Attempts to visualize lymphocytes latently infected with Marek’s Disease Virus in situ

by

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Abstract

Marek’s Disease (MD) is an avian lymphoma disease caused by an alphaherpesvirus, Marek’s Disease Virus (MDV). Live attenuated vaccines can protect birds from the disease but do not inhibit MDV infection. The co-infection of vaccine virus and virulent MDV perhaps contributed to the emergence of MDV strains that caused vaccine breaks. MDV establishes lytic and latent states of infection but it is during latency that cells can be transformed. This thesis strived to create a tool to visualize latently infected cells that would help reveal the transformation process and underlying mechanisms of the vaccine effect. Toward the goal, I constructed two recombinant MDVs that were designed to express a fluorescent protein during latency. The recombinant MDVs were able to replicate successfully in vitro and in vivo comparatively to parental MDV. They also expressed the fluorescent protein in the infected cells in vitro. However, the expression of the fluorescent protein was not confirmed in vivo and these recombinant MDVs did not cause lymphomas in infected birds.

Keywords: Marek’s Disease Virus; Latency; Lymphoma; Recombination Machinery; BAC; Fluorescent Protein
Dedication

To my parents who give me unconditional love
To my spouse who is my rock and gives me the strength
to find and follow my passion
To my friends who make sure I smile
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I would like to thank my parents, my brother and late grandparents for teaching me to dream big and always believing in me. Your love and support can be felt everyday and I carry your strength with me even though we are miles apart.

Finally I would like to thank my spouse, Adam Francis. Without you standing beside me I could never have completed this program. I will be eternally grateful for your love, care, support, and most importantly for giving me the courage to grow
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List of Acronyms

MDV  Marek’s Disease Virus
MD   Marek’s Disease
HSV-1 Human herpes-simplex Virus 1
VZV  Varicella zoster virus
KSHV Kaposi’s sarcoma-associated herpesvirus
CEF  Chicken Embryo Fibroblasts (primary cell culture)
BAC  Bacterial Artificial Chromosome
MSB-1 An MDV transformed chicken T cell line
SPF  Specific Pathogen Free
IE   Immediate Early genes
E    Early genes
L    Late genes
LAT  Latency Associated Transcript
EGFP Green Fluorescent protein
FISH Fluorescent In Situ Hybridization
Chapter 1.

An Introduction to Marek's Disease (MD)

Marek's disease virus (MDV) is an avian oncogenic herpesvirus that costs the agriculture industry USD$1-2 billion annually [1]. MDV causes Marek's Disease (MD), a T-cell lymphproliferative disease, which ultimately causes death. As MDV is extremely ubiquitous in the field, every battery chicken is vaccinated in ovo with an attenuated live vaccine. For over 40 years the MDV vaccine has been able to stop the formation of lymphomas; however, it does not stop chickens from becoming infected. MDV virulence is increasing and vaccine breaks are occurring in the field. Unfortunately, we do not fully understand a) the infection dynamics of MDV b) how MDV establishes T-cell lymphomas or c) how the vaccine works. MDV serves as an animal model for virus-induced tumour formation, so if the vaccine effect is uncovered it could aid the development of other anti-cancer vaccines. Our lack of knowledge is in part due to the strict cell-associated nature of the virus and absence of tools to study the different stages of MDV infection in vivo. If the underlying mechanisms of infection are not revealed, establishing novel vaccines to accommodate future vaccine breaks may be troublesome. This chapter will serve as an introduction to MDV by answering the following questions: What is MDV? How does MDV infect its host? What is MD and how is it controlled? Along the way, knowledge gaps will be highlighted and the chapter will conclude with information on research tools that are currently being utilized.

