount of the cDNA synthesis inhibitors in the samples such as phenol and guanidine thiocyanate salt. This problem is further enhanced because the RNA concentrations are very low. In order to determine the optimum RNA volume for the cDNA synthesis, different volumes of RNA (1 μ l, 2 μ l, 4 μ l, and 8 μ l) from 2×10^4 sorted mLANA negative cells were used in the cDNA synthesis and qRT-PCR reactions for detection of the cellular miRNA mmu-miR-191-5p and the cellular transcript SDHA. It was found that the qRT-qPCR reactions were inhibited when 8 μ l RNA were used for the qRT-PCR, whereas the lowest CT values, which corresponds to the highest abundance of the target, were observed when 4 μ l RNA were used in the qRT-PCR (Figure 4.4).

4.4 The mLANA positive splenocytes are latently infected

In order to confirm that the isolated mLANA positive splenocytes were latently infected, the expression profiles of viral gene transcripts were investigated using qRT-PCR. RTA (ORF50), an immediate early viral protein, is essential for lytic MHV-68 replication during *de novo* lytic infection or reactivation (Wu, Tong et al. 2001). The viral DNA polymerase (DNA pol) is an early viral protein and it is responsible for viral DNA synthesis during virus replication (Rochford, Lutzke et al. 2001). Therefore, RTA and DNA pol mRNAs were selected as markers for lytic viral replication. Although ORF73 (mLANA) is expressed during both latent and lytic infection, its function is mainly associated with latency; and thus, it was used as a marker for viral latency (Rochford, Lutzke et al. 2001, Fowler, Marques et al. 2003). The RNA isolated from the mLANA positive or mLANA negative splenocytes at 14 dpi or 21 dpi were reverse transcribed into cDNA and subjected to qRT-PCR for detection of RTA, DNA pol, and ORF73. The viral gene expression during latency was compared to that of the lytic replication in NIH 3T3 cells by running the qRT-PCR products on agarose gel (Figure 4.5).

The latent viral gene expression was very close to or below the limit of detection by qRT-PCR (~32-35 cycles) and thus an accurate quantification using the CT values was difficult. Therefore, the qRT-PCR products were analysed by gel electrophoresis (Figure 4.5). The ORF73 qRT-PCR product (129 bp) was detected across all the mLANA positive samples, but not in the mLANA negative samples (Figure 4.5). This does not only confirm the high efficiency of the splenocytes sorting based on mLANA expression, but also indicates that mLANA positive splenocytes isolated at both 14 and 21 dpi express ORF73 transcripts that are essential for viral latency. The

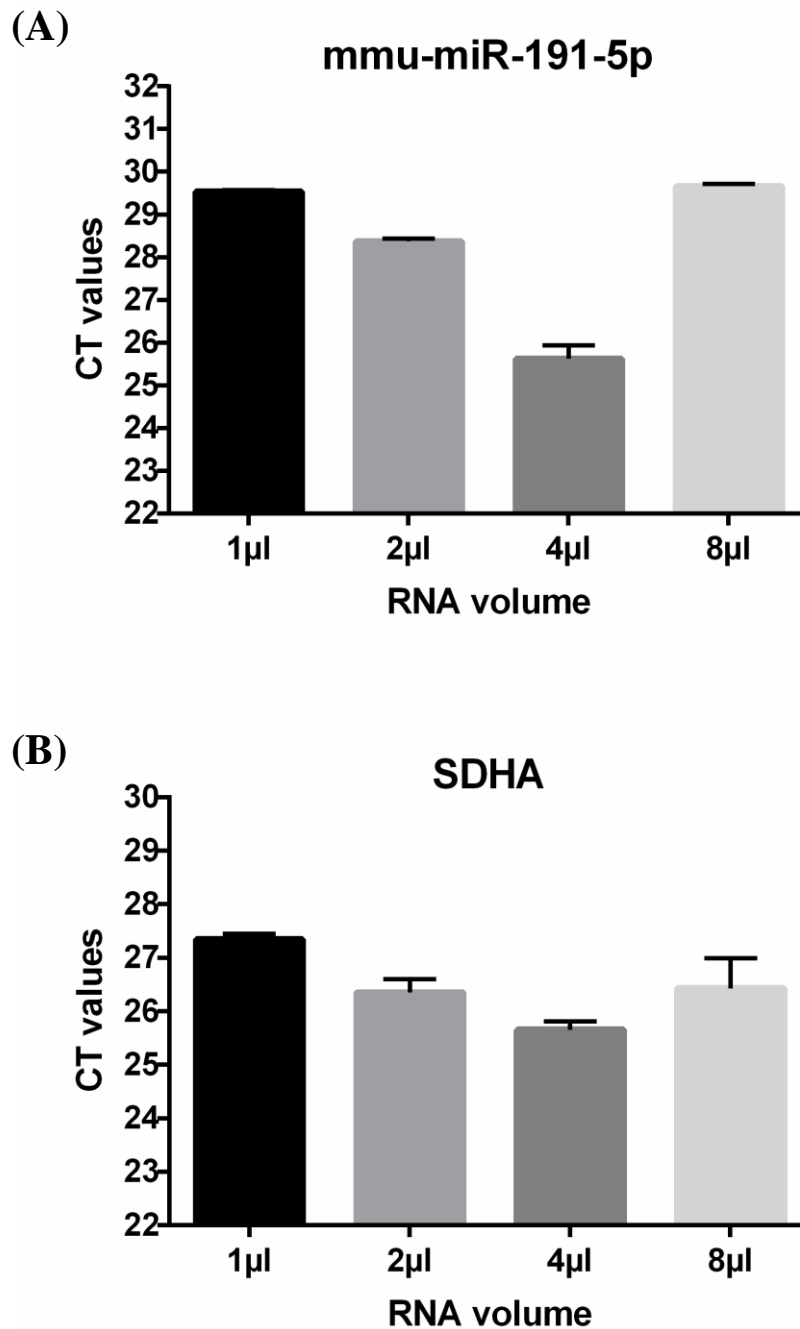


Figure 4.4 Optimisation of the RNA volume used in the cDNA synthesis.

RNA was extracted from 2×10^4 sorted mLANA negative cells. Different RNA volumes (1, 2, 4, or 8µl) were used for cDNA synthesis in a final reaction volume of 20µl. cDNAs were diluted 1/10 and subjected to qRT-PCR for determination of the abundance of the cellular miRNA mmu-miR-191-5p (A) or the cellular transcript SDHA (B). The CT values represent the abundance of the target gene transcript. The lower the CT value, the higher the abundance of the target. Error bars represent standard deviation of triplicate qRT-PCR reactions.

overall expression of RTA and DNA pol was higher at 14 dpi than at 21 dpi; however, it is much lower than NIH 3T3 cells as indicated visually by the intensity of the qRT-PCR product bands and the inconsistency of detection across individual mice.

At 14 dpi, which coincides with the highest latent virus titre in the spleen, the lytic gene transcripts RTA and DNA pol qRT-PCR products (138 bp and 107 bp respectively) were occasionally detected in some of the tested mLANA positive samples. In contrast, these transcripts were rarely detected at 21 dpi, which is expected as a result of viral latency programme and restriction of viral gene expression (Figure 4.5). These results indicate that the mLANA positive cells are latently infected. The occasional presence of RTA and DNA pol at 14 dpi in mLANA positive cells can be a result of sporadic reactivating virus that seeds new uninfected B cells in order to enhance the latency establishment and viral latent amplification. This explanation is consistent with a previous published study that showed that virus reactivation *in vivo* increases the frequency of latently infected splenocytes (Gargano, Forrest et al. 2009).

4.5 Latent expression profiles of MHV-68 encoded miRNAs in mLANA positive splenocytes

In order to investigate the expression levels of MHV-68 miRNAs in the latently infected splenocytes, qRT-PCR assays were carried out using miRNA specific primers. miRNAs expression levels at 14 and 21 dpi were determined (Figure 4.6) and normalised against the cellular miRNA mmu-miR-191-5p. The expression levels of the mmu-miR-191-5p represented by CT values at the 2 time-points are shown in figure 4.7. The expression levels of all of the miRNAs tested were higher at 14 dpi than at 21 dpi; however, to a varying degree.

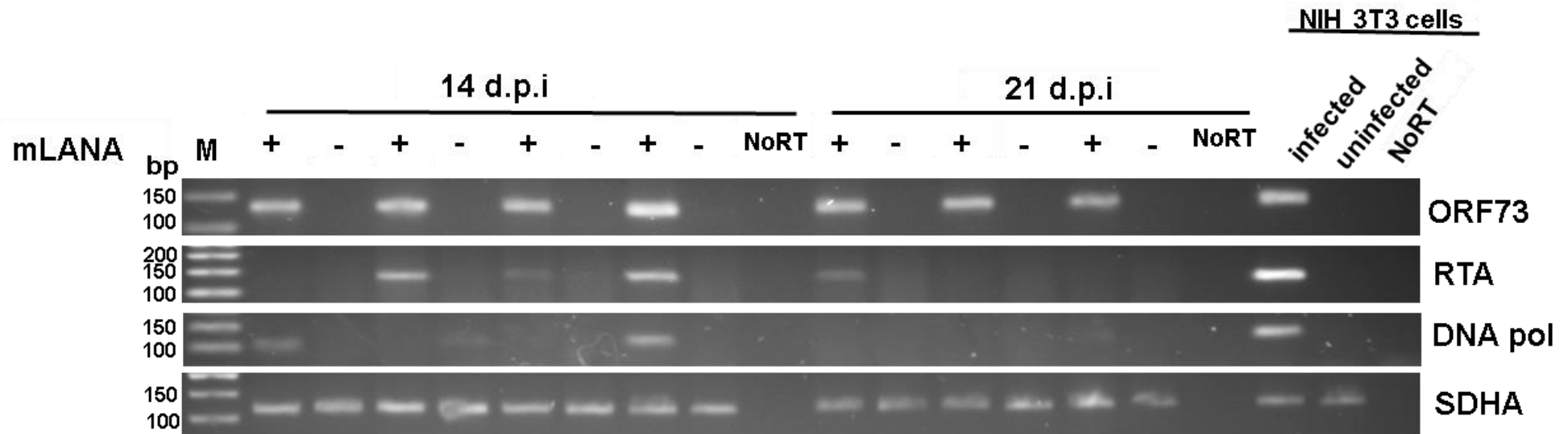


Figure 4.5 Agarose gel image showing MHV-68 mRNAs expression during latent infection *in vivo*. Four μ l of total RNA from mLANA positive (mLANA +) or mLANA negative (mLANA-) splenocytes at 14 dpi (4 mice) or 21 dpi (3 mice) were reverse transcribed into cDNA and subjected to qRT-PCR for detection of the indicated transcripts. The qRT-PCR products were analysed on 3% agarose gel. Five ng of RNA from NIH 3T3 infected with MHV-68 at MOI of 5 were used for similar qRT-PCRs as a representative of lytic infection. No reverse transcription controls (NoRT) were used to confirm the absence of contaminating genomic DNA in the RNA samples. SDHA was used as a cellular housekeeping gene. M, marker. bp, base pair.

The expression levels of mghv-miR-M1-1-3p, mghv-miR-M1-3-3p, mghv-miR-M1-5-5p, mghv-miR-M1-7-3p, mghv-miR-M1-8-5p, mghv-miR-M1-9-3p, and mghv-miR-M1-15-5p were 2-, 2.4-, 1.9-, 3.8-, 2.7-, 2.2-, and 2.3-fold higher at 14 dpi than at 21 dpi respectively (Figure 4.6). The higher miRNAs expression levels at 14 dpi were not statistically significant except for mghv-miR-M1-1-3p, mghv-miR-M1-7-3p, and mghv-miR-M1-8-5p, suggesting that these miRNAs in particular may play roles during the establishment of latency. Given the small changes in the miRNAs abundance levels, these roles can be subtle and involve fine tuning and slight adjustment of the expression of certain cellular and/or viral genes in order to optimise the virus microenvironment for establishment of latency.

4.6 Kinetics of MHV-68 miRNAs expression during different stages of latent infection *in vivo*

Although MHV68.ORF73 β la can provide a tool for detection and isolation of latently infected cells *in vivo*, the pathogenicity of this virus was different from the wild-type virus *in vivo* under the experimental conditions of this study. For example, the splenomegaly in the WT-MHV-68 infected mice was more prominent than that of the MHV68.ORF73 β la infected mice at 14 dpi by approximately 1.6-fold, which was statistically significant and consistent in independent experiments (Figure 4.8B). The bigger sizes of the spleens from the WT-MHV-68 infected mice at 14 dpi can be observed visually (Figure 4.8A). When the viral DNA genomic loads were determined by qPCR, it was found that the viral genomic DNA load in splenocytes from WT-MHV-68 infected mice was significantly higher than that of the MHV68.ORF73 β la infected mice by approximately 16.1 and 21.6-fold at 14 and 21 dpi, respectively (Figure 4.8C). This suggests that the MHV68.ORF73 β la virus is attenuated during the establishment of latent infection *in vivo*.

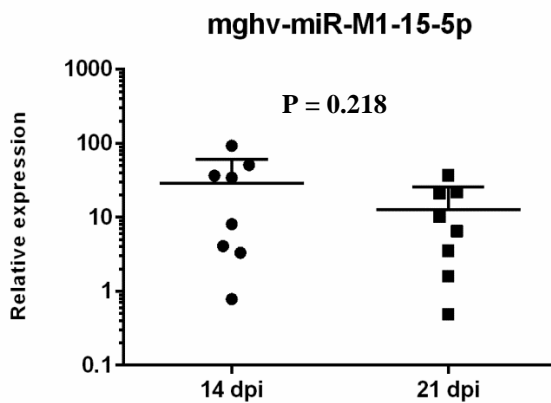
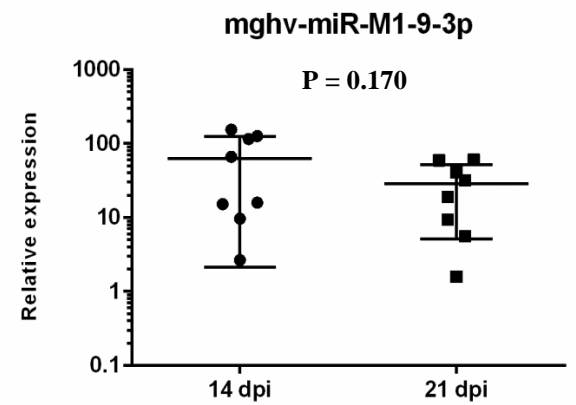
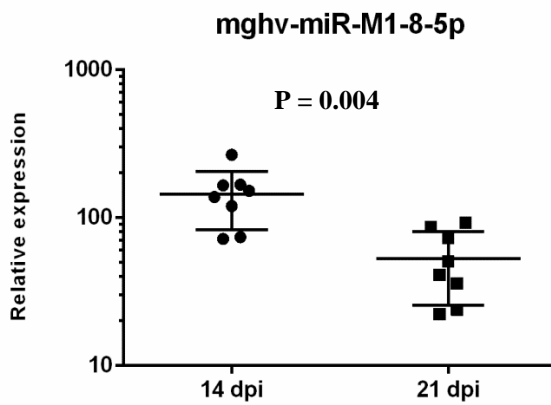
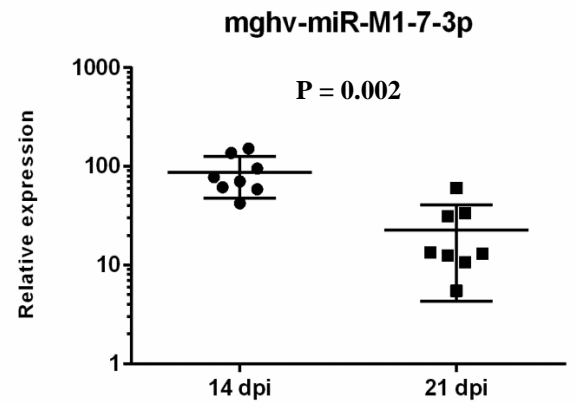
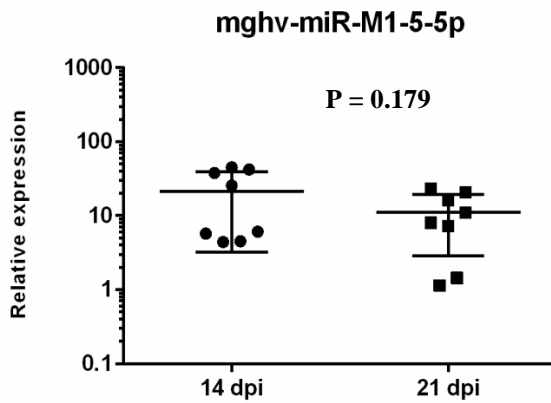
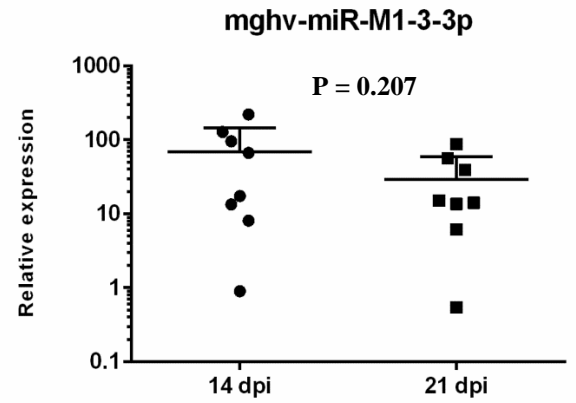
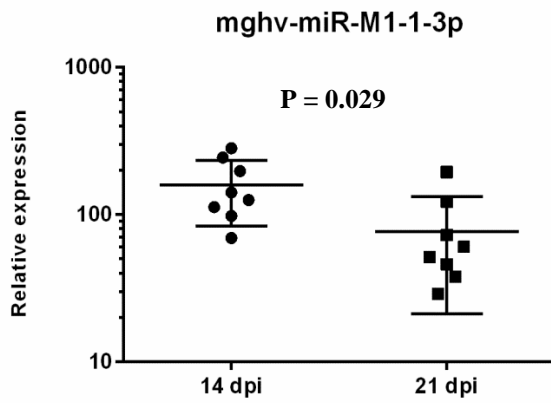


Figure 4.6 Expression of MHV-68 miRNAs during latency. BALB/c mice were infected with 4×10^5 pfu MHV68. ORF73 β la virus. The mLANA positive cells were isolated at 14 or 21 dpi and total RNA was extracted. Four μ l RNA were converted into cDNA and the viral miRNAs expression levels were determined by qRT-PCR. The relative expression was calculated as $2^{-\Delta CT} \times 10^2$ where $\Delta CT = CT_{\text{miRNA of interest}} - CT_{\text{reference miRNA}}$. mmu-miR-191-5p was used as a cellular reference miRNA in order to correct for any experimental variability. The error bars represent mean \pm SD of 2. The data were compiled from 2 independent experiments, with 3-5 mice were used per time-point per experiment. qRT-PCR was carried out in triplicates. P-values were calculated based on student's t test.

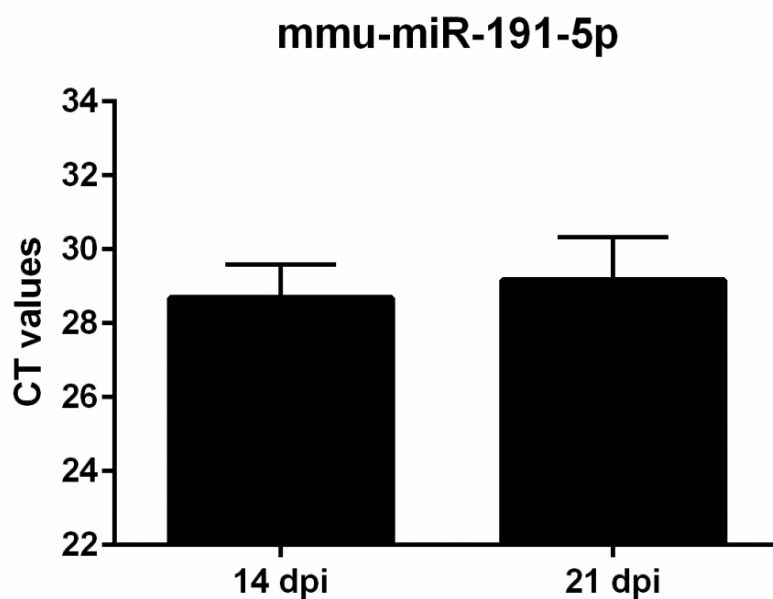
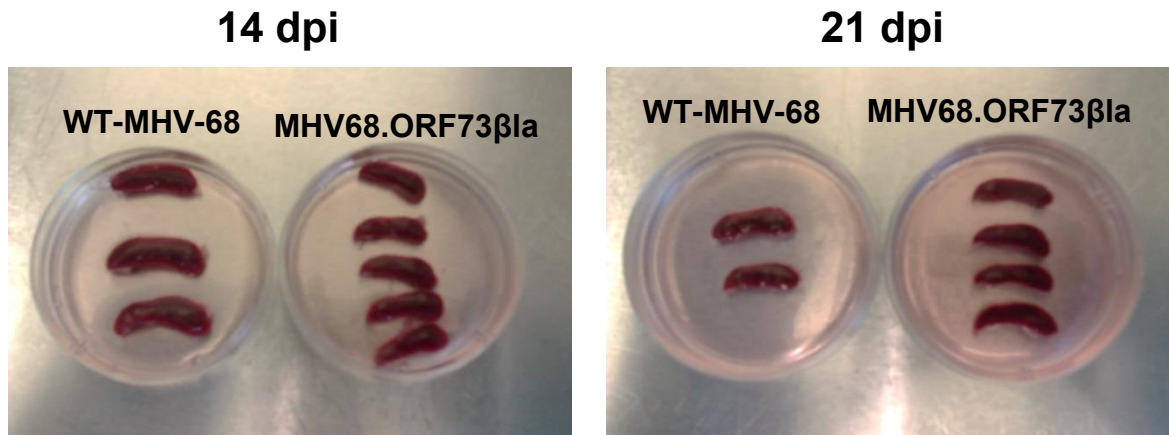
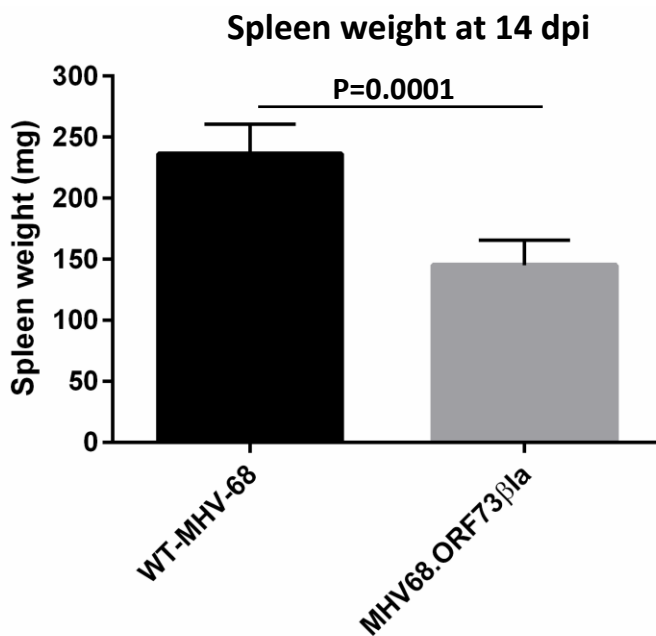


Figure 4.7 The expression levels of mmu-miR-191-5p in mLANA positive cells. mmu-miR-191-5p was used as a reference miRNA for the relative quantification of MHV-68 miRNAs in mLANA positive cells during latency (see figure 4.6). The mmu-miR-191-5p expression at 14 or 21 dpi was determined by qRT-PCR and presented as CT values. Error bars represent standard deviation of the mean of 2 independent experiments with 3-5 mice per time-point per experiment. qRT-PCR was performed in triplicates.