1.1. What is Marek's Disease Virus?

MDV is an alphaherpesvirus and as such falls under the family Herpesviridae. In order to understand MDV classification we should take a broad look at the Herpesviridae family to uncover common characteristics that viruses from within this family share.
Overview of Herpesviridae

MDV is one of more than 100 viruses in the family Herpesviridae. Herpesviruses infect a wide variety of hosts and have different disease characteristics; however, they do all contain similar traits. For example, they all have a double stranded linear DNA genome that is protected by an icosahedral capsid and, upon infection into its host, the virus either establishes a productive infection (lytic state) or enters into a state of dormancy (latent state). In lytic infection all viral proteins are expressed in a cascade and progeny virus particles are produced. Contrary to lytic infection, during latency the majority of viral proteins are not transcribed and viral progeny are not produced. In fact, in MDV the genes that are transcribed during latency include a Latency Associated Transcript (LAT) and meq oncogene. Occasionally latent viruses reactivate resulting in a second lytic infection. Despite these similar traits, herpesviruses are extremely host specific, infect different cellular targets and have a distinct genomic organization. It is these differences that have divided them into three subfamilies and thirteen genus groups. The three subfamilies are gammaherpesvirinae, betaherpesvirinae, and alphaherpesvirinae (Table 1-1):

Gammaherpesvirinae: This family cause lymphoproliferative diseases and examples that infect humans are Epstein-Barr virus (HHV-4 or EBV) and Kaposi’s sarcoma-associated herpesvirus (HHV-8 or KSHV). Both EBV and KSHV maintain latency in B cells and are commonly harmless. But in some cases, especially in immunocompromised individuals, they can cause cancer [2, 3].

Betaherpesvirinae: This family favours epithelial, monocytes, and fibroblasts as their target cells. Two betaherpesvirus species that infect humans are cytomegalovirus (CMV) and human herpesvirus-6 (HHV-6). CMV persists in about 50%-90% of the adult population and most do not show any symptoms. However in immunocompromised individuals CMV may cause multiple organ failure as well as vascular disease [4]. Similar to CMV, HHV6 persists harmlessly in the majority of the population, but in immunocompromised individuals HHV-6 has been linked with encephalitis, and seizures [5]. It is also suggested to be associated with multiple sclerosis but further examination is required [6].
**Alphaherpesvirinae:** This family infect epithelial and neuronal cells and are capable of establishing latent infection in sensory neurons. Species that infect humans include herpes simplex virus 1 and 2 (HSV-1 and HSV-2), which cause cold sore and genital herpes respectively, and varicella zoster virus (VZV) which causes the common chicken pox disease [5]. VZV is the only human herpesvirus that has an available vaccine. VZV vaccination consists of a live attenuated virus vaccine that was introduced in 1995 [7, 8]. Even though MDV is biologically similar to gammaherpes viruses, it is genetically comparable to viruses such as HSV-1 and VZV therefore it is placed within the alphaherpesvirinae subfamily (see section 1.1.3).
### Herpesviridae subfamilies, genus and examples of species that are referred to in this chapter

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Genus</th>
<th>Examples of Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gammaherpesvirinae</strong></td>
<td>Lymphocryptovirus</td>
<td>Epstein-Barr virus (HHV-4)</td>
</tr>
<tr>
<td></td>
<td>Rhadinovirus</td>
<td>Kaposi’s sarcoma-associated herpesvirus (KSHV/HHV-8)</td>
</tr>
<tr>
<td></td>
<td>Macavirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Percavirus</td>
<td></td>
</tr>
<tr>
<td><strong>Betaherpesvirinae</strong></td>
<td>Cytomegalovirus</td>
<td>Human cytomegalovirus (HCMV/HHV-5)</td>
</tr>
<tr>
<td></td>
<td>Roseolovirus</td>
<td>Human herpesvirus-6 (HHV-6)</td>
</tr>
<tr>
<td></td>
<td>Proboscivirus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Muromegalovirus</td>
<td></td>
</tr>
<tr>
<td><strong>Alphaherpesvirinae</strong></td>
<td>Iltovirus</td>
<td>Gallid herpesvirus 1</td>
</tr>
<tr>
<td></td>
<td>Mardivirus</td>
<td>*1) MDV serotype 1; MDV-1/Gallid herpesvirus 2 (oncogenic)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>**2) MDV serotype 2; MDV-2/Gallid herpesvirus 3 (non virulent)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>***3) MDV serotype 3; MDV-3/Meleagrid herpesvirus (Herpesvirus of turkeys, HVT)</td>
</tr>
<tr>
<td></td>
<td>Simplexvirus</td>
<td>Human herpes simplex virus -1 and -2 (HSV1, HSV 2)</td>
</tr>
<tr>
<td></td>
<td>Varicellovirus</td>
<td>Varicella zoster virus (VZV/HHV-3)</td>
</tr>
<tr>
<td></td>
<td>Scutavirus</td>
<td></td>
</tr>
</tbody>
</table>