(A)



(B)



(C)

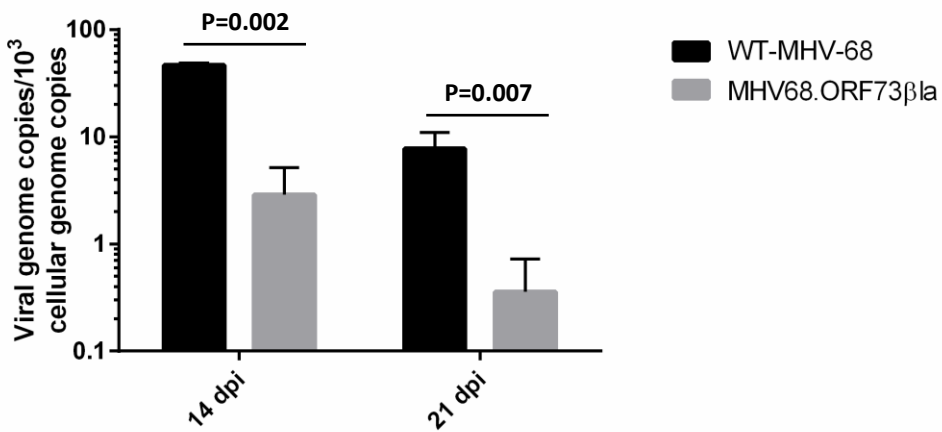


Figure 4.8 The MHV-68.ORF73 β la virus is attenuated during latent infection *in vivo*.

BALB/c mice were infected with 4×10^5 WT-MHV-68 or MHV-68.ORF73 β la virus intranasally. (A) At 14 or 21 dpi, mice were sacrificed and the spleens were harvested and the splenomegaly was observed. (B) The spleen weights were determined at 14 dpi as a measurement of splenomegaly. (C) The genomic DNA was extracted from the splenocytes of infected mice and the absolute copy numbers of the viral genomic loads were determined by qPCR using RTA primers and 100 ng genomic DNA as a template. The viral genomic copy numbers were normalised to 10^3 copies of the cellular genomic load using the SDHA primers. The error bars represent the mean \pm SD. Data represent two independent experiments, using 2-5 mice per group per time-point. P-value was determined using unpaired student's t test.

The rationale of using the MHV68.ORF73 β la virus was to enrich for latently infected splenocytes by selectively isolating them from the uninfected population, aiming to facilitate the quantification of viral miRNAs. However, the small numbers of the isolated mLANA positive splenocytes, which is most likely due to the small numbers of MHV-68 ORF73 β la-infected cells, caused difficulty in recovering RNA of good quality and quantity for the qRT-PCR analysis. This observed attenuation of MHV68.ORF73 β la virus during latency *in vivo*, made us decide to use the WT-MHV-68 virus for expression profiling of the viral miRNAs during latent infection. This can allow for further confirmation of the findings obtained from the MHV68.ORF73 β la virus using an independent virus as well as investigating the miRNA expression levels at earlier and later time-points post-infection, during which the numbers of infected cells are very small.

In order to evaluate the expression kinetics of MHV-68 miRNAs during the course of latent infection *in vivo* using the WT virus, BALB/c mice were infected intranasally with 4×10^5 pfu WT-MHV-68 virus. The spleens were harvested at 10, 14, 21, and 32 dpi. The use of WT-MHV-68 virus for profiling the viral miRNAs during different stages of latency *in vivo* is possible; however, the potential changes of the miRNAs expression can be merely due to the changes of the viral genomic loads during the course of latent infection. For this reason parallel RNA and DNA samples were extracted for quantification of viral miRNAs as well as viral DNA genomic loads. The levels of viral miRNAs expression were then standardised against the levels of viral genomic loads.

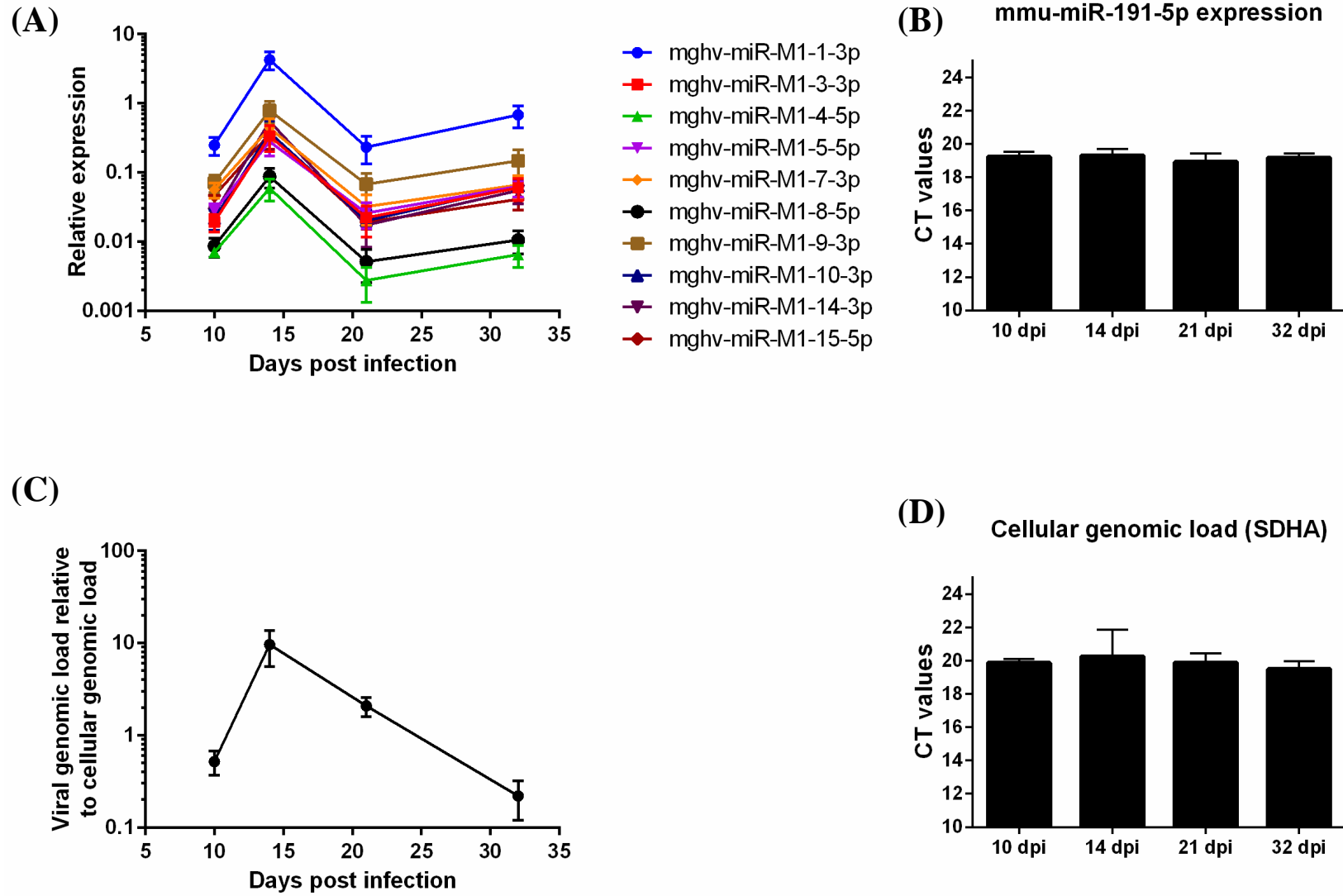


Figure 4.9 The expression kinetics of MHV-68 encoded miRNAs and viral genomic load during latent infection *in vivo*. BALB/c mice were infected intranasally with 4×10^5 WT-MHV-68 virus. Spleens were harvested at 10, 14, 21, and 32 dpi and single-cell suspensions were prepared and parallel RNA and DNA extractions were carried out on the same animal. Total RNA (500 ng) was converted into cDNA and subjected to qRT-PCR using miRNA specific primers. The viral miRNAs expression levels were quantified relative to the reference miRNA mmu-miR-191-5p (A and B). The viral DNA genomic loads (RTA primers) were quantified relative to the cellular genomic levels (SDHA primers) using 100 ng template DNA (C and D). Error bars in A and C represent the mean \pm SEM from 5 mice per group per time-point. Error bars in B and D represent mean \pm SD of the CT values from 5 mice per group per time-point. qRT-PCR reactions were carried out in triplicates.

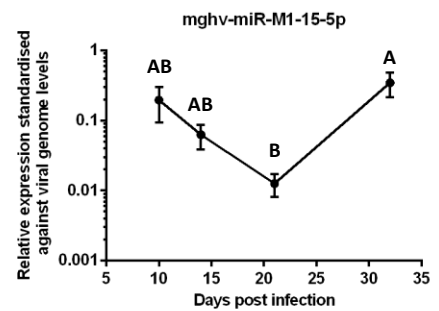
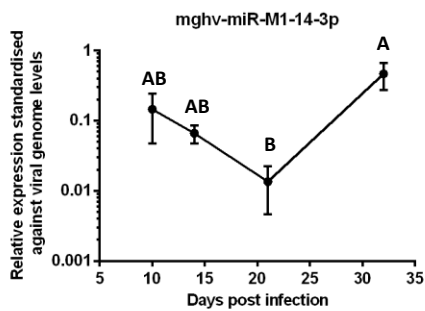
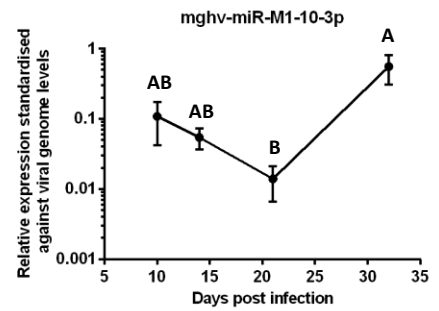
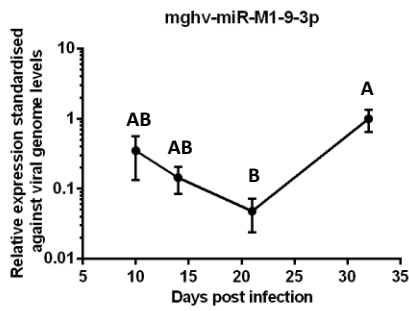
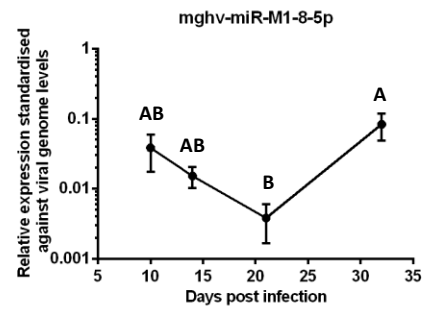
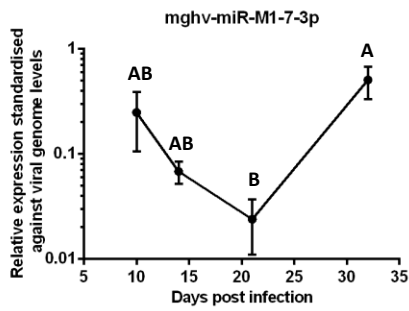
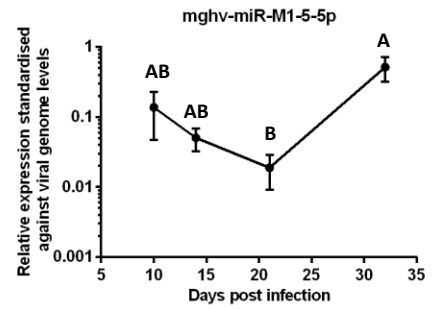
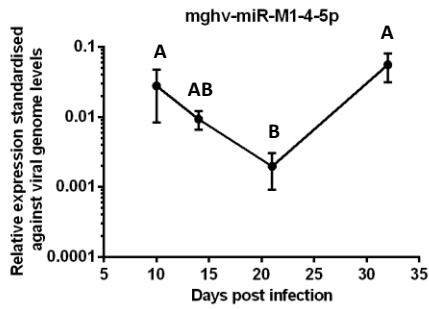
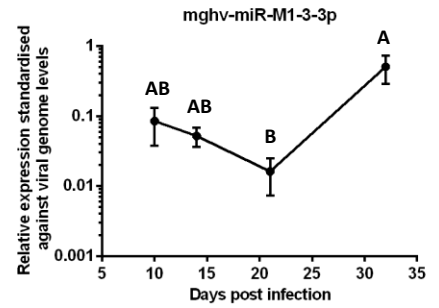
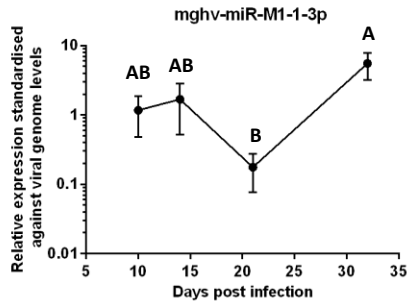


Figure 4.10 The kinetics of MHV-68 miRNAs expression normalised against the genomic viral load during latent infection *in vivo*. The data shown in this figure are from the same experiment as figure 4.9, but here the normalised levels of miRNAs expression were standardised against the normalised viral genomic loads. Error bars represent mean \pm SEM. The means that do not share a letter are significantly different ($P < 0.05$). The statistical significance was determined by one-way ANOVA and Tukey's comparison test on log-transformed data using Minitab 16 statistical software. Data were compiled from 5 mice per time-point per group. The qRT-PCR reactions were performed in triplicates.

Interestingly, all of the tested miRNAs showed similar expression kinetics pattern (Figure 4.9A). The miRNAs expression levels are shown as relative expression to the cellular reference miRNA mmu-miR-191-5p (Figure 4.9B). The viral genomic loads were normalised to the cellular genomic loads (Figure 4.9D). The miRNAs expression levels increased between 10 and 14 dpi relative to the expression of the cellular miRNA mmu-miR-191-5p, which coincides with increase of the viral latent genomic load in the spleen detected by qPCR (Figure 4.9C). The relative miRNAs expression levels decreased between 14 and 21 dpi. Surprisingly, despite the decrease of the viral genomic DNA load between 21 and 32 dpi by approximately 9.5-fold, there was slight increase of the miRNAs expression levels by approximately 2-3-fold at 32 dpi over 21 dpi (Figure 4.9A and C). The normalised miRNAs expression levels were standardised against the normalised viral genomic levels in order to rule out the effect of the viral load differences between different time-points on the changes of the viral miRNAs expression levels. Although, there was a non-significant decrease in the abundance of the viral miRNAs between 10 dpi and 21 dpi, except for mghv-miR-M1-4-5p, this decrease was consistent among all the tested miRNAs (Figure 4.10).

Due to the large variation between individual mice, a larger experiment will be needed to detect possible statistically significant differences at these time points. The miRNAs expression levels increased over time between 21 and 32 dpi. This increase in expression was statistically significant in all of the miRNAs tested (Figure 4.10). These findings suggest that the regulation of the expression of MHV-68 miRNAs expression may differ at different stages of latent infection and that the miRNAs may play different roles during latency. Due to time constraints this experiment was done once and another experiment will be needed to confirm these results.

4.7 Discussion

The roles of the gamma-herpesviruses encoded miRNAs during the latent infection are not fully understood. Characterisation of the expression patterns of these miRNAs during latency can be helpful to further dissect their functions. In this study, a marked virus, MHV68.ORF73 β la, that carries a β -lactamase gene fused in-frame downstream of the mLANA expressing gene ORF73 was used to detect and isolate latently infected splenocytes (mLANA positive splenocytes) at 14 dpi and 21 dpi by flow cytometry, aiming at quantifying the viral miRNAs expression levels during the early establishment of latency and during latency maintenance. The MHV-68 miRNAs expression levels were quantified in the mLANA positive cells at 14 and 21 dpi. It was found that the expression levels of all of the miRNAs tested showed decrease in the expression levels over time between 14 and 21 dpi. This is consistent with the findings in the current study, where The WT-MHV-68 virus was used to evaluate the expression kinetics of the MHV-68 miRNAs at 10, 14, 21, and 32 dpi in total splenocytes. The miRNAs expression levels of the WT-MHV-68 decreased over time between 10 and 21 dpi. Interestingly, the miRNAs expression levels then increased between 21 and 32 dpi, suggesting that the MHV-68 latency is a complex dynamic process. It is plausible that there are different stages of MHV-68 latency: (i) the stage of early establishment of latency in spleen (between 10 to 21 days post infection) when the miRNAs can play an important role in silencing viral gene expression and inhibiting lytic infection possibly by targeting viral transcription activators, particularly RTA, in a way similar to that of the KSHV miRNA, miR-K12-9*, which targets a sequence in the 3' UTR of KSHV RTA (Bellare and Ganem 2009). MHV-68 miRNAs can also possibly down-regulate expression of cellular genes involved in supporting lytic infection. This strategy has been reported for other

gamma-herpesvirus miRNAs. For example, the KSHV-encoded miR-K12-1 has been shown to down-regulate I κ B α , a suppressor of NF- κ B complexes resulting in promoting NF- κ B-dependent viral latency (Lei, Bai et al. 2010). Furthermore, the EBV miRNA, BART-miR-18-5p, has been shown to down-regulate the expression of the cellular protein MAP3K2 leading to decreased viral lytic gene expression and virus production (Qiu and Thorley-Lawson 2014).

The MHV-68 miRNAs expression decreased over time between 10 and 21 dpi possibly to allow for basal expression levels of lytic genes particularly RTA in order to allow for a minimal level of reactivation, resulting in virus spread to neighbouring uninfected cells; (ii) the stage of latency maintenance which involves increase of miRNAs expression (after 21 dpi in spleen) in order to suppress any lytic reactivation beyond the basal level and possibly to increase the longevity of infected cells through anti-apoptotic functions.

In the present study, each animal was analysed individually and samples from different animals were not pooled in order to get an insight into individual variations between animals. In a previous published study involving the use of MHV68.ORF73 β la virus for evaluating the kinetics of mLANA expression in B cell subsets the samples from different mice were pooled and analysed (Nealy, Coleman et al. 2010). This possibly facilitated the gene expression analysis in that study. In the same study the MHV68.ORF73 β la virus showed a significant decrease in virus spread among cells during the multi-step growth curve analysis, approximately 2 log difference in virus titre at 24 and 48 hours post infection in NIH 3T12 cells as compared to the WT virus, a phenotype that is observed in the MHV-68 mutant virus lacking mLANA expression (Moorman, Willer et al. 2003, Forrest, Paden et al.

2007), suggesting that the C-terminal fusion of β -lactamase can affect the mLANA function. However, in contrast to the current study, no attenuation was reported during latent infection *in vivo* in Nealy *et. al.* study (Nealy, Coleman et al. 2010).

It would have been interesting if the miRNA expression levels were assayed in the latently infected splenocyte population at time points later than 21 dpi; however, very small numbers of infected splenocytes are expected to be isolated later than 21 dpi using the MHV68.ORF73 β la virus because of its attenuation and it was thought to be not worth trying. Future experiments can involve the use of a marked MHV-68 virus, which can establish and maintain latency at levels similar to that of the WT virus, for isolation of latently infected splenocytes during late latency and for determination of the miRNAs expression after 32 days post infection in these cells.

Chapter 5: Functional analysis of the MHV-68-encoded miRNAs

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5 Chapter 5: Functional analysis of the MHV-68-encoded miRNAs

5.1 Introduction

An important feature of gammaherpesvirus latency is the restricted gene expression that allows the virus to evade the immune system and to remain in the host throughout its lifetime. However, the viruses need to encode functions which modulate the viral gene expression and the cellular environment to maintain latent infection. Protein expression in latently infected cells can be a target of the immune system, hence expression of miRNAs provides the gammaherpesviruses with a powerful tool to manipulate the cell and maintain latency. A number of studies have used cross-linking immunoprecipitation (CLIP) of the RNA-induced silencing complex (RISC) and validation of miRNAs targets to identify potential functions of the miRNAs encoded by the human gammaherpesviruses EBV and KSHV (Dolken, Malterer et al. 2010, Haecker, Gay et al. 2012, Ramalingam, Kieffer-Kwon et al. 2012, Riley, Rabinowitz et al. 2012, Skalsky, Corcoran et al. 2012, Zhu, Haecker et al. 2013). These studies have identified viral miRNAs targets that are involved in cell cycle regulation, apoptosis, and immune recognition and thus enhance viral latency and survival in the host. Some viral miRNAs have also been proposed to directly target the viral transcripts in order to suppress lytic genes and promote latency (Kincaid and Sullivan 2012, Riaz, Dry et al. 2014).

Although some of these studies indicate that the miRNAs encoded by the human gammaherpesviruses can target both cellular and viral targets, the functions of these miRNAs remain not fully characterised in the context of natural host infection due to

the strict host specificity of gammaherpesviruses. MHV-68 is genetically related to the human gammaherpesviruses, EBV and KSHV (Barton, Mandal et al. 2011), and it provides a tractable small animal model for studying the potential functions of gammaherpesvirus miRNAs during the natural course of infection (primary lytic infection, establishment of latency, maintenance of latency, and reactivation).

The aim of this chapter was to investigate the potential roles played by the MHV-68 miRNAs in the virus pathogenesis. For this purpose, an MHV-68 mutant virus, lacking 9 miRNAs associated with vtRNAs1-5, was constructed using homologous recombination within the MHV-68 bacterial artificial chromosome (BAC). This miRNA mutant virus (designated MHV-68.ΔmiRNAs) was used along with its wild-type counterpart to investigate:

- 1- The role of the miRNAs in virus productive replication *in vitro* and *in vivo*
- 2- The kinetics of latency establishment *in vivo*
- 3- The efficiency of virus reactivation from latency *ex vivo*
- 4- The ability of the virus to maintain long-term latency in the spleen
- 5- The virus-induced B cell activation and proliferation in spleen

The construction of the MHV-68.ΔmiRNAs virus was successful and the virus lacked the expression of the deleted miRNAs *in vitro* and *in vivo*. Although the miRNA mutant virus and its wild-type counterpart showed similar ability to replicate productively, to establish latent infection *in vivo* and to reactivate *ex vivo* during early latency and as late as 28 dpi, the MHV-68.ΔmiRNAs virus showed higher *ex vivo* reactivation efficiency than the WT virus during late latency (49 dpi). At this time point, an increase in the expression levels of the viral lytic mRNAs in the spleens of MHV-68.ΔmiRNAs virus infected mice, particularly the M3 transcript that is abundantly expressed during lytic infection and encodes a chemokine-binding

protein (Parry, Simas et al. 2000, Rochford, Lutzke et al. 2001), demonstrated a key role for MHV-68 miRNAs in maintaining long-term latency and control of the transition from latent state to lytic state during late latency.

While this study was underway, two groups published papers using a similar approach. These studies suggested that the MHV-68 miRNAs are dispensable for the productive virus infection and the establishment of latency *in vivo* and that they can be involved in increasing the numbers of the MHV-68-infected memory B cells and in the development of lethal pneumonia in IFN γ -deficient mice (Feldman, Kara et al. 2014, Diebel, Oko et al. 2015).

5.2 Construction of an MHV-68 mutant virus lacking the expression of 9 miRNAs

In order to investigate the potential roles of the MHV-68 miRNAs during the virus life cycle, a recombinant virus carrying mutations that deleted 9 miRNAs, designated MHV-68. Δ miRNAs, was constructed (Figure 5.1). The WT-MHV-68 BAC (Adler, Messerle et al. 2000) was used as a backbone for generating the MHV-68. Δ miRNAs virus by two-step lambda red-mediated recombination (Tischer, von Einem et al. 2006). The mutations targeted the deletion of 9 miRNAs (mghv-mir-M1-1, mghv-mir-M1-10, mghv-mir-M1-2, mghv-mir-M1-3, mghv-mir-M1-4, mghv-mir-M1-5, mghv-mir-M1-6, mghv-mir-M1-7, and mghv-mir-M1-12), while keeping intact the vtRNA genes and putative open reading frames. The positions of the nucleotide mutations within the MHV-68 genome are shown in Table 5.1.

After cloning and recombination, the generated miRNA mutant MHV-68 BAC clones were screened by PCR in order to determine whether the mutated region

showed the expected size by gel electrophoresis followed by Sanger sequencing of this region (see Materials and Methods). All the expected mutations were present and the intact sequences between the mutations were correct when aligned against the wild-type sequence (Figure 5.2). In order to confirm that the genome structure within the MHV-68.ΔmiRNAs BAC is similar to that of the parental WT-MHV-68 BAC and that there is no genomic rearrangement in the MHV-68.ΔmiRNAs BAC, the anatomy of the viral genomic structure within the intact BAC DNA was determined by restriction enzyme digest using XhoI and EcoRI enzymes (Figure 5.3A). It was found that the genomic structure of the MHV-68.ΔmiRNAs BAC was similar to that of the WT-MHV-68 BAC. Having confirmed the correct mutant sequence and the proper genomic structure, the MHV-68.ΔmiRNAs BAC DNA and its wild-type counterpart were transfected into NIH 3T3 cells expressing Cre recombinase in order to remove the BAC vector sequences by Cre-*loxP* recombination and to produce MHV-68.ΔmiRNAs virus and its parental WT-MHV-68 virus (see Materials and Methods).

In order to confirm that the generated miRNA mutant virus carried all the miRNAs deletions, NIH 3T3 cells were infected with MHV-68.ΔmiRNAs virus or WT-MHV-68 virus at MOI of 5 for 24 hours. Total genomic DNA was extracted and PCR amplification of the DNA fragment containing the miRNAs mutations was carried out. Uninfected cells were used as a negative control. As expected, when the PCR products were analysed by agarose gel electrophoresis, it was found that the amplified DNA fragments showed the expected sizes of 1,719 bp and 1,360 bp for the WT-MHV-68 and the MHV-68.ΔmiRNAs-infected samples, respectively. This corresponds to a difference of 359 bp between the wild-type and miRNA mutant sequences due to deletion of the miRNAs (Figure 5.3B).

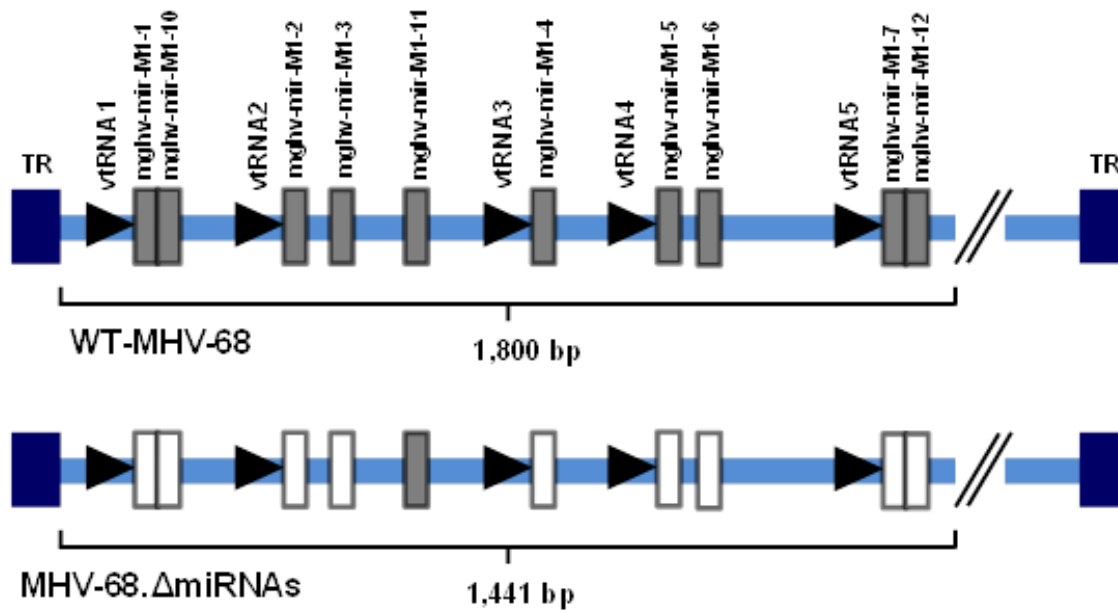


Figure 5.1 Schematic of the genomic organisation of the MHV-68 tRNAs and its associated miRNAs within the genomes of WT-MHV-68 and MHV-68.ΔmiRNAs viruses. The vtRNA genes are represented as black triangles. The miRNA WT sequences are represented as grey rectangles. The miRNA mutant sequences are represented as white rectangles. The names of the genes are indicated. The miRNA genes are named according to the miRBase nomenclature. TR, terminal repeat.

| Feature | Position | Position of deleted or mutated sequences | Deleted feature |
|----------------|-----------|---|-----------------|
| vtRNA1 | 127-196 | - | - |
| mghv-mir-M1-1 | 197-260 | 197-316 | mghv-mir-M1-1 |
| mghv-mir-M1-10 | 261-322 | | mghv-mir-M1-10 |
| vtRNA2 | 488-561 | - | - |
| mghv-mir-M1-2 | 562-627 | 607-614 | mghv-mir-M1-2 |
| mghv-mir-M1-3 | 638-698 | Point mutations: C673A, G676C, G679A, A682C, A685C, A688C, T691A, and G694A | mghv-mir-M1-3 |
| mghv-mir-M1-11 | 759-811 | - | - |
| vtRNA3 | 894-955 | - | - |
| mghv-mir-M1-4 | 956-1049 | 1029-1036 | mghv-mir-M1-4 |
| vtRNA4 | 1182-1254 | - | - |
| mghv-mir-M1-5 | 1255-1314 | 1255 – 1373 | mghv-mir-M1-5 |
| mghv-mir-M1-6 | 1319-1381 | | mghv-mir-M1-6 |
| EcoRI site | 1517-1522 | A1518T | EcoRI site |
| EcoRI site | 1570-1575 | A1571T | EcoRI site |
| vtRNA5 | 1588-1659 | - | - |
| mghv-mir-M1-7 | 1660-1719 | 1660 – 1764 | mghv-mir-M1-7 |
| mghv-mir-M1-12 | 1720-1789 | | mghv-mir-M1-12 |

Table 5-1 MHV-68 vtRNAs and miRNAs genomic locations and the positions of the deleted sequences in the MHV-68.ΔmiRNAs virus.

CLUSTAL 2.1 multiple sequence alignment

```

WT  GCGCGCCCTGGAGATCCGGGGCTGATGCTGGGTTTCAGGAGTTCAGGGCGGCCACCATTTC 60
Mut  GCGCGCCCTGGAGATCCGGGGCTGATGCTGGGTTTCAGGAGTTCAGGGCGGCCACCATTTC 60
*****

WT  GATGCAAATGTTGGGTGTGTGCACCCCACTATCTCTGGTTCTGCAAAGCTTGCTTTTTTGT 120
Mut  GATGCAAATGTTGGGTGTGTGCACCCCACTATCTCTGGTTCTGCAAAGCTTGCTTTTTTGT 120
*****

WT  GCTGGTGCCAGAGTAGCTCAATTGGTAGAGCAACAGGTCACCGATCCTGGTGGTTCTCGG 180
Mut  GCTGGT GCCAGAGTAGCTCAATTGGTAGAGCAACAGGTCACCGATCCTGGTGGTTCTCGG 180
*****

WT  TTCAAGTCCGAGCTCTGGTCGAGGAAGTGGGTCCAACCTTATCCCTATAGTGTGATAGAA 240
Mut  TTCAAGTCCGAGCTCT----- 196
*****

WT  ATGCCGTA CTTCC TTTATAATTAAGAACCCCTCAGTGCAATCACTTGTCTGCATTTGGAG 300
Mut  -----

WT  TGATTACACGGAAGGTTCTTTTGT TTTGGGACTTGCTGCTTTGATATAGCCAGGCTTTT 360
Mut  -----TCTTTTGT TTTGGGACTTGCTGCTTTGATATAGCCAGGCTTTT 240
*****

WT  ATGTGCAACTTTCTATTTTTCTACTATGCAAAGACCCTTGATTATTGAGAGTCTAGCAA 420
Mut  ATGTGCAACTTTCTATTTTTCTACTATGCAAAGACCCTTGATTATTGAGAGTCTAGCAA 300
*****

WT  CCTCATGCAAATGTCTTTTAAAGACCCGTAGGTTTCATCAAGCCCAACAGATACTATTT 480
Mut  CCTCATGCAAATGTCTTTTAAAGACCCGTAGGTTTCATCAAGCCCAACAGATACTATTT 360
*****

WT  ACATAAGGCCAGGGTAGCTCAATCGGTAGAGCAGCGGTTCTGGAGTCCGCTGGTTCTCG 540
Mut  ACATAAG GCCAGGGTAGCTCAATCGGTAGAGCAGCGGTTCTGGAGTCCGCTGGTTCTCG 420
*****

WT  GTTCAAGCCCAGCCCTGGTTGAGAGGGGAGTGTGTGGTCTGTAGAGAGACATGAGTTG 600
Mut  GTTCAAGCCCAGCCCTGGTTGAGAGGGGAGTGTGTGGTCTGTAGAGAGACATGAGTTG 480
*****

WT  ATCGGCAGACCCCTCTCCCCTCTTTCCCTCTTTACGAAGTAGCGAACCTCTGCTCACT 660
Mut  ATCGGC -----TCTCCCCTCTTTCCCTCTTTACGAAGTAGCGAACCTCTGCTCACT 532
*****

WT  GCCCGGGCCCTCCGGGAGGTGAGCAGGAGTTGCGCTTTTCTTTCTCTGTTTGT TTTTGC 720
Mut  GCCCGGGCCCTCAGGCAGATGCGCCGCGTAGCACTTTTCTTTCTCTGTTTGT TTTTGC 592
*****

WT  CATAACCAAGTCTATTGGCAGCTGCCAATCTCACCTGACAGCTGTCAGGGGTACATG 780
Mut  CATAACCAAGTCTATTGGCAGCTGCCAATCTCACCTGACAGCTGTCAGGGGTACATG 652
*****

WT  AGAGAACTTCATGTAACCCCTGACAGCTGTCAACCTAATCCTGACCGTGAGAACTTTTCA 840
Mut  AGAGAACTTCATGTAACCCCTGACAGCTGTCAACCTAATCCTGACCGTGAGAACTTTTCA 712
*****

WT  GACTCATATCGAAAAATGGGTAACAACCTCTGAAGGAACTGTGTCTTTTACAGCGTCGGGG 900
Mut  GACTCATATCGAAAAATGGGTAACAACCTCTGAAGGAACTGTGTCTTTTACAGCGTCGGGG 772
*****

WT  TAGTCAAATGGTAGAGTGGCAGGCCAACATAGCCAGCAGATCTCGGTTCAAACCCGAGC 960
Mut  TAGTCAAATGGTAGAGTGGCAGGCCAACATAGCCAGCAGATCTCGGTTCAAACCCGAGC 832
*****

WT  CCTGACCATCGAGGAGCACGTGTTATTCTAGACCTCTCTCCAGCTAAGATAGCATGTGC 1020

```

vtRNA1

mgHV-mir-M1-1 & mgHV-mir-M1-10 mutations

vtRNA2

mgHV-mir-M1-2 mutation

mgHV-mir-M1-3 mutation

vtRNA3

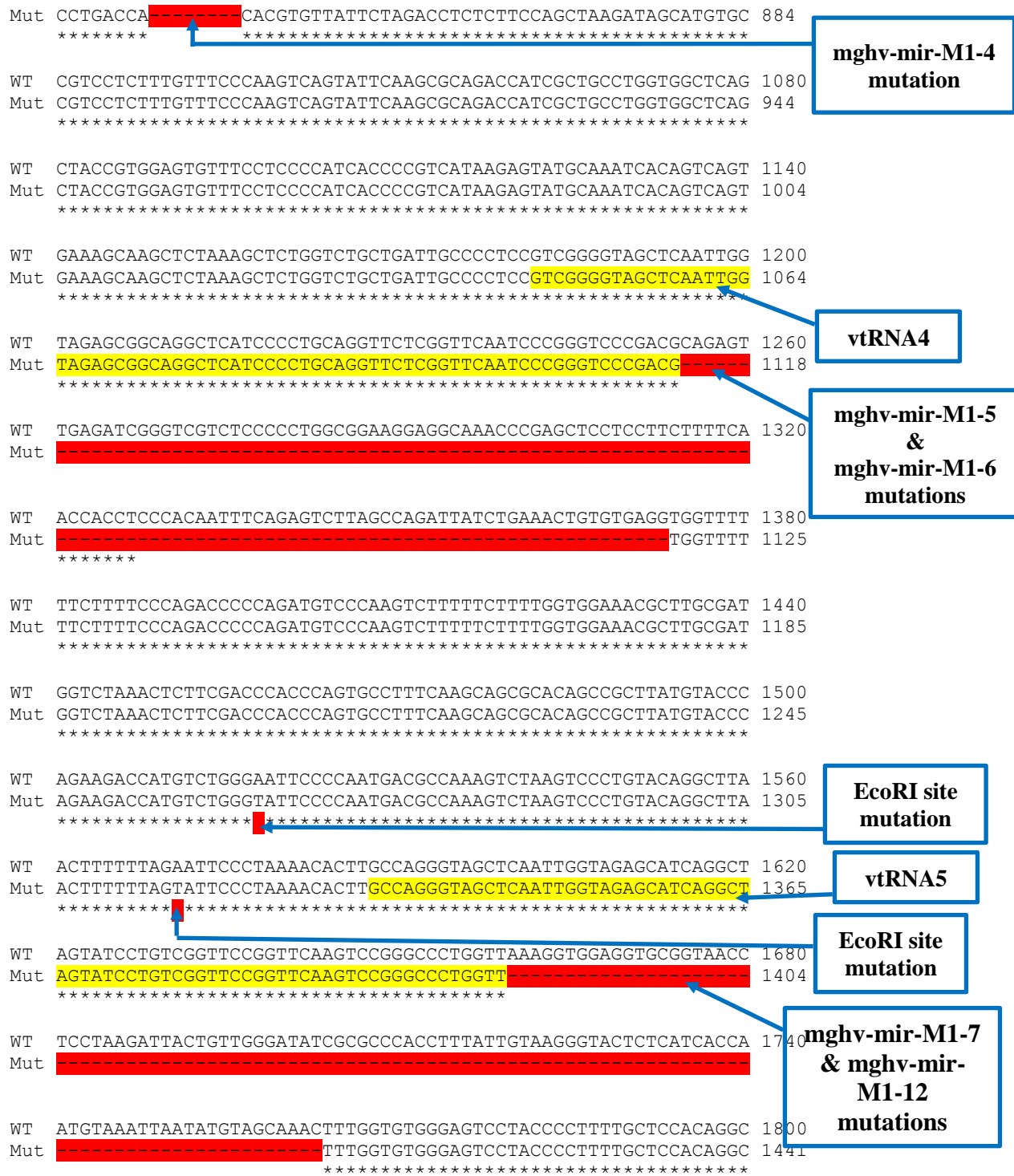


Figure 5.2 Clustal 2.1 multiple sequence alignment.

Alignment of the consensus sequence of the MHV-68.ΔmiRNAs BAC (Mut) against the WT-MHV-68 sequence (WT). All the non-aligned sequences are indicated including the 9 miRNAs and EcoRI sites. The vtRNA sequences in the MHV-68.ΔmiRNAs BAC were identical to that of the WT-MHV-68.

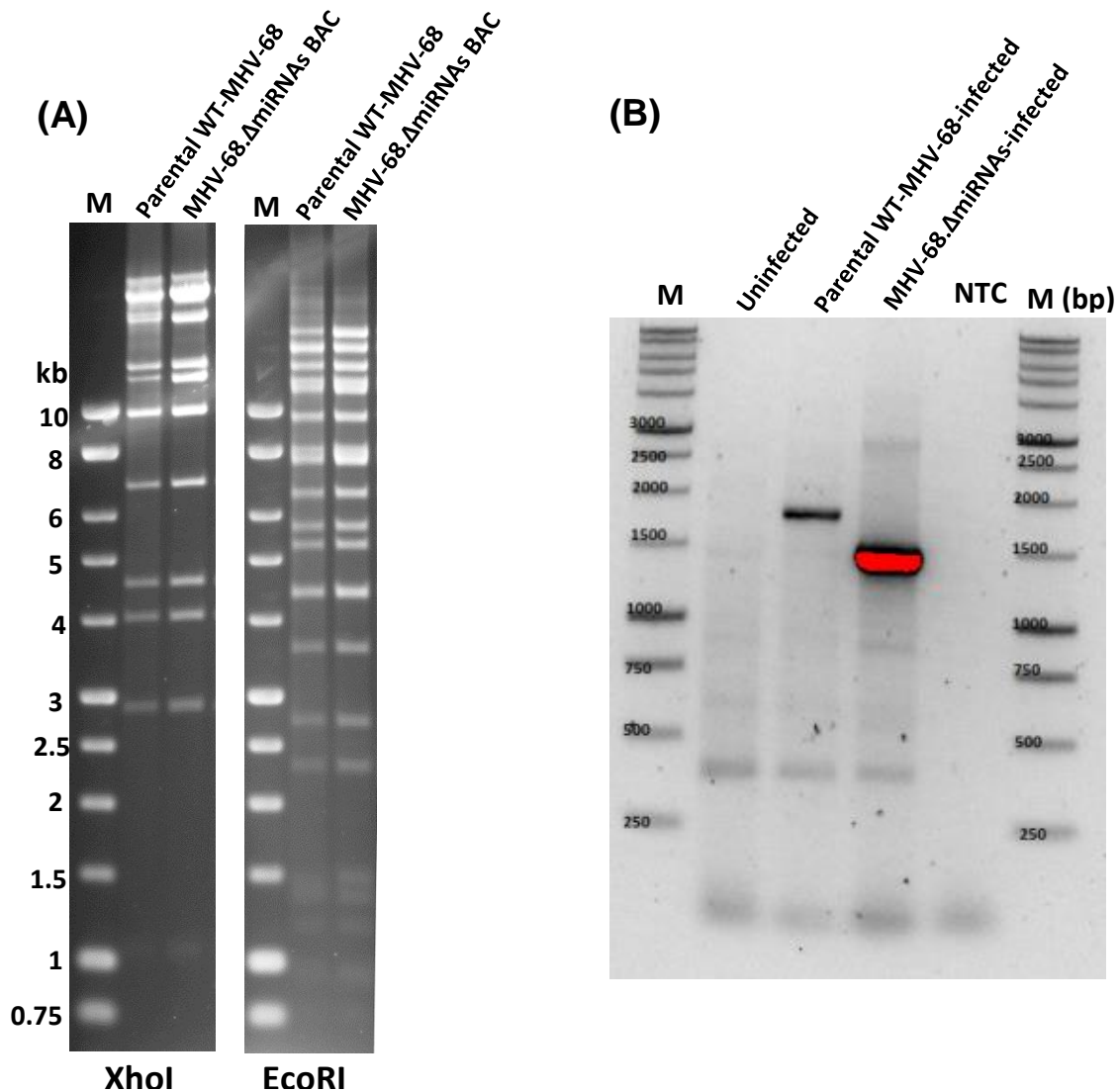


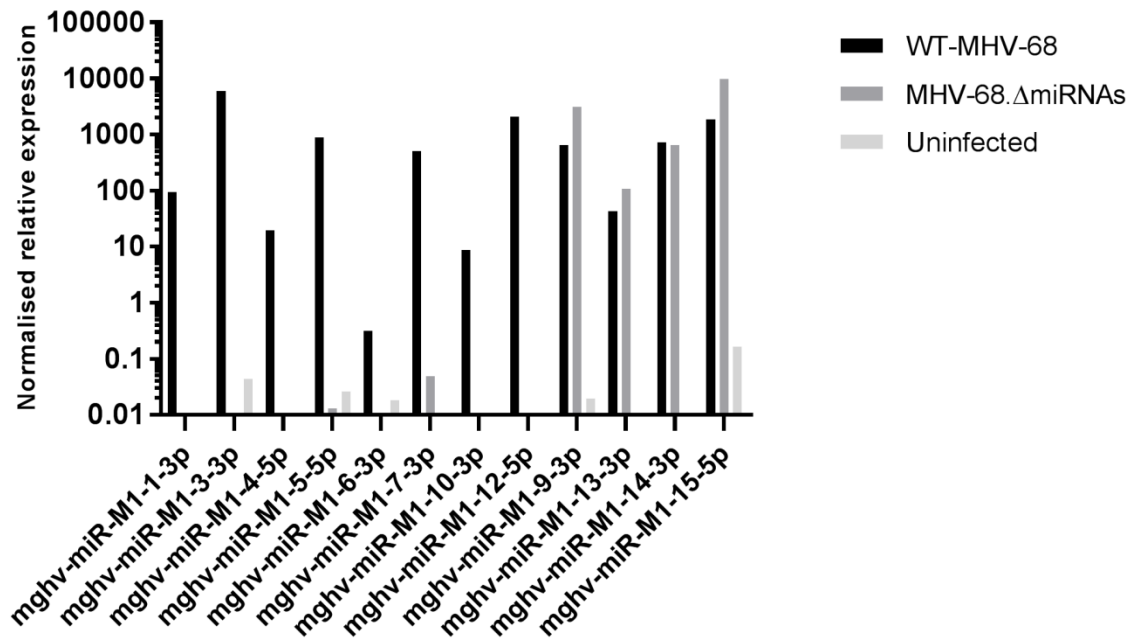
Figure 5.3 Analysis of BAC stability and miRNAs deletion.

(A) Agarose gel electrophoresis of restriction digests of the parental WT MHV-68 and the MHV-68.ΔmiRNAs BACs. BAC DNA was digested with either XhoI or EcoRI enzyme overnight at 37°C followed by analysis of the resultant DNA fragments on 0.8% agarose gel at 30 volt for 16 hours. (B) Agarose gel electrophoresis of the amplified PCR products that carries either WT miRNA sequence or miRNA sequence with deletion mutations. NIH 3T3 cells were infected with MHV-68.ΔmiRNAs virus or its wild-type counterpart at MOI of 5 for 24 hours. Genomic DNA (100 ng) from infected cells was used as a template. DNA extracted from uninfected cells was used as a negative control. NTC, no template control. Molecular size markers (M) are shown beside each gel. bp, base pairs. kb, kilobase pairs.

5.3 The MHV-68.ΔmiRNAs virus lacks the expression of the deleted miRNAs

In order to confirm that the miRNA mutations within the MHV-68.ΔmiRNAs genome eliminated the miRNAs expression, NIH 3T3 were infected with MHV-68.ΔmiRNAs or WT-MHV-68 viruses at MOI of 0.001 for 6 days. qRT-PCR was carried out for detection of virus encoded miRNAs (Figure 5.4A). The mutations resulted in elimination of the mature miRNAs expression in cells infected with MHV-68.ΔmiRNAs virus. The miRNAs expression in these cells was comparable to the background level in the uninfected cells. The melting curve analysis showed that the apparent expression of low levels of some deleted miRNAs in the MHV-68.ΔmiRNAs-infected cells and uninfected cells is due to non-specific products when compared to the melting curves of the WT-MHV-68-infected cells (Figure 8.3 in the appendix).

Notably, the intact miRNAs such as mghv-miR-M1-9-3p, mghv-miR-M1-13-3p, mghv-miR-M1-14-3p, and mghv-miR-M1-15-5p showed similar expression levels in cells infected with MHV-68.ΔmiRNAs or WT-MHV-68 virus (Figure 5.4A). qRT-PCR was also carried out for detection of mature MHV-68 miRNAs in spleen at 14 days following infection of BALB/c mice intranasally with 4×10^5 PFU of MHV-68.ΔmiRNAs or WT-MHV-68 viruses (Figure 5.4B). None of the deleted miRNAs were detected in splenocytes from the mouse infected with the MHV-68.ΔmiRNAs virus, whereas the intact miRNAs were expressed at levels comparable to the WT-MHV-68 virus.



(B)

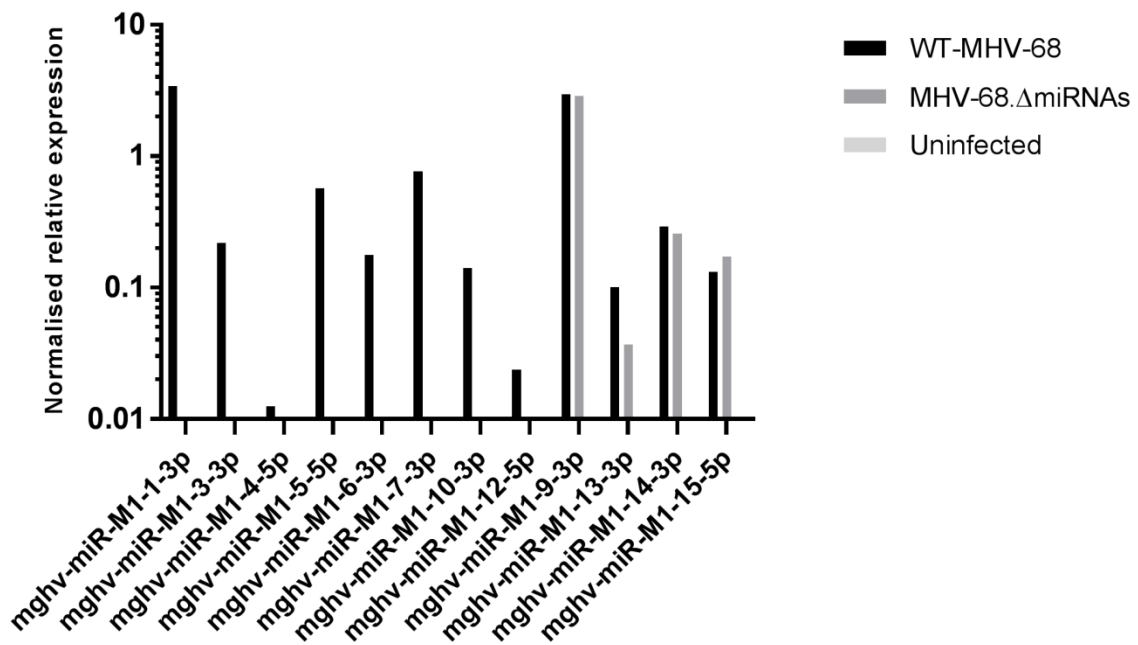
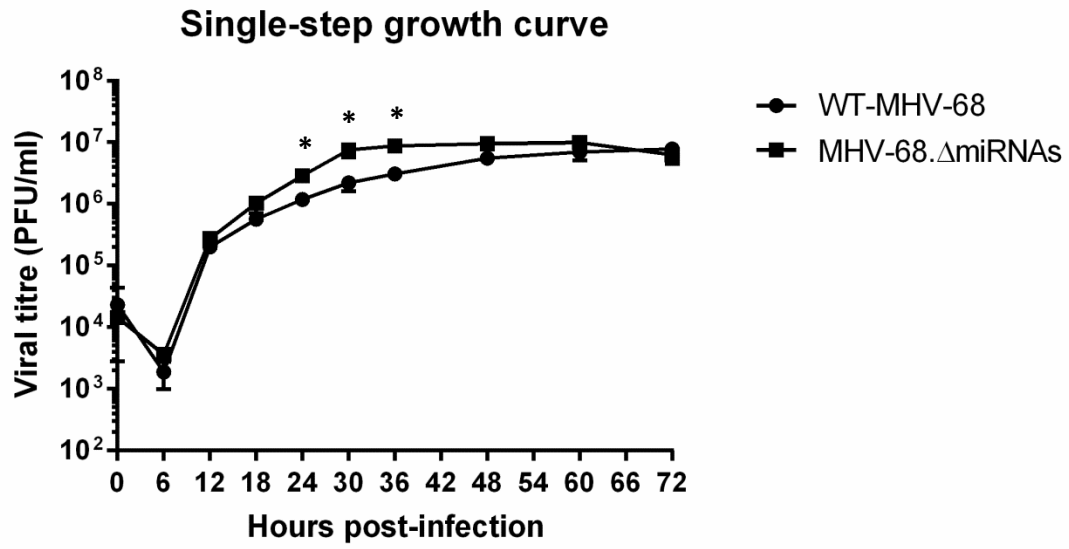


Figure 5.4 miRNA deletion mutations within the MHV-68.ΔmiRNAs virus genome resulted in elimination of miRNAs expression. (A) qRT-PCR for detection of mature miRNAs was carried out using RNA from NIH 3T3 cells infected with WT-MHV-68 or MHV-68.ΔmiRNAs viruses for 6 days at MOI of 0.001. Uninfected cells were used as a negative control. mmu-miR-191-5p was used as an endogenous cellular miRNA for normalisation. (B) qRT-PCR was performed for detection of MHV-68 miRNAs in spleens of BALB/c mice 14 dpi. The mice were infected intranasally with 4×10^5 PFU of WT-MHV-68 or MHV-68.ΔmiRNAs viruses. The viral miRNAs expression were normalised to the cellular miRNA mmu-miR-191-5p. Splenocytes from an uninfected mouse were used as a negative control.

(A)



(B)

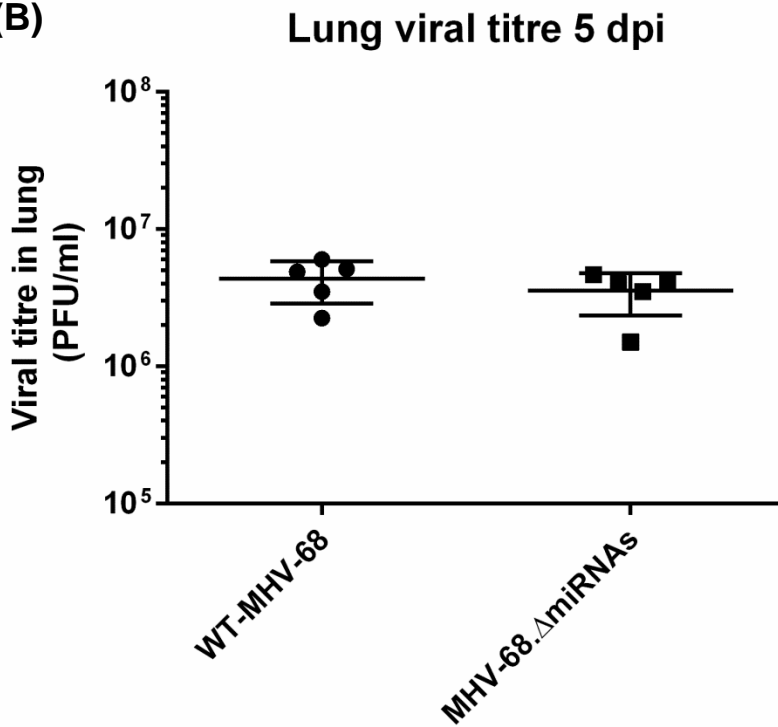


Figure 5.5 The deleted miRNAs are dispensable for virus lytic replication.

(A) Single-step virus replication growth curve was analysed by infecting NIH 3T3 cells at MOI of 5 with WT-MHV-68 or MHV-68.ΔmiRNAs viruses. Cells and supernatants were harvested at the indicated time-points post-infection and subjected to 3 cycles of freezing and thawing in order to disrupt the cells and release the virus. Viral titres were determined by plaque assay. The error bars represent SD ± mean of 2 experiments with 2 replicates per experiment. *, P<0.05 calculated by unpaired student's t-test. (B) Viral titres in the lung were measured by plaque assay 5 days after infecting BALB/c mice intranasally with 4×10^5 PFU with WT-MHV-68 or MHV-68.ΔmiRNAs viruses. Each symbol represents individual animal. The error bars represent SD ± mean.

5.4 The deleted miRNAs are not essential for lytic replication *in vitro* and *in vivo*

In order to determine whether the MHV-68 miRNAs play a role during productive infection, a single-step analysis of virus replication was carried out using NIH 3T3 cells infected with WT-MHV-68 or MHV-68.ΔmiRNAs virus at MOI of 5 for time intervals from 0-72 hours post-infection (Figure 5.5A). Viral titres were assessed by plaque assay. There was a slight but statistically significant increase in the titres of MHV-68.ΔmiRNAs virus as compared to that of the WT-MHV-68 virus during some intermediate time-points; however, the replication of the miRNA mutant virus was almost identical to the WT-MHV-68 virus at early time-points and during the peak of viral titres (Figure 5.5A).

This indicates that the deletion of the miRNAs did not interfere with the ability of the virus to replicate productively *in vitro*. In order to determine whether the miRNAs may alter virus replication *in vivo*, BALB/c mice were infected intranasally with 4×10^5 PFU WT-MHV-68 or MHV-68.ΔmiRNAs viruses. The viral titres in the lungs of infected mice 5 dpi were assessed using plaque assay (Figure 5.5B). It was found that the replication of the miRNA mutant virus was comparable to the WT virus in the lungs of the infected animals, indicating that the deleted miRNAs are not essential for lytic replication *in vivo* at this time-point post infection.

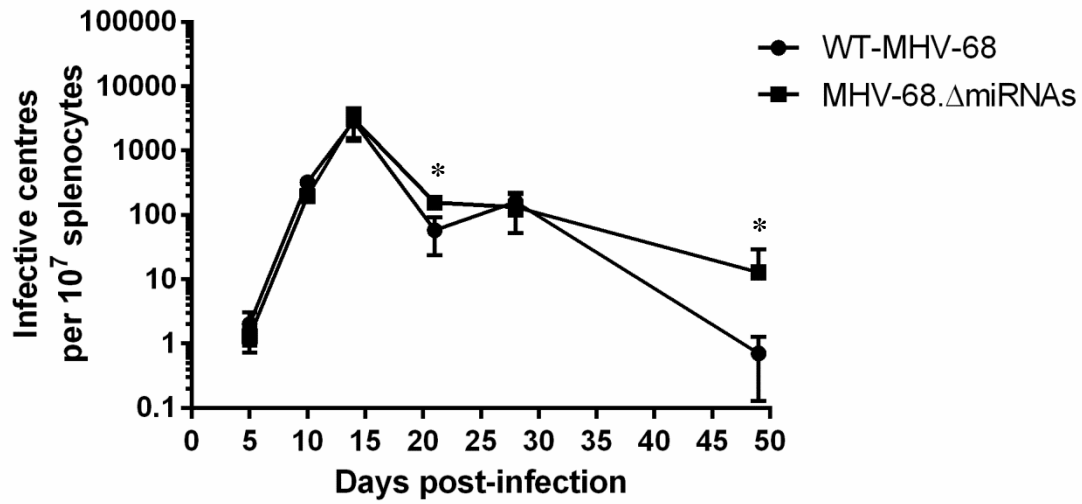
5.5 Investigating the potential functions of the MHV-68 miRNAs during the latent virus infection *in vivo*

In order to evaluate the MHV-68.ΔmiRNAs virus ability to establish, maintain, and reactivate from latency, splenocytes harvested at different time points during latency from mice infected with WT-MHV-68 or MHV-68.ΔmiRNAs viruses were subjected to parallel assays. These assays were aimed at determining the ability of the virus to reactivate from latency, detecting the presence of produced infectious virus, measuring the viral DNA genomic load, and measuring the level of viral lytic and latency-associated mRNA expression.

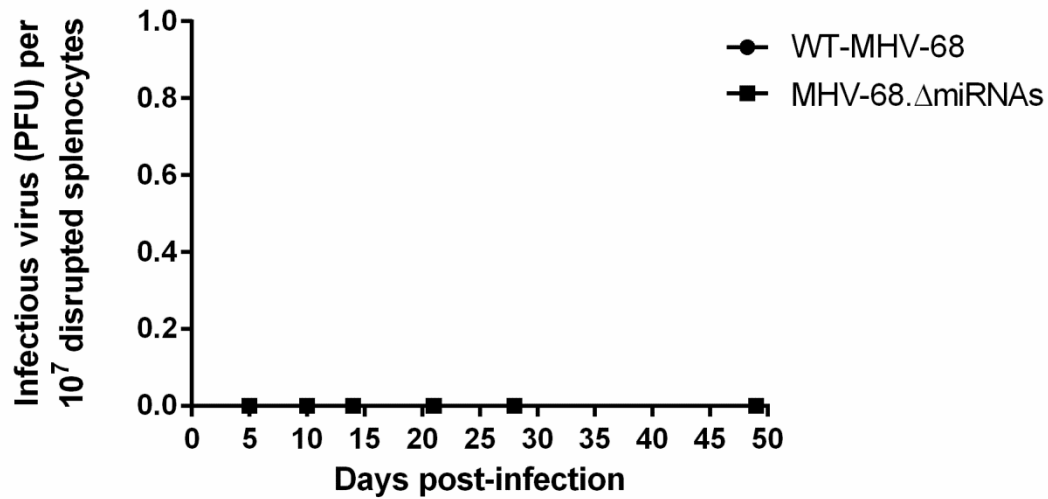
5.5.1 MHV-68 miRNAs are not essential for establishment of latency, but control the virus reactivation during late latency

In order to determine the ability of the MHV-68.ΔmiRNAs virus to reactivate from latency, infective centre assays were performed and spontaneous *ex-vivo* reactivation was measured. For this purpose, BALB/c mice were infected intranasally with 4×10^5 PFU of WT-MHV-68 or MHV-68.ΔmiRNAs viruses. Splenocytes were harvested at 5, 10, 14, 21, 28, and 49 dpi and were co-cultivated with BHK-21 cells for 5 days to allow virus reactivation from splenocytes. The number of infective centres (virus plaques) was determined by plaque assay as a measurement of the number of the cells containing reactivatable virus (Figure 5.6A). During early latent infection and the establishment of latency, the number of infective centres in splenocytes from MHV-68.ΔmiRNAs-infected mice was comparable to that of the WT-MHV-68-infected mice except at day 21 post infection when there was a slight (2.7-fold) but significant increase in the number of the infective centre with the MHV-68.ΔmiRNAs virus as compared to the WT-MHV-68 virus; however, this difference disappeared at 28 dpi (Figure 5.6A). Interestingly, at 49 dpi, during late latency, there was a statistically significant ($P=0.024$) increase in the reactivation level in the

(A)



(B)



(C)

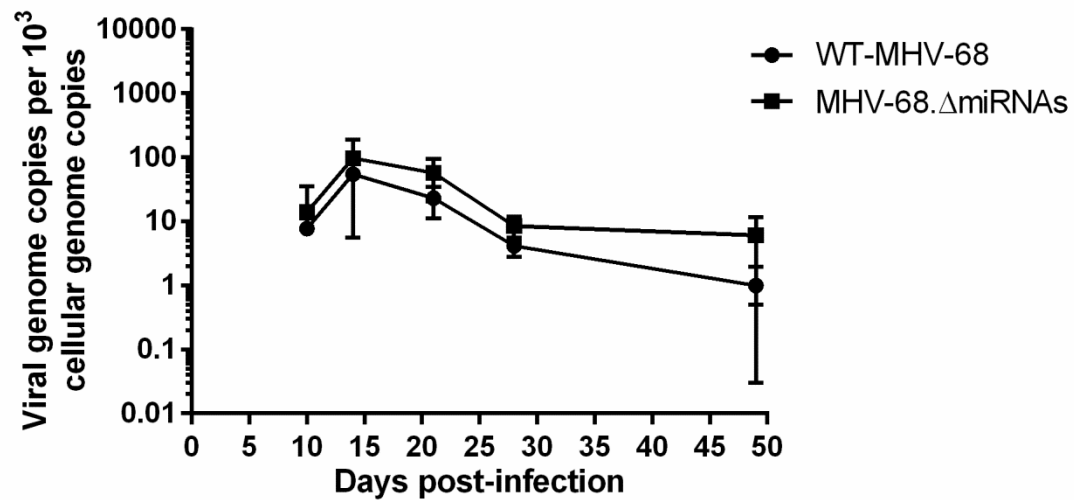


Figure 5.6 MHV- 68 miRNAs are not essential for establishment of latency, but can control reactivation during long-term latency.

BALB/c mice were infected with 4×10^5 PFU of MHV-68. Δ miRNAs virus or WT-MHV-68 virus intranasally. Four to five mice were used per group per time-point. (A) Infective centre assays were carried out using single-splenocyte suspensions at the indicated time-points post-infection. Different numbers of splenocytes (10^5 , 10^6 , and 10^7) from mice infected with WT-MHV-68 virus or the miRNA deletion mutant virus at the indicated time-points were co-cultivated with 10^6 BHK-21 cells for 5 days at 37°C as described in the materials and methods. The virus infective centres were counted and data are presented as infective centres per 10^7 infected splenocytes, Error bars represent mean values \pm SD. (B) The presence of produced infectious virus was determined by disruption of parallel splenocyte by 3 freeze-thaw cycles and adding an equivalent volume of 10^7 splenocytes to 10^6 BHK-21 cells for 4 days at 37°C before counting the virus plaques. (C) Viral DNA genomic load was determined at the indicated time-points by qPCR. Genomic DNA was extracted from infected splenocytes and 100 ng DNA was used as a template for quantification of the viral genome levels using ORF50 (RTA) primers. The data are presented as viral genome copy numbers normalised against 10^3 cellular genome copy numbers (SDHA primers). The error bars represent mean values \pm SD. The Statistically significant differences from the WT virus were determined by Mann-Whitney test and are indicated by an asterisk. *, $P < 0.05$.

splenocytes from mice infected with the MHV-68.ΔmiRNAs virus by approximately 18.2-fold higher than that of the mice infected with the WT-MHV-68 virus (Figure 5.6A). Parallel splenocyte samples were disrupted by 3 cycles of freezing and thawing and were used to measure the amount of any produced infectious virus (Figure 5.6B). Disruption of the splenocytes prevents the virus from reactivation without damaging the intact virus particles (Weck, Kim et al. 1999). No virus plaques were detected in samples from any group at any time-point (Figure 5.7B). In order to determine the viral DNA genomic load in the infected splenocytes, qPCR was carried out using DNA extracted from splenocytes from mice infected with WT-MHV-68 or MHV-68.ΔmiRNAs viruses at 10, 14, 21, 28, and 49 dpi (Figure 5.6C). There was no statistically significant difference between the viral genomic loads in the spleens of mice infected with the WT-MHV-68 virus or the MHV-68.ΔmiRNAs virus at any time-point; however, there was a trend towards higher viral genomic load in the mice infected with the miRNA mutant virus than those infected with the WT virus (approximately 6-fold difference). These results indicate that the MHV-68 miRNAs are not essential for the establishment of latency, but suggest they may play a role in controlling reactivation and promoting latency maintenance at later time-points (e.g. 49 dpi).

5.5.2 The lack of MHV-68 miRNAs resulted in increased levels of viral lytic gene expression during late latency

Because the *ex vivo* reactivation level of MHV-68.ΔmiRNAs virus was higher than that of the WT-MHV-68 virus at 49 dpi, it was important to compare the expression levels of the lytic genes of the two viruses at this time-point. During latency, particularly late latency, the viral gene expression is highly restricted and the basal

expression levels are usually below or close to the detection limit of the qRT-PCR assays (~ 32-35 cycles); therefore, accurate quantification by directly measuring the absolute mRNA copy numbers is difficult. In order to quantify viral gene expression at 49 dpi, the level of viral mRNA expression in splenocytes from mice infected with MHV-68.ΔmiRNAs or WT-MHV-68 were determined as fold change compared to the qRT-PCR background signal in splenocytes from uninfected mice (Figure 5.7). Expression levels of the immediate-early RTA, early DNA pol, and late ORF65 transcripts were determined (Figure 5.7A, B, and C). In addition, the expression levels of the latency-associated mRNA ORF73 (mLANA) and the M3 transcript were also determined (Figure 5.7D and E).

Strikingly, the expression level of the M3 transcript, that encodes a secreted broad spectrum chemokine-binding protein that is abundantly expressed during lytic infection and early latency (Parry, Simas et al. 2000, Rochford, Lutzke et al. 2001), in mice infected with MHV-68.ΔmiRNAs virus was 4.7-fold higher than the background level and 5.5-fold significantly higher than its expression in mice infected with the WT-MHV-68 virus (Figure 5.7E). Although the difference in expression levels of RTA, DNA pol, ORF65, and ORF73 between the mice infected with MHV-68.ΔmiRNAs or WT-MHV-68 viruses were not statistically significant, the abundance of these transcripts was always higher in the mice infected with the miRNA mutant virus. For example, in 2 mice infected with MHV-68.ΔmiRNAs virus, the expression level of ORF65 mRNA, a late transcript that is proposed to encode a capsid protein (Wu, Tong et al. 2001), was approximately 7- and 9-fold higher than the background level, whereas none of the mice infected with the WT-MHV-68 showed expression of ORF65 mRNA higher than the background level

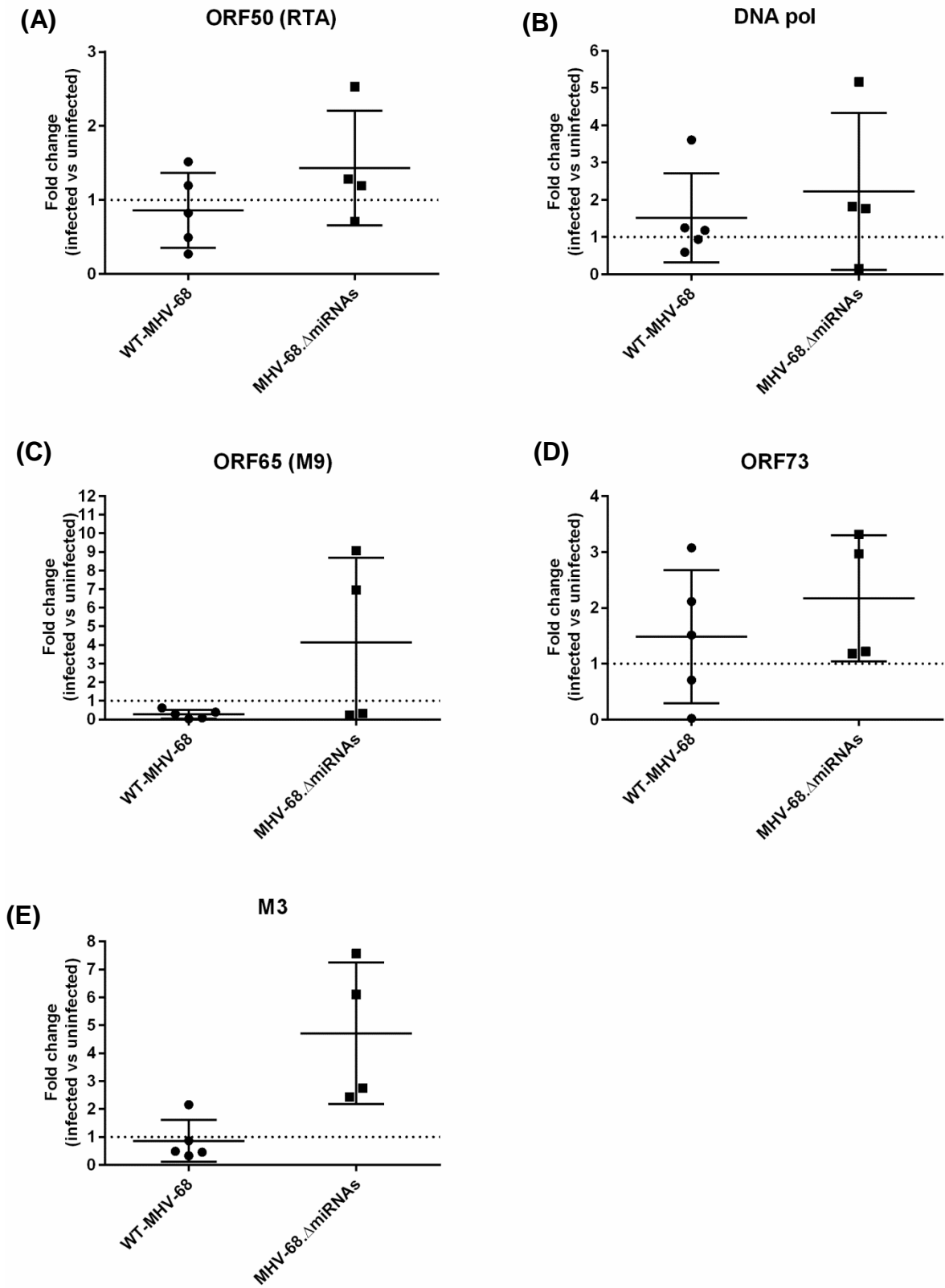


Figure 5.7 Expression of viral mRNAs at 49 dpi in the spleen.

qRT-PCR was carried out in order to detect the presence of lytic and latency-associated viral gene expression during late latency (49 dpi). RNA from splenocytes recovered from mice infected with WT-MHV-68 virus (n=5) or MHV-68.miRNAs virus (n=4) were extracted and 500 ng RNA were used for cDNA synthesis. qRT-PCR assays were carried out following 1:10 dilution of the cDNAs as described in Materials and Methods using primers specific for RTA (A), viral DNA pol (B), ORF65 (C), ORF73 (D), and M3 (E). qRT-PCR using RNA from splenocytes of an uninfected mouse was used to determine the CT values for any non-specific products produced. Data are presented as fold change of expression levels in infected samples as compared to the background level in the uninfected samples that is considered 1 (dotted line) based on the $2^{-\Delta\Delta CT}$ method. In cases when the qRT-PCR amplification curve did not cross the cycle threshold, the CT value was considered 40. The cellular reference mRNA, SDHA, was used as an endogenous control for normalisation of any experimental variability. Each symbol represents an individual animal. Error bars represent mean values \pm SD. Statistical significance was determined by Mann-Whitney test. *, $p < 0.05$.

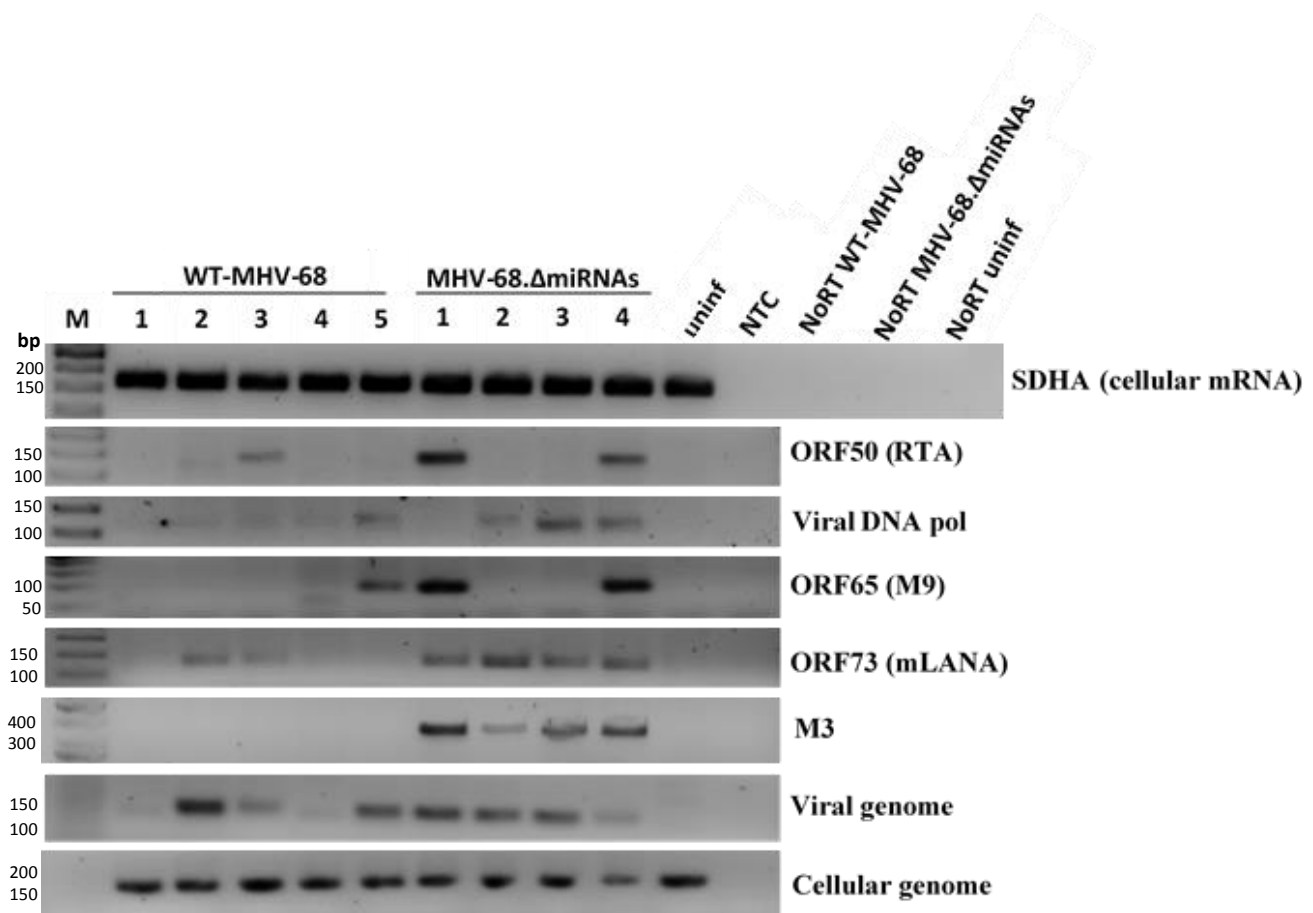


Figure 5.8 Viral mRNA expression and detection of viral genomic DNA during late latency (49 dpi) in the spleen.

qRT-PCR products of the assays described in figure 5.8 and qPCR products of the assays described in figure 5.7C (49 dpi) were analysed by agarose gel electrophoresis. qRT-PCR and qPCR reactions were carried out in triplicates in 20 μ l final volume per reaction. The qRT-PCR or qPCR products were pooled from the 3 reactions and 20 μ l were loaded on 3% NuSieve gel. The expected qRT-PCR product sizes are as follow: RTA (138 bp), ORF73 (129 bp), DNA pol (107 bp), ORF65 (84 bp), M3 (381 bp), and SDHA (157 bp). The numbers above the gel represent individual mice. M, marker. NTC, no template control. Uninf, uninfected. NoRT, no reverse transcriptase control. RNA aliquots from spleens from each group were pooled and 500ng RNA were used to synthesise the NoRT controls. Colour was inverted for clarity.

(Figure 5.7D). These experiments were done once and further work is needed to confirm these results and a larger number of animals may be required to establish statistically significant differences.

In order to confirm that the qRT-PCR CT values of the above mentioned viral transcripts were representing true qRT-PCR targets, the qRT-PCR products of these transcripts were analysed by agarose gel electrophoresis (Figure 5.8). The agarose gel electrophoresis analysis showed

the expected sizes of the qRT-PCR products for the mRNAs and it was consistent with the qRT-PCR relative quantification in terms of mRNAs abundance. These results indicate that the splenocytes from mice infected with the miRNA mutant virus were undergoing lytic cycle infection at 49 dpi and that the lytic genes were expressed at levels higher than the basal levels in the splenocytes from mice infected with the WT virus at 49 dpi. The lack of produced infectious virus at this time-point is most likely due to the fact that the level of lytic infection is very low and therefore beyond the limit of detection of the conventional plaque assay or because the majority of lytically infected cells would be rapidly eliminated by the immune system.

Increased reactivation of MHV-68 virus in the spleen was previously shown to be accompanied by increased virus replication in the lung (Gargano, Forrest et al. 2009). In order to determine whether the increased virus reactivation in the splenocytes from mice infected with MHV-68.ΔmiRNAs virus was accompanied by increase in the level of virus replication in the lung, the lungs were harvested from the mice infected with MHV-

68.ΔmiRNAs or WT-MHV-68 viruses and qPCR assays were carried out using genomic DNA extracted from these lungs for quantification of viral genomic loads. Interestingly, the MHV-68.ΔmiRNAs genomic load was significantly higher than that of the WT-MHV-68 by approximately 2.6-fold (Figure 5.9). Collectively, these results indicate that the MHV-68 miRNAs are important for controlling unwanted reactivation and initiation of virus lytic cycle during the maintenance of latency.

5.5.3 The lack of MHV-68 miRNAs did not alter viral mRNA expression during the establishment of latency in spleen at 14 dpi and as late as 28 dpi

In order to determine whether the MHV-68 miRNAs may change the viral mRNA expression pattern during the establishment of latency and later time-points of latency, qRT-PCR was performed on RNA extracted from mice infected with the miRNA mutant virus or its WT counterpart at 14, 21, and 28 dpi for quantification of RTA, ORF73, ORF65, and M3 transcripts (Figure 5.10). It was found that the differences between tested viral mRNAs expression levels in splenocytes from mice infected with the MHV-68.miRNAs or WT-MHV-68 viruses were not statistically significant at any time-point.

5.5.4 Investigating B lymphocyte activation and proliferation in spleens from mice infected with the MHV-68.ΔmiRNAs virus

In order to determine whether the MHV-68 miRNAs are involved in the virus-induced B cell activation in the spleen, BALB/c mice were infected with 4×10^5 PFU of MHV-68.ΔmiRNAs or the WT virus and spleens were harvested at 5, 10, 14, 21, or 28 dpi. Single-cell suspensions were prepared and B lymphocytes were labelled

with the anti-CD19 antibody and anti-CD69 antibody was used as a marker of B cell activation for the flow cytometric analysis (Figure 5.11). Mediastinal lymph nodes (MLN) were harvested from 3 mice at 5 dpi and the cells were pooled and used as a positive control for B cell activation. There were no significant differences in B cell activation between in B cells from mice infected with the miRNA mutant virus or the WT virus at any time-point post infection (Figure 5.12). The percentage of activated B cells (CD19+CD69+) was approximately 11% of total B cells in the MLN at 5 dpi (Figure 5.12). However, the percentage of activated B cells in spleens of wild type and MHV-68.ΔmiRNAs virus infected mice during the peak of viral latency (14 dpi) was approximately 1.8% of the total B cells analysed and was statistically non-significant relative to that of the uninfected (approximately 1.3 % of total B cells).

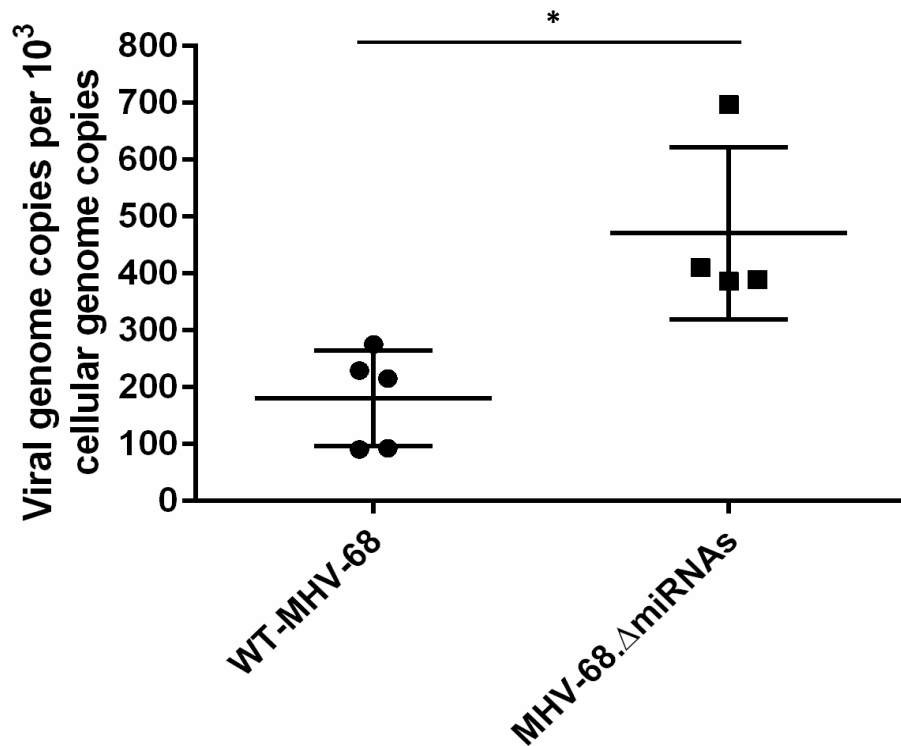


Figure 5.9 Viral DNA genomic load in the lung at 49 dpi.

DNA (100ng) extracted from lungs of mice infected with WT-MHV-68 or MHV-68.ΔmiRNAs viruses were used to quantify the viral genomic load by qPCR. The data are presented as viral genome copy numbers (RTA primers) per 10³ cellular genome copy numbers (SDHA primers). Error bars represent means \pm SD. Each symbol represents individual animal. The statistical significance was determined using Student's t test. *, $P < 0.05$.

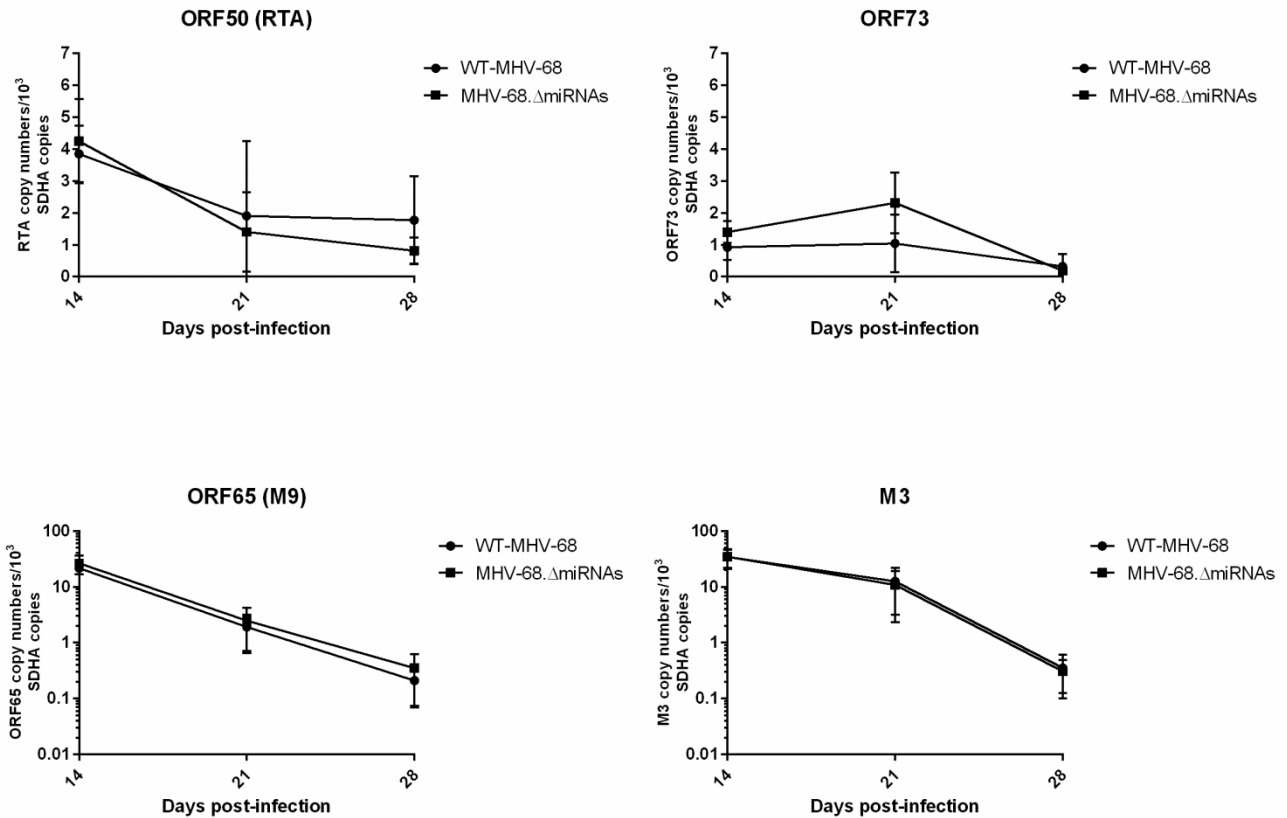


Figure 5.10 Viral mRNA expression during latent infection in the spleen.

qRT-PCR assays were carried out to quantify the expression of RTA, ORF73, ORF65, and M3 transcripts in the spleens of mice infected with WT-MHV-68 or MHV-68.ΔmiRNAs viruses at 14, 21, and 28 dpi. RNA (500ng) extracted from the splenocytes of infected animals were reverse transcribed into cDNA for the qRT-PCR. The data are presented as viral transcript copy numbers per 10³ endogenous transcript SDHA copy numbers. Error bars represent mean values ± SD. Five mice per group per time-point were used.

Published data indicate that expression of CD69 on B cells is significantly elevated compared to uninfected mice at this time point (Stevenson and Doherty 1999). This suggests that the CD69 antibody was not used at optimum concentration or under optimum conditions and casts doubt on the reliability of the data. Therefore further work is needed to evaluate the role of the miRNAs in B cell activation. MHV-68 miRNAs are not essential for the virus-induced B lymphocyte proliferation *in vivo*.

In order to determine whether the MHV-68 miRNAs may play a role in the virus-induced B cell proliferation in the spleen, BALB/c mice were infected with 4×10^5 PFU of the MHV-68. Δ miRNAs virus or the WT virus and spleens were harvested at 5, 10, 14, or 28 dpi. A single-cell suspension was prepared and B lymphocytes were labelled with the anti-CD19 antibody and anti-ki67 antibody which is a marker of cellular proliferation (Figure 13.A). There was a small but statistically significant increase in the B cell proliferation in mice infected with the MHV-68. Δ miRNAs virus as compared to mice infected with the WT virus at 14 dpi, this difference disappeared at 28 dpi and was not detected at the earlier time-points (Figure 5.13.B). The weight of the spleens was used as a measurement of splenomegaly (Figure 5.13.C). There were no statistically significant differences between the weights of the spleens from mice infected with the WT-MHV-68 or the MHV-68. Δ miRNAs viruses at any time-point. As splenomegaly is due to the increase in cell numbers of both T and B cells in the spleen it is possible that measurement of spleen weight is not sensitive enough to detect differences in B cell proliferation.

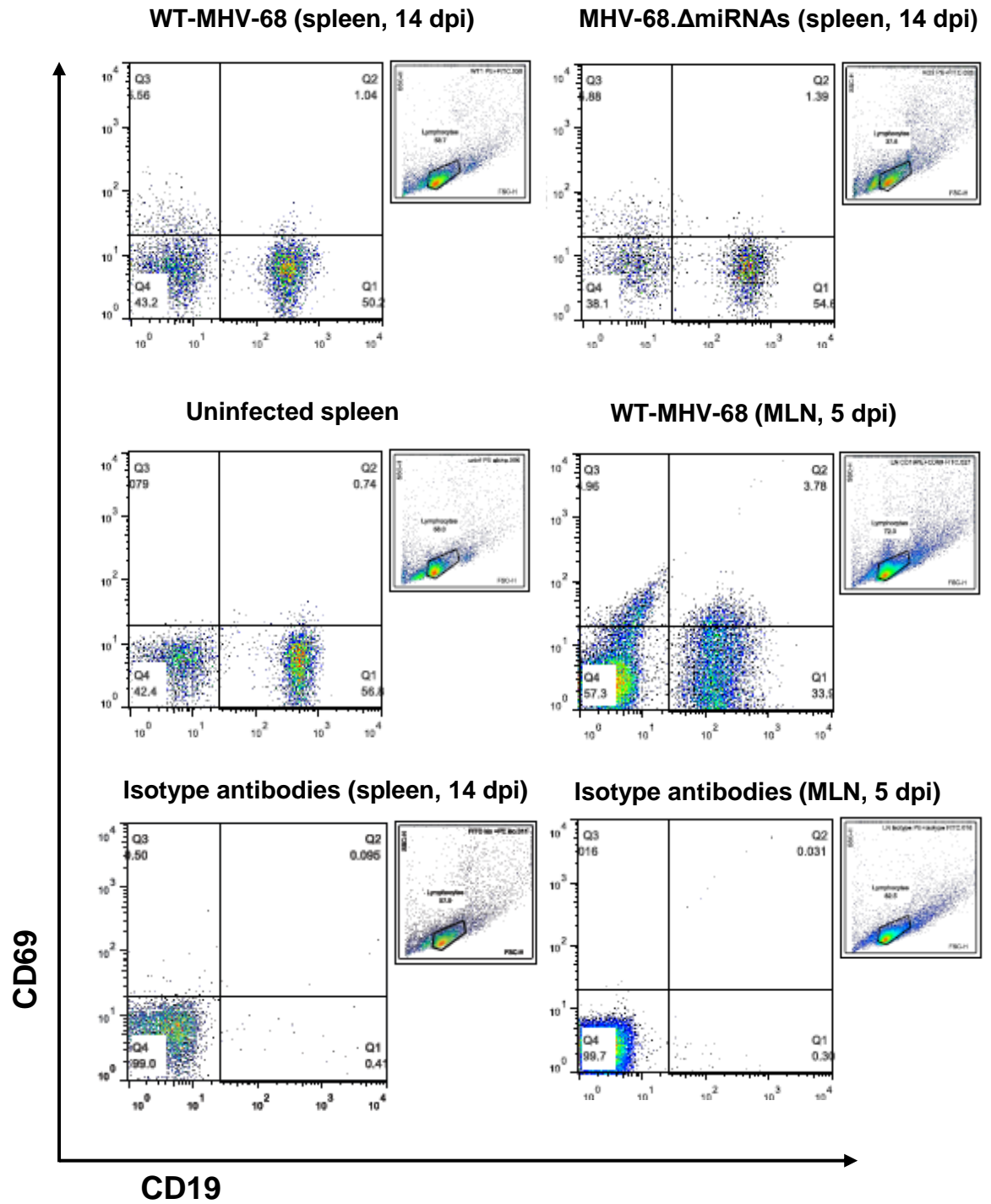


Figure 5.11 Representative flow cytometric analysis for measurement of B cell activation in mice spleens at 14 days following WT-MHV-68 or MHV-68.ΔmiRNAs infections (n=5).

Single-cell suspensions from spleens or lymph nodes from infected mice were labelled with anti-CD19-PE antibody (B cell marker) and anti-CD69-FITC antibody (B cell activation marker) for detection of activated B cells (CD19+CD69+) in the lymphocyte population. The small plots on the right side of the main plots show the gating for the lymphocyte population in single cell suspensions (boxed gates) based on forward and side scatter of laser light. Isotype antibodies were used with cells pooled from all the samples as a negative control for detection of non-specific background signals caused by the primary antibodies. Splenocytes from uninfected mice were used as a negative control for B cell activation (n=2). The mediastinal lymph nodes (MLN) from mice infected with WT-MHV-68 at 5 dpi were used as a positive control for B cell activation (n=3, pooled).

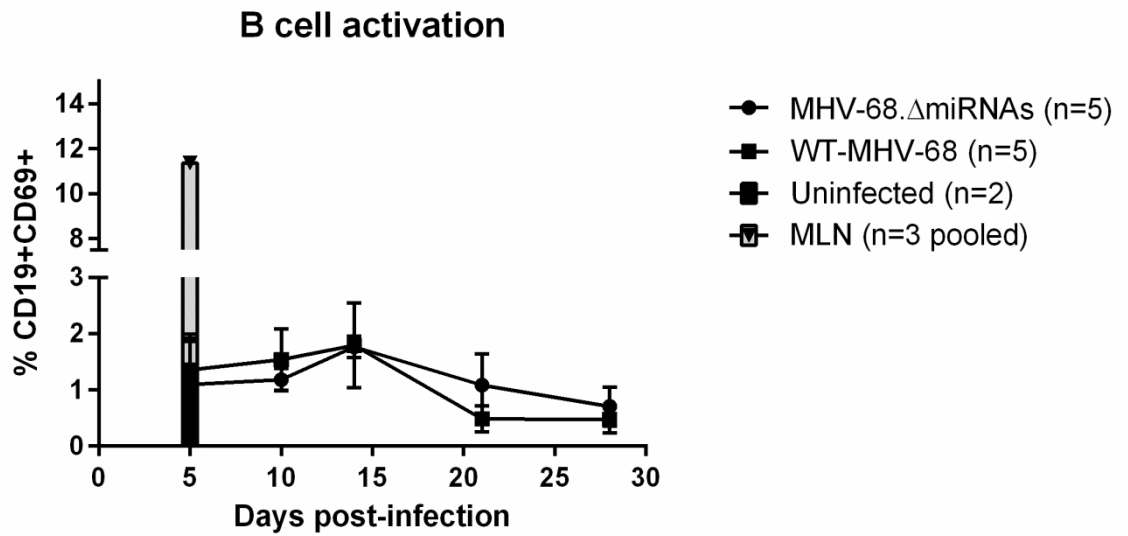


Figure 5.12 B cell activation in the spleen.

Expression of CD69 on CD19+ B cells as a measurement of B cell activation following infection of BALB/c mice with the miRNA mutant virus or its WT counterpart at the indicated time-points. The data represent the per cent of CD69+CD19+ cells of the total CD19+ B cells. Cells from mediastinal lymph nodes (MLN) of 3 mice infected with the WT-MHV-68 virus at 5 dpi were harvested at 5 dpi, pooled, and used as a positive control for B cell activation (grey bar). Cells harvested from spleens of uninfected mice were used as a negative control for B cell activation (black bar).

5.6 Discussion

MHV-68 provides a unique small animal model for studying the possible functions of gammaherpesvirus miRNAs during the natural course of infection. Such functions are difficult to assess in the human gammaherpesviruses due to the narrow host range of herpesviruses. In order to examine the potential roles of MHV-68 miRNAs during the virus life cycle, a recombinant virus lacking 9 pre-miRNAs was constructed using homologous recombination on the backbone of the MHV-68 wild-type BAC. The miRNAs expression was successfully abolished and the miRNAs were not detected by qRT-PCR in NIH 3T3 fibroblasts and in splenocytes from mice infected with the miRNA mutant virus. In order to determine the potential roles of MHV-68 miRNAs in the virus life cycle, a series of *in vitro* and *in vivo* assays were carried out.

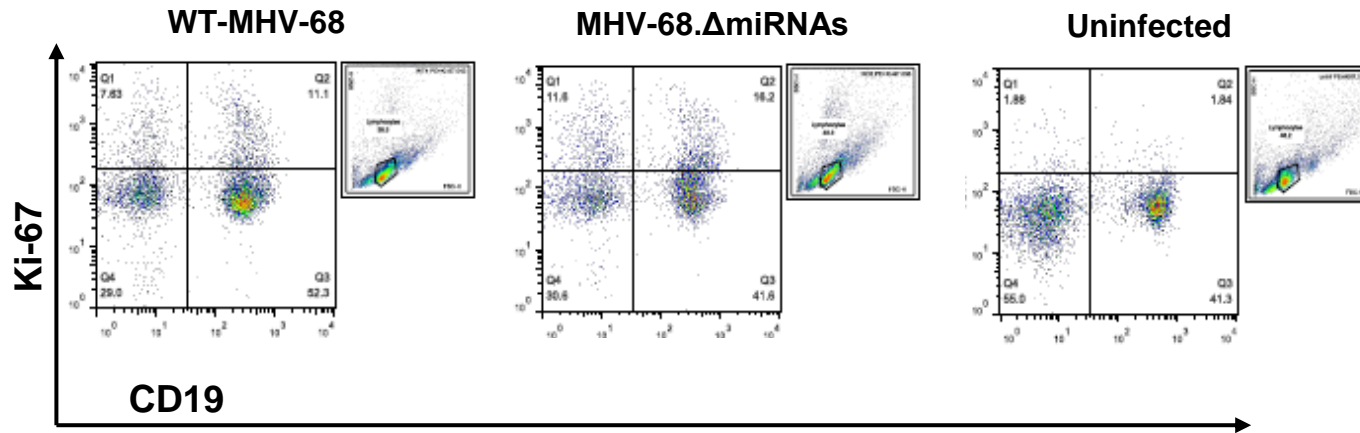
The MHV-68.ΔmiRNAs virus replicated productively *in vitro* to levels that are similar to that of the WT virus; however, there was minor but statistically significant increase in MHV-68.ΔmiRNAs virus titres in some intermediate time-points in the single-step growth curve. The exact reason for that is not clear, but it is possible that there was increased MHV-68.ΔmiRNAs virus release at these time-points possibly because the miRNAs may play roles in restricting lytic infection. During *in vivo* lytic infection in the lung, the MHV-68.ΔmiRNAs virus replicated to levels comparable to that of the WT virus. These results indicate that the MHV-68 miRNAs are not essential for the lytic replication.

It was found that the lack of MHV-68 miRNAs did not alter the ability of the virus to establish latent infection *in vivo*. In addition, the *ex vivo* reactivation of the

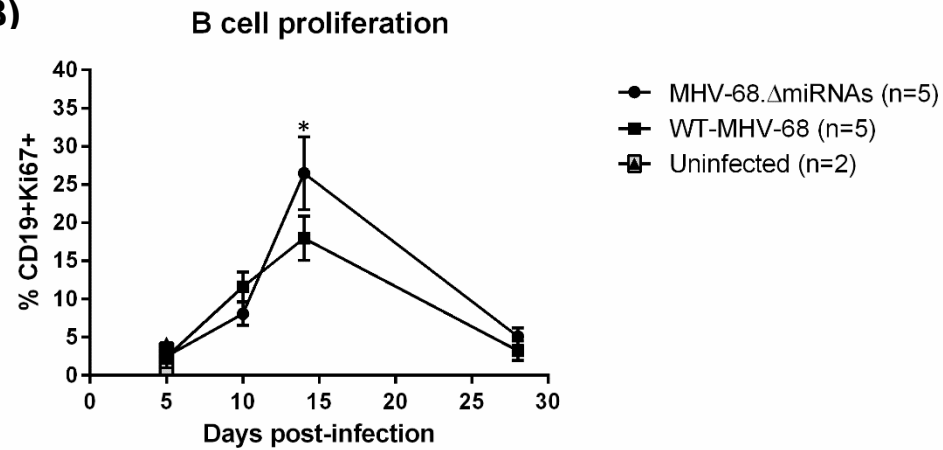
splenocytes infected with the MHV-68.ΔmiRNAs virus was comparable to that infected with the WT virus during early latency and at 28 dpi and the viral genomic loads of both viruses were similar at these splenocytes infected with the MHV-68.ΔmiRNAs virus was comparable to that infected with the WT virus during early latency and at 28 dpi and the viral genomic loads of both viruses were similar at these time-points. However, during late latency (7 weeks post-infection), the reactivation level of the MHV-68.ΔmiRNAs-infected splenocytes was significantly higher ($P=0.024$) than the WT-MHV-68-infected splenocytes.

The mRNA levels of RTA, DNA pol, ORF65, and ORF73 in the splenocytes from mice infected with the MHV-68.ΔmiRNAs were higher than that of the mice infected with the WT MHV-68 at 49 dpi, although statistically non-significant. Notably, M3, which is an early-late transcript that encodes a secreted chemokine-binding protein (Parry, Simas et al. 2000, Rochford, Lutzke et al. 2001, Ebrahimi, Dutia et al. 2003), was the most highly expressed transcript among the tested viral mRNAs during MHV-68.ΔmiRNAs virus infection at 49 dpi, whereas its expression was below level of detection during the WT-virus infection at the same time-point. It has been previously shown that the 600 bp of the sequence upstream of the M3 TATA box contain an RTA responsive element and that M3 is regulated by RTA in the absence of other viral factors (Martinez-Guzman, Rickabaugh et al. 2003). The same study also showed that M3 was one of the most highly expressed transcripts in BHK-21 cells infected with a recombinant MHV-68 virus overexpressing RTA. This may explain why M3 was the transcript whose expression increased the most at 49 dpi in the splenocytes from mice infected with the MHV-68.ΔmiRNAs virus, which were undergoing an increased level of reactivation and lytic cycle progression.

(A)



(B)



(C)

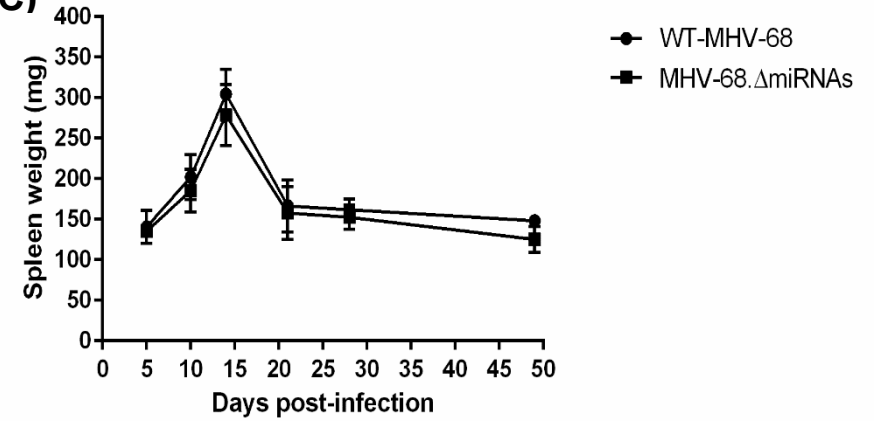


Figure 5.13 B cell proliferation in the spleen.

(A) Representative flow cytometric analysis for measurement of B cell proliferation in mice spleens at 14 days following WT-MHV-68 or MHV-68.ΔmiRNAs infections (n=5). Single-cell suspensions from spleens of infected mice were labelled with anti-CD19-PE antibody and anti-ki67 antibody (B cell proliferation marker) for detection of proliferating B cells (CD19+ki67+) in the lymphocyte population. The small plots on the right side of the main plots show the gating for the lymphocyte population in single cell suspensions (boxed gates). Splenocytes from uninfected mice were used as a negative control for B cell proliferation (grey bar, n=2). (B) Measurement of B cell proliferation using anti-ki-67 antibody for labelling proliferating CD19+ B cell following mice infection at the indicated time-points with either MHV-68.ΔmiRNAs or WT-MHV-68 viruses. Data represent the per cent of CD19+ki67+ cells of the total CD19+ cells. P-value was determined by Student's t test. *, P<0.05. (C) BALB/c mice were infected intranasally with 4×10^5 PFU of WT-MHV-68 or MHV-68.ΔmiRNAs viruses. Spleens were harvested and weighed at the indicated time-points. Data are represented as mean \pm SD. Four to five mice were used per group per time-point.

In support of this, the results also show an increase in the MHV-68.ΔmiRNAs viral genomic load. No infectious virus was detected by plaque assay in these spleens but this may be because the levels are too low to be detected by this method or because the immune system had rapidly cleared the lytically infected cells. Taken together, the increased level of reactivation and the higher levels of lytic mRNA expression support the hypothesis that the miRNAs play a role in control of latent-lytic switch at this late time point. This finding is consistent with a recently published study in which an MHV-68 lacking all the miRNAs and vtRNAs showed higher viral genomic load in the spleen at 42 dpi by qPCR; however, the authors could not detect *ex-vivo* reactivation (Steer B *et al.*, 2016).

Interestingly, when the viral genomic loads in the lungs from mice infected with either the MHV-68.ΔmiRNAs virus or its WT counterpart virus at 49 dpi were determined, there was significant increase in MHV-68.ΔmiRNAs genome load compared to that of the WT-virus. It is not clear whether the reactivating virus in the spleen re-infected the lung or the latently infected cells in the lung underwent reactivation. Measuring the level of infectious virus, if any, and determining the viral mRNA expression profiles in the lung at this time-point could be helpful in gaining further insight into this. Given the higher reactivation rate and the presence of lytic transcripts at higher levels during MHV-68.ΔmiRNAs infection than during WT-virus infection in spleen at 49 dpi, it is reasonable to assume that the miRNAs can function as control valves that prevent precocious reactivation and control exit from long-term latency.

When the expression levels of the viral mRNAs, ORF50, ORF73, ORF65, and M3, were determined at earlier time-points than 7 weeks post-infection such as 14, 21, and 28 dpi in the splenocytes from mice infected with the miRNA mutant or WT viruses, there were no significant differences in the expression of these mRNAs at any time-point. Although, the similar mRNA levels does not guarantee similar protein levels because the miRNAs may target mRNAs via translational repression rather than mRNA degradation, the absence of a prominent phenotype at these time-points suggests that either the tested mRNAs are not targeted by the miRNAs that were deleted from the MHV-68.ΔmiRNAs virus or the miRNA-mediated translational inhibition, if any, is too slight to cause a pronounced phenotype.

One of the prominent features of MHV-68 infection is the virus-induced B lymphocyte proliferation. It has been shown that EBV-encoded miRNAs can promote cell cycle progression and induce proliferation of primary human B cells (Seto, Moosmann et al. 2010, Vereide, Seto et al. 2014). In this study, the B cell proliferation was examined in splenocytes of mice infected with the WT or the miRNA mutant virus during various time-points of latency. It was found that the deleted miRNAs are dispensable for the virus-induced B cell proliferation *in vivo* at days 5, 10, and 28 post infection; however, there was slight but statistically significant increase in B-cell proliferation in splenocytes from mice infected with the miRNA mutant virus as compared to that of the WT virus at 14 dpi. This is a surprising finding because it was thought that the MHV-68 miRNAs may play a role in enhancing B cell proliferation in a manner similar to that of the EBV miRNAs (Seto, Moosmann et al. 2010). miRNAs from the same virus can have contradictory functions. For example, EBV-miR-BART15-3p has been shown to inhibit cell proliferation and to induce apoptosis in a gastric cancer cell line, whereas the

majority of the BART miRNAs promote proliferation in this cell line (Choi, Lee et al. 2013), but the total effect of the EBV miRNAs is towards increased cell proliferation. It is possible that one or more miRNAs that were deleted in the MHV-68.ΔmiRNAs virus may play a role in inhibiting B cell proliferation in a manner similar to that of EBV-miR-BART15-3p miRNA. In the MHV-68.ΔmiRNAs virus, 9 miRNAs were deleted while the other 6 miRNAs were intact and this may have slightly shifted the total effect towards more B cell proliferation in the mice infected with this mutant virus. It is important to note that due to time constraints the *in vivo* experiments using the MHV-68.ΔmiRNAs virus were carried out once and further work is needed to confirm the role of the miRNAs during the latent infection.

Whilst it is tantalising that the miRNA mutant virus displayed a hyper-reactivation phenotype at 49 dpi, a revertant virus will be needed in order to confirm that this phenotype is due to the miRNAs deletions and not any other random mutation within the virus genome. Alternatively, a whole-genome high-throughput sequencing of the MHV-68.ΔmiRNAs genome could confirm that the MHV-68.ΔmiRNAs genome does not carry any unintended mutations. When the leftmost end of the original BAC derived WT MHV-68 genome was sequenced, it was found that the first 56 bp are missing. It would therefore be useful to use WT-MHV-68 field strain as an additional control in future experiments.

Chapter 6: Discussion

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6 Chapter 6: Discussion

6.1 Expression of MHV-68 encoded miRNAs

To establish a qRT-PCR platform for detection and quantification of the MHV-68 mature miRNAs expression during latency *in vivo*, expression of a defined set of previously confirmed or predicted mature miRNAs was first determined in cells lytically or latently infected *in vitro*. In this study, it was shown that the pattern of miRNAs expression depends on both the phase of infection and the infection model employed. For instance, mghv-miR-M1-9-3p was among the least abundant miRNAs during lytic infection NIH 3T3 cells, but it was one of the most abundant miRNAs during latent infection in NS0 cells and in splenocytes from infected mice, suggesting that this miRNA may play a more fundamental role during latent infection. Although mghv-miR-M1-14-3p was one of the least expressed miRNAs in latently infected NS0 cells, it was one of the highly expressed miRNAs in splenocytes, suggesting that cell lines are not necessarily valid models for characterisation of miRNAs that are expressed *in vivo*. These differences can be due to differential processing of the miRNAs transcripts. This is consistent with the presence of post-transcriptional regulatory pathways that control cell type-specific maturation of viral and cellular miRNAs (Dolken, Perot et al. 2007, Michlewski, Guil et al. 2008). In addition, the stability of the miRNAs can be influenced by the protection by the RISC Argonaute proteins and the presence of the target mRNAs (Chatterjee and Grosshans 2009, Kai and Pasquinelli 2010), which may be present at levels that are different between NS0 cells and splenocytes.

All the tested miRNAs were detected as early as 2 hpi except mghv-miR-M1-4-5p, indicating that the miRNAs are transcribed early after infection. This is consistent with previous published studies that demonstrated the immediate early transcription kinetics of the vtRNAs, which contain RNA pol III promoters that drive the miRNAs expression (Ebrahimi, Dutia et al. 2003, Marques, Efstathiou et al. 2003, Pfeffer, Sewer et al. 2005, Cliffe, Nash et al. 2009). It can also be speculated that MHV-68 miRNAs may be packaged within the virion particles in a similar way to that of the vtRNAs (Cliffe AR et al., 2009). Although MHV-68 miRNAs are thought to play a major role during latency, the detection of MHV-68 miRNAs early after lytic infection suggests a role played by these miRNAs during this stage of infection. This role is not yet clear. It is possible that the miRNAs can counteract innate immunity immediately after infection by targeting signaling pathways involved in cellular sensing of viral DNA.

Interestingly, there was a large variation in the expression levels of individual miRNAs within the same cell type. For example, the expression level of the most abundant miRNA in infected NIH 3T3 cells, mghv-miR-M1-7-3p, was approximately 10,400-fold higher than the expression level of the least abundant miRNA, mghv-miR-M1-4-5p in the same cell line. The expression level of the most abundant miRNA, mghv-miR-M1-1-3p, in infected NS0 cells and splenocytes from infected mice was approximately 4,460- and 68-fold higher than that of the least abundant miRNA, mghv-miR-M1-4-5p, respectively. This large variation in miRNAs expression levels could be due to differences in the stability of individual miRNAs within the same cell type and/or variation in the efficiency of miRNA maturation from the primary transcript.

Interestingly, miRNAs that are associated with the same vtRNA and are transcribed by from the same RNA pol III promoter showed different expression profiles. For example, the expression level of mghv-miR-M1-1-3p, which is processed from a primary miRNA that is associated with vtRNA1, was 331-, 450-, and 11-fold higher than that of mghv-miR-M1-10-3p, which is processed from a primary miRNA that is associated with the same vtRNA, in NIH 3T3 cells at 24 hpi, NS0 cells at 5 dpi, and splenocytes at 14 dpi respectively. This suggests that these miRNAs may undergo regulation at the post-transcriptional level.

While this work was in progress, Feldman and colleagues described a similar quantitative study of MHV-68 miRNAs expression in a number of cell lines and in splenocytes from infected mice (Feldman, Kara et al. 2014). Their overall conclusions are broadly similar to those of the present work. For example, they showed that the MHV-68 miRNAs expression is dependent on cell type and stage of infection and that the individual miRNAs expression levels varied widely within the same cell line. The authors also showed that the expression of the majority of the 17 tested mature miRNAs was induced after lytic infection of NIH 3T12 fibroblasts with peak of expression at 16, 24, or 48 hpi.

Despite these similarities, there were a number of discordant results between the two studies. For instance, mghv-miR-M1-7-3p and mghv-miR-M1-1-3p were the most abundant miRNAs during NIH 3T3 fibroblasts lytic infection in the current study, whereas they were among the least abundant miRNAs during NIH 3T12 fibroblasts lytic infection in their study. In addition, although the mghv-miR-M1-1-3p and mghv-miR-

M1-9-3p were the most highly expressed miRNAs in splenocytes from infected mice at 14 dpi in the present study, they were among the least abundant miRNAs in splenocytes from infected mice at 16 dpi in their study. These differences are likely due to the different qRT-PCR platforms used in these studies. The TaqMan stem loop PCR assays were used in their study for quantification of the mature MHV-68 miRNAs, whereas the poly (A) tailing followed by universal RT reaction and qPCR assays were used in the present study for this purpose. The stem-loop PCR assays cannot detect previously unpublished alternate mature miRNAs sequences and miRNA isoforms (isomiRs) that may be present as a result of post-transcriptional modification. In contrast, the SYBR green qRT-PCR assays can detect previously unpublished alternate mature miRNAs sequences, particularly 3' isomiRs. It is also likely that the different mouse strains used in the 2 studies (C57BL6/J mice in Feldman study and BALB/c mice in the current study) may lead to differences in miRNAs expression levels; possibly as a result of differential miRNAs processing between the 2 mouse strains.

6.2 MHV-68 miRNAs and *in vitro* reactivation

PMA is a potent inducer of MHV-68, KSHV, and EBV reactivation in latently infected cells through induction of protein kinase C activity and RTA expression (Davies, Grand et al. 1991, Renne, Zhong et al. 1996, Miller, Heston et al. 1997, Zhu, Cusano et al. 1999, Wang, Wu et al. 2004, Forrest and Speck 2008). Interestingly, the expression of the latency associated ORF73 mRNA was induced after PMA treatment, which is consistent with a previous finding that ORF73 expression was induced following PMA-induction in latently infected murine B cell line (Forrest and Speck 2008). In support of

these findings, Forrest and colleagues (Forrest, Paden et al. 2007) found that an ORF73-null MHV-68 virus was deficient in lytic infection in murine fibroblasts and in lungs from infected mice, indicating a role played by mLANA protein during lytic infection. The same study proposed that this role may be played through the mLANA dependent regulation of p53 transcription factor that enhances MHV-68 gene expression and lytic replication. It is therefore likely that mLANA may play similar roles following PMA-induced reactivation of latently infected NS0 cells.

The expression kinetics of the MHV-68 encoded miRNAs were evaluated following PMA-induced virus reactivation in NS0 cells. Determination of the ratio of the miRNAs expression level to the viral genome level over time following reactivation revealed that a number of the miRNAs was down-regulated, while others were up-regulated after the PMA-induced reactivation, indicating that the expression of the MHV-68 miRNAs is differentially regulated during virus reactivation *in vitro* and that the miRNAs may have various roles in modulating virus reactivation.

6.3 The expression of MHV-68 miRNAs is independent of virus DNA replication

There has been evidence that the MHV-68 vtRNAs that are associated with the miRNAs have immediate-early expression kinetics (Ebrahimi, Dutia et al. 2003). The MHV-68 mature miRNAs could be detected as early as 2 hpi during lytic infection in NIH 3T3 cells (chapter 3). In the present study, an antiviral thionucleoside analogue that can inhibit MHV-68 viral DNA replication without interfering with viral latency, 4'-S-EtdU

(Barnes, Dyson et al. 1999), was used to inhibit productive viral DNA replication in MHV-68-infected NS0 cells followed by evaluation of viral mRNAs and miRNAs expression. Although the NS0 cell line supports MHV-68 latent infection, approximately 5 % of the infected cells undergo reactivation and lytic gene expression (Nash, Dutia et al. 2001).

It was found that the miRNAs expression levels in the 4'-S-EtdU-treated and untreated MHV-68 infected NS0 cells at 48 hours post-treatment were similar, indicating that the expression of the MHV-68 miRNAs is independent of viral DNA synthesis. It is therefore plausible to suggest that these miRNAs may play roles during latent virus infection when the viral DNA replication is highly restricted. It is important to note that NS0 cells predominantly support latent infection and there are only approximately 5% of the cells that may undergo lytic replication. It is possible that the inhibition of lytic replication in infected NS0 cells by 4'-S-EtdU may not be sufficient for detecting differences in miRNAs expression due to the small proportion of lytically infected cells. However, the decrease of viral genomic load level and the late transcript, ORF65, expression level following 4'-S-EtdU treatment of infected NS0 cells indicates that this approach can be valid.

6.4 Kinetics of MHV-68 mature miRNAs expression during latency *in vivo*

A recombinant MHV-68 virus that carries a β -lactamase gene fused to ORF73, MHV-68. ORF73 β la, was used to detect and isolate latently infected spleen cells (β -lactamase

positive/mLANA positive) *in vivo* by flow cytometry, allowing for assessing the expression levels of the viral miRNAs in these cells. Although, this virus was shown to establish latency at comparable levels to that of the wild-type virus (Nealy, Coleman et al. 2010). In the current study, MHV-68.ORF73 β la latent genomic load and splenomegaly were significantly lower than that of the wild-type virus.

Given the latency attenuation of MHV-68.ORF73 β la virus and the difficult, albeit successful, recovery of enough mLANA+ cells for the qRT-PCR analyses at day 14 and 21 post infection, it was thought that the attenuation of MHV-68.ORF73 β la virus latency would hamper the assessment of viral miRNAs expression levels at time-points later than 21 dpi because the viral genomic load drops dramatically over time after establishment of latency and it was expected that the frequency of infected splenocytes from mice infected with MHV-68.ORF73 β la virus would be lower than those of the wild-type virus. Therefore, the WT-MHV-68 virus was used to evaluate the expression kinetics of MHV-68 miRNAs at 10, 14, 21, and 32 dpi during latency in the spleen.

At the early stage of latency (10 dpi), MHV-68 miRNAs were expressed at high levels as compared to day 14 and 21 post-infection. It can be speculated that the MHV-68 miRNAs are expressed at high levels during this early stage of latency in order to help establish latent infection by shutting down lytic viral gene expression and possibly by down-regulating cellular gene expression involved in supporting lytic infection. The MHV-68 miRNAs expression was down-regulated over time (between 10 and 21 dpi) possibly to allow for some level of reactivation and spread of virus to the adjacent uninfected cells. At a later stage of latency (between 21 and 32 dpi) the miRNAs expression levels increased again possibly to inhibit unwanted lytic reactivation and to

maintain cell survival. This explanation is consistent with the finding that splenocytes from mice infected with MHV-68.ΔmiRNAs virus exhibited higher *ex-vivo* reactivation than the WT-MHV-68 (section 5.5.1).

The increase of miRNAs abundance after 21 dpi can possibly be due to selective expression of the miRNAs in certain splenocyte subpopulations whose distribution increases over time. It was previously reported that the MHV-68 latent gene transcription is dependent on the differential stage of B cells and the spleen cell type and that the MHV-68 genome was only detected in newly formed and germinal-centre B cells beyond 21 dpi (Marques, Efstathiou et al. 2003). Another study which investigated the expression of the latency associated mLANA protein in mature B cell subsets (naïve follicular, germinal-centre, and memory B cells) throughout a 90-day infection course showed that the memory B cells constituted the vast majority of mLANA-expressing cells at 16 dpi, whereas the germinal-centre B cells represented the majority of the mLANA-expressing cells from 28 dpi to 90 dpi and that the naïve follicular B cells constituted a small proportion of the mLANA-expressing cells at all the time-points (Nealy, Coleman et al. 2010).

It can be speculated that the MHV-68 miRNAs act as fine-tuners of gene expression during different stages of virus latency. It is also suggested that the regulation of MHV-68 miRNAs expression is dependent on the stage of latent infection and that the day 32 post infection constitutes a previously uncharacterised important stage for establishing a long-term latency. The latter suggestion is reminiscent of the observation that the highest frequency of genome-positive cells expressing the latency-associated mLANA protein occurred at 28 dpi in a 90 day-infection course experiment (Nealy, Coleman et al. 2010).

6.5 MHV-68 encoded miRNAs are dispensable for productive virus replication

Since the discovery of viral miRNAs, the assessment of their functions has been a field of extensive research, particularly in the herpesvirus field. However, few studies have investigated these functions in natural host. The engineered miRNA deletion mutant viruses, lacking the miRNAs expression, provide a very powerful tool to examine viral miRNAs function both *in vitro* and *in vivo*. The first *in vivo* study of a phenotype for a recombinant miRNA mutant virus revealed that a miRNA mutant murine cytomegalovirus, lacking 2 viral miRNAs, exhibited virus production attenuation in the salivary glands of the infected mice (Dolken, Krmpotic et al. 2010), highlighting the importance of the natural host infection model. MHV-68 provides an authentic natural host-virus infection system for studying potential gammaherpesvirus miRNAs functions during the different aspects of *in vivo* infection (primary lytic infection, latency establishment, latency maintenance, and reactivation). In the present study, a recombinant MHV-68 virus lacking 9 miRNAs, MHV-68.ΔmiRNAs, was constructed in order to investigate the potential functions of the miRNAs with specific focus on the latent infection *in vivo*.

The lytic replication of the MHV-68.ΔmiRNAs virus was comparable to that of the WT-MHV-68 virus *in vitro* and *in vivo*. This is consistent with previous studies that demonstrated that a spontaneous MHV-68 deletion mutant virus (MHV-76) lacking ~9.5 kb in the left end of the genome, which includes the unique genes M1, M2, M3, M4, and the vtRNAs and their associated miRNAs, exhibited normal productive replication *in vitro* and *in vivo*, but it was cleared more rapidly from the lung and showed attenuation

in the latency establishment in spleen (Macrae, Dutia et al. 2001, Clambey, Virgin et al. 2002). These results are also consistent with recent published studies that showed that miRNA mutant viruses lacking all of the miRNAs exhibited similar levels of lytic replication to that of the WT virus (Feldman, Kara et al. 2014, Diebel, Oko et al. 2015). Nevertheless, these findings do not exclude any potential role of these miRNAs in post-transcriptional regulation of lytic transcripts. The miRNAs may function as rheostats that fine-tune gene expression levels; indeed, a number of herpesvirus miRNAs suppress leaky lytic transcripts expression during latent infection (Ebert and Sharp 2012, Kincaid and Sullivan 2012, Grey 2015). These functions may not be relevant during virus productive replication that is associated with high levels of lytic gene expression.

6.6 MHV-68 miRNAs and B-cell proliferation

It was found that the spleens from MHV-68.ΔmiRNAs-infected mice developed splenomegaly that is comparable to that of the spleens from mice infected with the WT virus during latency establishment. This was evidenced by the weight of the spleens (Figure 5.13). It is important to note the spleen weight is not a very sensitive method for determining subtle changes in B lymphocyte proliferation because it also measures T lymphocytes and other cell types besides B cells. FACS analysis showed that although there was a slight but statistically significant increase (1.5-fold) in proliferation of B cells from the mice infected with the miRNA mutant virus at 14 dpi, which coincides with maximal B cell proliferation, there was no significant difference at earlier and later time-points. This was an unexpected finding as it was hypothesised that MHV-68 miRNAs may play roles in promoting B-cell proliferation in a way reminiscent of EBV miRNAs

(Seto, Moosmann et al. 2010) . It was reported that the unique M2 protein of MHV-68 was able to induce B cell proliferation and differentiation of infected B cells in an IL-10 dependent manner (Siegel, Herskowitz et al. 2008). Therefore, the proliferation of MHV-68-infected B cells is likely induced by viral proteins rather than viral miRNA, not to mention the important role of CD4⁺ T cells in inducing B cell proliferation and splenomegaly (Usherwood, Ross et al. 1996).

6.7 MHV-68 miRNAs are not essential for latency establishment, but help the virus lay low during the maintenance of long-term latency *in vivo*

The MHV-68.ΔmiRNAs virus established *in vivo* latent infection comparable to that of the WT virus, indicating that the deleted miRNAs are not required for latency establishment. These findings are consistent with recent published studies (Feldman, Kara et al. 2014, Diebel, Oko et al. 2015).

Strikingly, although the *ex vivo* reactivation of the miRNA mutant virus was identical to that of the WT virus during the establishment of latency (14 dpi), it was approximately 18-fold higher than that of the WT virus during the maintenance of latency (49 dpi) despite the similar viral DNA genomic loads for both viruses at all the time points, indicating that the *ex vivo* reactivation was representing the ability of the infected cells to reactivate from latency rather than the amount of latent virus. Consistent with this hyper-reactivation phenotype, there was an increase of the MHV-68.ΔmiRNAs lytic gene expression as compared to that of the WT virus at 49 dpi, indicating the presence of viral lytic cycle. Future experiments may be required to investigate the proportions of

episomal and linear forms of the virus genome at this time-point and later time-points in order to determine the ratio between latent and lytic replication. The presence of lytic transcripts also raises the question as to whether latent infection is maintained in the long term or whether on-going reactivation eventually leads to disease in infected animals. This could be investigated by maintaining infected animals for longer periods or by infecting transgenic mice with compromised immune systems such that they might be more susceptible to infection. It would also be of interest to investigate the ability of MHV-68.ΔmiRNAs virus to establish latency in cell lines.

There was no difference in the gene expression levels, viral DNA genomic loads, or *ex vivo* reactivation at earlier time-points (14, 21, and 28 dpi) except a slight increase in the *ex vivo* reactivation of the MHV-68.ΔmiRNAs-infected splenocytes over that of the WT-MHV-68-infected splenocytes at 21 dpi. Taken together, it is suggested that the MHV-68 miRNAs are not required for latency establishment, but they play a key role in controlling virus reactivation and exit from latency during the maintenance of latency.

The similar mRNA levels of RTA, ORF73, ORF65, and M3 between the miRNA mutant virus and the WT virus at time-points earlier than 49 dpi suggest that the mRNAs of these genes may not be targets for the deleted viral miRNAs; however, it is important to note that the protein expression levels of these genes were not determined and the miRNAs may exert their functions via translational repression leaving intact target mRNA rather than mRNA degradation (Filipowicz, Bhattacharyya et al. 2008); indeed, the work of Riaz *et. al.* has revealed that Ovine herpesvirus-2 miRNAs reduced the target ORF20 and ORF73 protein levels without affecting their mRNA levels (Riaz, Dry et al. 2014).

Despite the accentuated reactivation of the MHV-68.ΔmiRNAs-infected splenocytes at 49 dpi, no produced infectious virus was detected by plaque assay. It is possible that the immune system had cleared the lytically infected splenocytes or that the plaque assay is not sensitive enough to detect very low level of infectious virus. Interestingly, the DNA genomic load of MHV-68.ΔmiRNAs virus was significantly higher than that of the WT virus in the lung at 49 dpi. The lung was reported as an important site for MHV-68 persistent infection in intact and B cell deficient mice (Stewart, Usherwood et al. 1998). It is therefore suggested that MHV-68 miRNAs control virus reactivation from latency in both spleen and lung during late latency. It is possible that the increase in MHV-68.ΔmiRNAs genomic load in the lung at 49 dpi is due to the transfer of reactivating virus from the spleen to infect the lung or due to increase in the reactivation of latently infected epithelial cells, or both. Additional experiments are required to examine the presence of infectious virus as well as the levels of lytic gene expression in the lungs from the mice infected with MHV-68.ΔmiRNAs at 49 dpi and compare it to that of the WT virus.

The finding that the MHV-68 miRNAs control virus reactivation and enhance long-term latency is not surprising. Lei *et. al.* demonstrated that an miRNA encoded by KSHV, miR-k1, directly inhibited the expression of IκBα and thus increased the NF-κB activity that led to inhibition of lytic viral gene expression and productive replication, a function that is important for promoting latency (Lei, Bai et al. 2010). Consistent with these findings, another study showed that overexpression of miR-K3 in KSHV latently infected cells caused an increase in RTA expression and that miR-K3 directly targets NF-κB inhibitor for suppression (Lu, Li et al. 2010). In addition, in the same study, it

was demonstrated that blocking miRNAs biogenesis in KSHV latently infected cells through knocking down the Dicer or Argonaute proteins led to increased lytic viral gene expression, indicating the importance of KSHV miRNAs in controlling reactivation and promoting latency.

Lu *et al.* reported that a KSHV miRNA mutant virus lacking 10 miRNAs displayed comparable numbers of KSHV-positive cells to that of the WT virus. Latency gene transcription and viral DNA genome copies were also similar to that of the WT virus. However, the mRNA expression levels of the key lytic switch, RTA, were significantly lower in cells infected with the miRNA mutant KSHV than those infected with the WT virus due to targeting of the RTA mRNA by miR-K12-5 miRNA (Lu, Stedman *et al.* 2010). The work of Bellare *et al.* showed that the KSHV miR-K9* down-regulated RTA expression and helped inhibit virus lytic reactivation (Bellare and Ganem 2009). Additionally, it was reported that a KSHV miRNA deletion mutant virus lacking miR-K12-3 and miR-K12-11 miRNAs showed increased reactivation and more sensitivity to reactivation inducers as compared to the WT virus, a phenotype resulting from the miRNA-induced inhibition of cellular transcription factors that activate KSHV RTA (Plaisance-Bonstaff, Choi *et al.* 2014). The miRNA BART-miR-18-5p of the human gamma herpesvirus EBV was reported to suppress the expression of the cellular MAP kinase kinase kinase 2 (MAP3K2), which is involved in signalling pathways that facilitate viral lytic infection, and thus inhibit virus reactivation from latency (Qiu and Thorley-Lawson 2014).

While this work was in progress, 3 studies investigating the phenotype of a miRNA mutant MHV-68 virus lacking 14 miRNAs with intact vtRNAs (Feldman, Kara et al. 2014) and the phenotype of a mutant MHV-68 virus lacking all the vtRNAs and their associated miRNAs (Diebel, Oko et al. 2015) were published. Consistent with the results in the present study, they found that the mutant viruses have similar levels of lytic replication to that of the WT virus and that the miRNAs are non-essential for the development of splenomegaly and the establishment of latent infection *in vivo*. They reported that deletion of the miRNAs from the MHV-68. ORF73 β la virus led to modest decrease in the number of mLANA+ memory B cells as compared to that of the WT MHV-68. ORF73 β la virus, whereas the opposite effect was observed in naïve B cells during the establishment of latency. They also reported that deletion of the miRNAs caused significant reduction in the virus pathogenicity in a viral pneumonia model using interferon gamma deficient mice. Diebel *et al.* showed that expression of vtRNA1 alone in the absence of its associated miRNAs and other vtRNAs and miRNAs partially restored the lethal pneumonia phenotype seen in the immunodeficient mice infected with the WT virus, indicating that vtRNAs can play a role in virus pathogenicity. Consistent with the hyper-reactivation phenotype of the miRNA mutant virus, Steer et al. have shown that the viral genomic load of a mutant MHV-68 virus lacking all viral miRNAs and vtRNAs in splenocytes from infected mice was higher than that of the WT virus-infected mice at 42 dpi (Steer et al, 2016).

What are the molecular mechanisms behind the hyper-reactivation phenotype of the MHV-68. Δ miRNAs virus during late latency *in vivo*?

The exact molecular mechanisms behind this phenotype are unknown. However, it can be speculated that the MHV-68 miRNAs may hijack cellular signalling pathways in a manner reminiscent of that of the miRNAs encoded by KSHV and EBV viruses, particularly the activation of the NF- κ B pathway, which has an important role in maintaining gamma-herpesvirus latency and suppression of lytic reactivation (Brown, Song et al. 2003, Bellare and Ganem 2009, Krug, Collins et al. 2009, Lei, Bai et al. 2010, Lu, Li et al. 2010, Lu, Stedman et al. 2010, Plaisance-Bonstaff, Choi et al. 2014, Qiu and Thorley-Lawson 2014). Members of the NF- κ B family of transcription factors have low activity in various cell types due to their association with the NF- κ B family of inhibitors that promote their retention in the cytoplasm; however, upon suppression of the NF- κ B inhibitor, NF- κ B is activated and accumulated in the nucleus (Hayden and Ghosh 2008).

Intriguingly, NF- κ B is particularly active in the mature murine B lymphocytes, which are the major reservoir of MHV-68 latency, and constitutively present in the nucleus due to the instability of the NF- κ B inhibitor in these cells (Miyamoto, Chiao et al. 1994, Flano, Kim et al. 2002). Following gammaherpesvirus infection, NF- κ B is further elevated in the latently infected B lymphocytes by the latency associated viral proteins (Hammarskjold and Simurda 1992, Laherty, Hu et al. 1992, Schwarz and Murphy 2001, Guasparri, Keller et al. 2004, Gewurz, Mar et al. 2011, Lavorgna and Harhaj 2012, Ersing, Bernhardt et al. 2013). It is therefore plausible to hypothesise that over time

during latent infection when the MHV-68 protein levels decrease dramatically, the viral miRNAs expression levels increases (see chapter 4) in order to compensate for the loss of the roles played by the viral proteins in exploiting host cell pathways in order to maintain long-term latency and prevent undesired reactivation. By this way the virus can evade the immune response elicited by the viral proteins while maintaining the important latency enhancement functions through its miRNAs, which cannot be detected by the host immune system, and thus the virus can remain under the radar of the immune surveillance.

We can surmise that the MHV-68 proteins and miRNAs may have some redundant functions related to promoting latency, but during late latency when the protein expression is highly restricted, the miRNAs are expressed and act as safety valves that prevent undesired disruption of latency. This speculation can explain the absence of a striking phenotype of the MHV-68.ΔmiRNAs virus during the early latency, whereas it displayed an accentuated reactivation phenotype during late latency.

Another possibility is that the miRNAs may target some viral lytic genes for translational repression, particularly RTA. For instance, the 3' UTR of RTA carries a predicted target site for mghv-miR-M1-1-3p. The similar lytic gene transcription levels in spleen cells from mice infected with the miRNA mutant MHV-68 and those infected with the WT MHV-68 virus during the establishment of latency does not guarantee similar protein expression levels. The absence of a pronounced phenotype during the establishment of latency is possibly because the inhibition of protein expression by the miRNAs was too subtle to cause a noticeable phenotype, but the phenotype is more

pronounced over time when the viral protein levels decrease and the viral miRNAs levels increase. The finding that the MHV-68. Δ miRNAs was able to establish latent infection similar to that of the WT virus does not exclude possible subtle roles of the miRNAs during the establishment of latency. Indeed, a previous study reported that an MHV-68 mutant virus lacking 14 miRNAs displayed modest decrease in frequency of the MHV-68-positive memory B cells as compared to that of the WT virus during latency establishment (Feldman, Kara et al. 2014), suggesting a role played by these miRNAs in increasing the numbers of MHV-68-infected memory B cells during latency establishment.

6.8 Possible future work

The role of the miRNAs in controlling reactivation can be scrutinized in cell lines supporting latent infection such as NS0 cell line. In addition, investigating the sensitivity of the MHV-68. Δ miRNAs to reactivation inducers could be carried out in order to measure the ability of this virus to reactivate *in vitro* as compared to the WT virus.

Given the significant increase in the genomic load of the MHV-68. Δ miRNAs virus in the lung during late latency (49 dpi) as compared to the WT virus, it will be interesting to evaluate the level of virus reactivation in the lung epithelial cells as well as the level lytic gene expression at this time-point and probably at earlier time-points.

Analysing the levels of lytic viral protein expression in the spleen, particularly during late latency can confirm whether the increase of the MHV-68. Δ miRNAs lytic transcripts

is accompanied by increase in the level of protein expression. Measuring the NF- κ B activation level at 49 dpi will give insight into whether the MHV-68 miRNAs may modulate the NF- κ B pathway in a manner similar to that of the KSHV miRNAs and whether the NF- κ B signalling pathway is involved in the hyper-reactivation phenotype of the MHV-68. Δ miRNAs virus at this stage of infection.

Developing a marker miRNA mutant MHV-68 virus that is not attenuated *in vivo* could be a very powerful tool in investigating the previously mentioned potential roles of the miRNAs through isolating the latently infected cells and thus enriching for these rare cells, particularly during late latency. In addition, a marker virus could allow for investigation the potential miRNAs functions in B cell subpopulations and other cell types such as macrophages and dendritic cells during the course of latent infection *in vivo*. A marker miRNA mutant MHV-68 virus that carries a reporter gene fused to a lytic viral gene could be designed in order to monitor virus reactivation *in vivo*. For example, previous studies showed the use of a recombinant MHV-68 virus expressing firefly luciferase, which is driven by the M3 promoter, for real-time monitoring of the virus replication and reactivation in the same live animal non-invasively using a bioluminescence imaging technique (Hwang, Wu et al. 2008, Reese, Wakeman et al. 2014). Such a technique could be useful in studying the role of the MHV-68 miRNAs in controlling reactivation *in vivo* provided that the latency establishment and maintenance are not altered due to the presence of the reporter gene.

7. References

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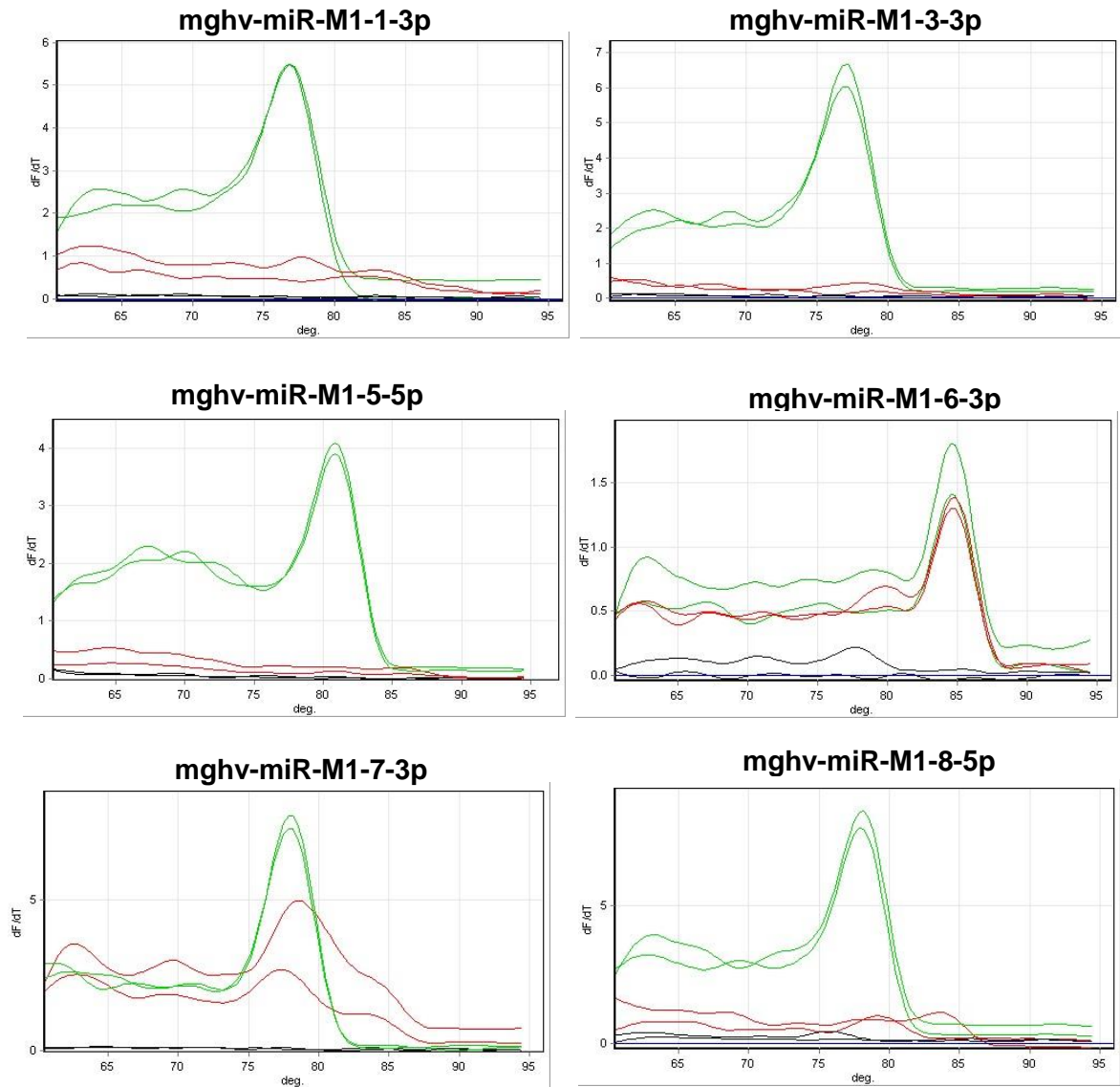
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8. Appendix



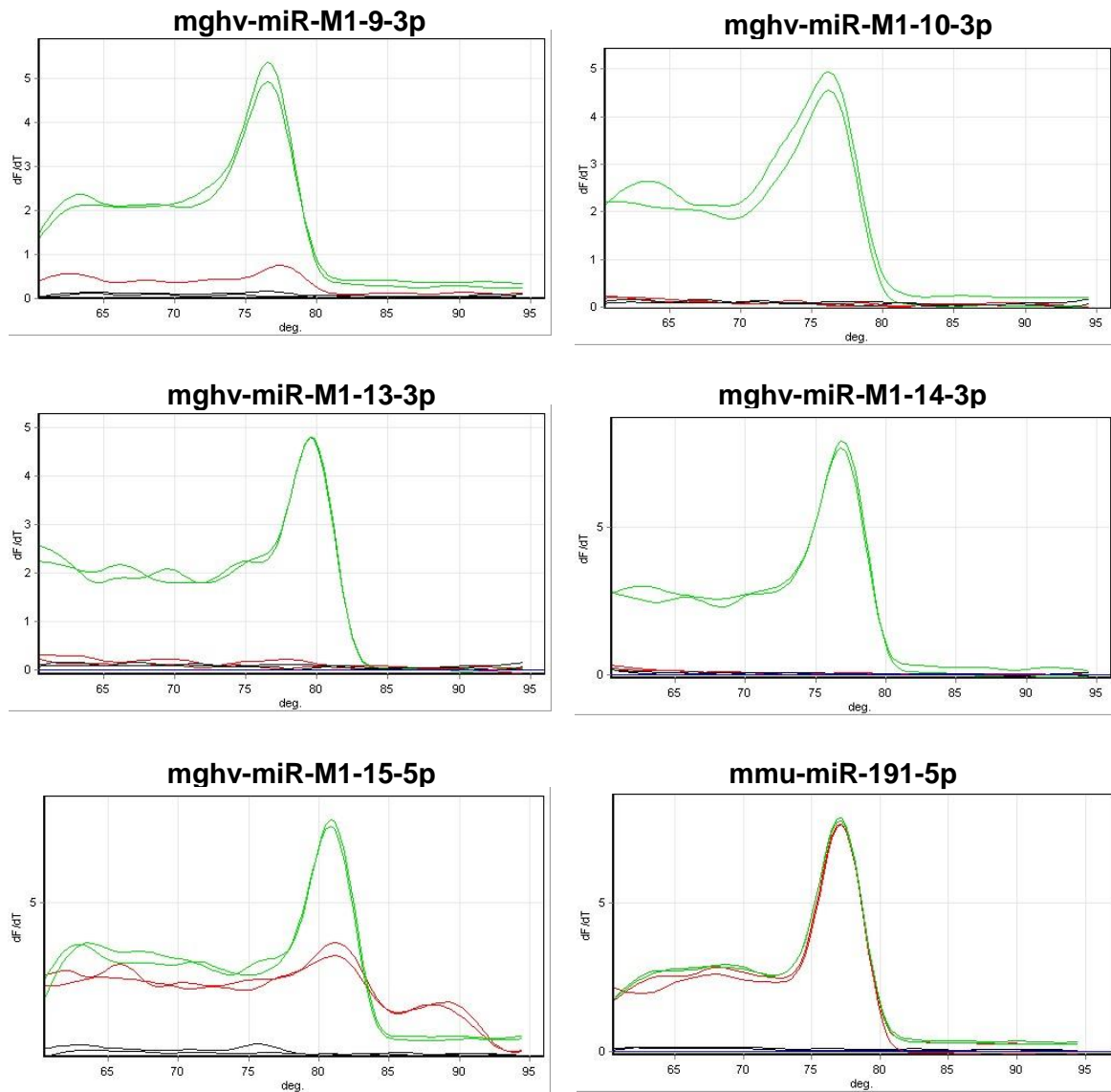


Figure 8.1 qRT-PCR melting curves for MHV-68 miRNAs and cellular mmu-miR-191-5p expression in BHK-21 cells. qRT-PCR was carried out using RNA extracted from cells infected with MHV-68 at MOI of 5 for 24 hours (green) or from uninfected cells (red). No template control (black) is a sample in which water was added instead of the template cDNA.

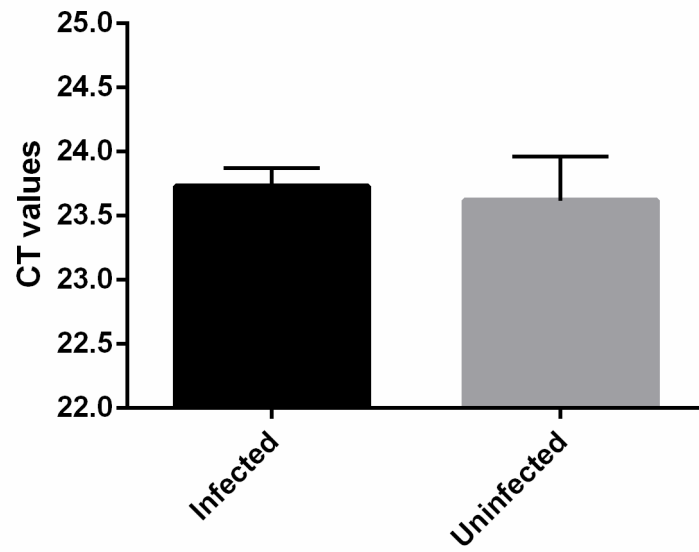
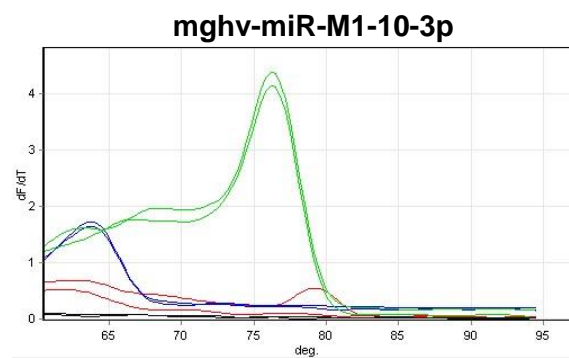
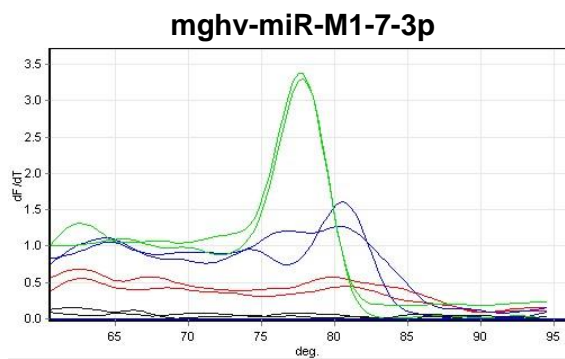
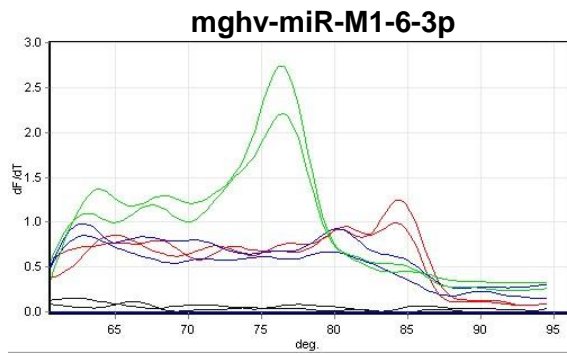
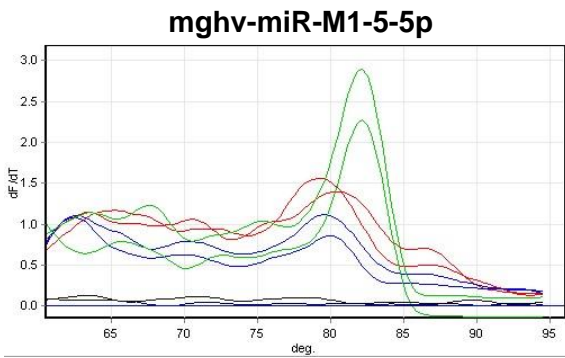
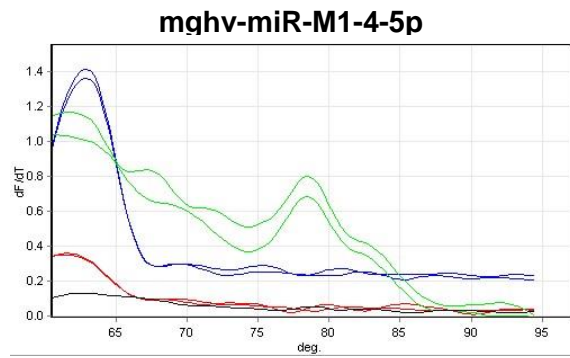
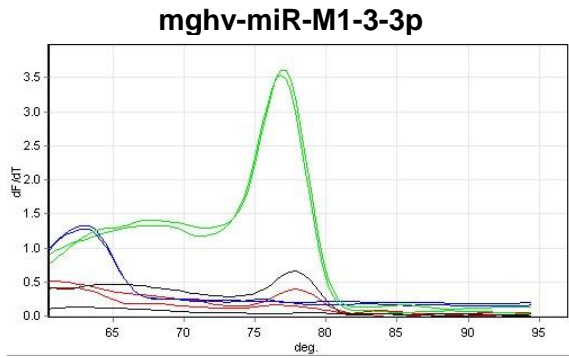
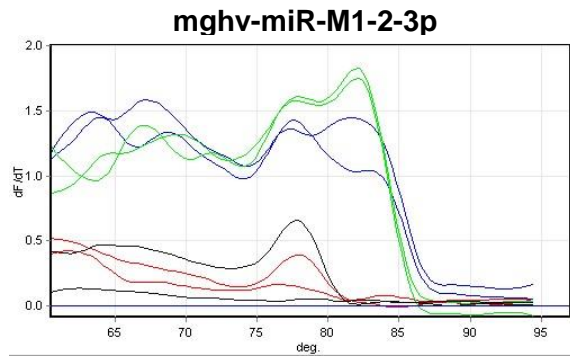
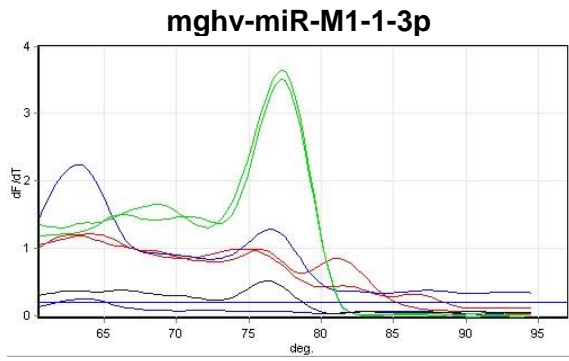


Figure 8.2 mmu-miR-191-5p expression in BHK-21 cells. qRT-PCR was carried out using RNA extracted from BHK-21 cells infected with MHV-68 at MOI of 5 for 24 hours or from uninfected cells. Error bars represent mean \pm SD. Data were compiled from 2 independent experiments.

Appendix



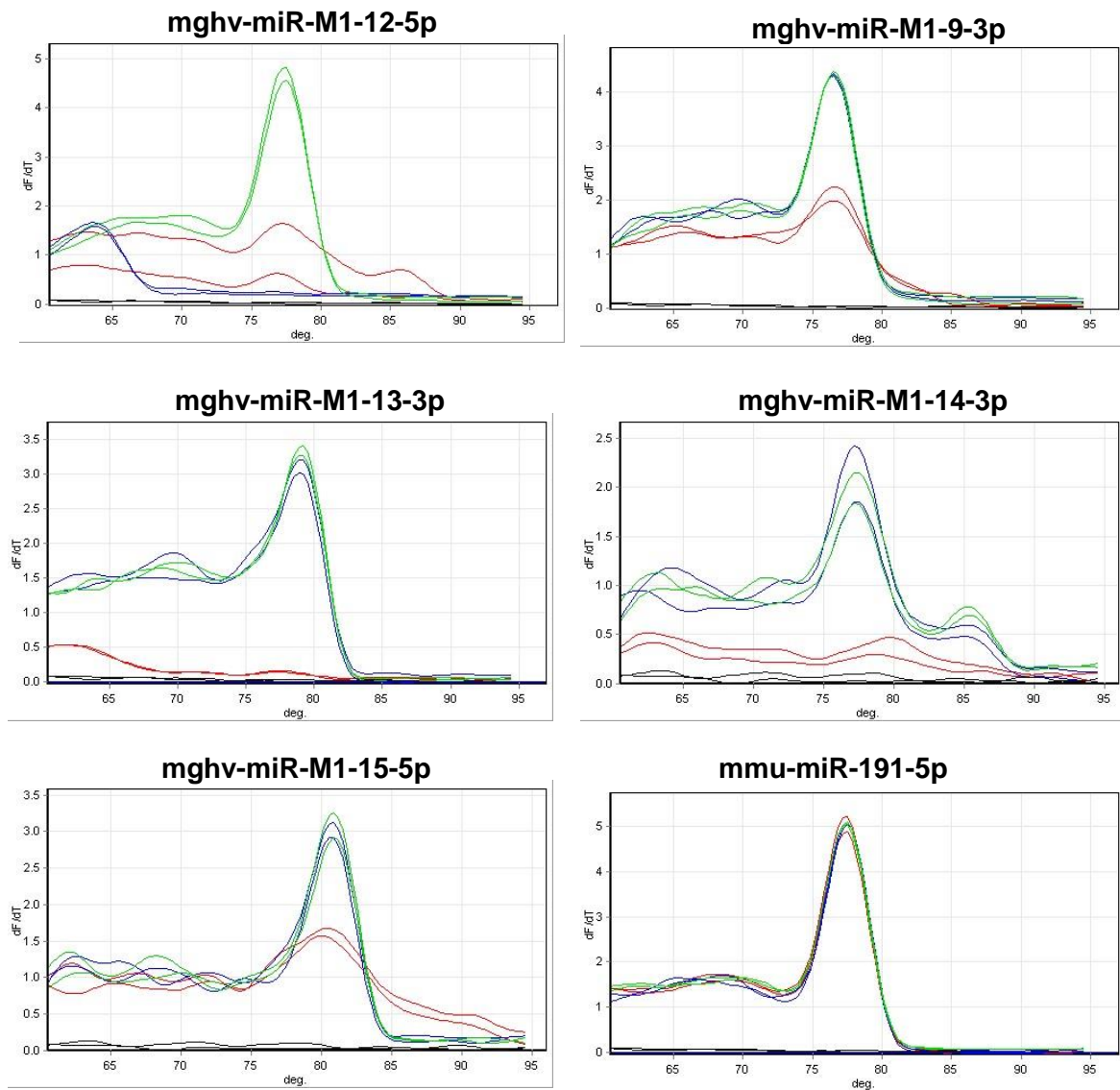


Figure 8.3 qRT-PCR melting curves of MHV-68 miRNAs expression in NIH 3T3 cells infected with either WT MHV-68 virus or MHV-68.ΔmiRNAs virus. qRT-PCR was performed using RNA from NIH 3T3 cells infected with either WT MHV-68 virus (green) or MHV-68.ΔmiRNAs virus (blue) at MOI of 0.001 for 6 days. Uninfected control (red) and no template control (black) were used to monitor contamination and primer-dimer formation that could produce false positive results.