http://ictvonline.org/virusTaxonomy.asp
* Oncogenic Marek’s Disease virus
** Generation 2 MD vaccine
***Generation 1 MD vaccine

### 1.1.2. Marek’s Disease Virus Taxonomy

As previously stated, MDV is classified under the family alphaherpesvirus, genus Mardivirus (Table 1-1). There are three serotypes within the MDV Mardivirus genus that were distinguished by analyzing viral DNA or using either polyclonal or monoclonal antibodies (Table 1-2). The three serotypes also differ in pathogenicity details of which are found below [9]:

4
**Serotype 1 (MDV-1/Gallid herpesvirus 2):** This includes all oncogenic strains of MDV and three pathotypes have been characterized. MDV pathotypes were established because there was an emergence of unexplained MD incidence in vaccinated chickens. Pathotype is defined by virulence which is evaluated by observing the frequency of disease induction [10] (see section 1.3.2). Strains with lowest virulence are designated as virulent (v) (strains such as JM), followed by very virulent (vv) (strains Md5 and RB-1B) [11, 12]. Finally, strains have been isolated that are more virulent than vvMDV and were named very virulent plus (vv+) (RK-1, 648A). It is important to note that the increasing virulent pathotypes go hand in hand with the frequency of vaccine breaks, which will be discussed later in section 1.3.2. Unfortunately, some vv+ strains are resistant to current vaccination protocols.

Virulent MDV strains become attenuated by cell culture passage. Passage of MDV is essential for many experimental procedures such as viral amplification. Therefore it is always extremely important to note what cellular passage is being used in all experimental procedures, especially when the pathogenicity is required.

**Serotype 2 (MDV-2/Gallid herpesvirus 3):** This is a genetically distinct naturally non-pathogenic MDV. Strains include SB-1 and HPRS24. MDV-2 SB-1 strain is currently being used along with MDV-3 as a bivalent vaccine against MDV-1, which will be discussed later in section 1.3.2.

**Serotype 3 (MDV-3)/Meleagrid herpesvirus:** MDV-3 is actually a herpesvirus of turkeys (HVT). MDV-3 is not oncogenic to chickens, but it does cause viremia (virus is able to enter the bloodstream of host). MDV-3 was used as the first vaccine against MDV-1 (section1.3.2).
Table 1-2 The Organization of Serotypes that are Within the MDV Group

<table>
<thead>
<tr>
<th>MDV Serotype</th>
<th>Also Known As</th>
<th>Pathotypes, Strains and Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serotype 1</strong></td>
<td>MDV-1/ Gallid herpesvirus 2</td>
<td>Virulent (v)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strains: JM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Very virulent (VV)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strains: Md5, RB-1B</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Very virulent + (VV+)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strains RK-1, 648A</td>
</tr>
<tr>
<td><strong>Serotype 2</strong></td>
<td>MDV-2/Gallid herpesvirus 3</td>
<td>Naturally non-pathogenic.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strains include SB-1, HPRS24, HN-1</td>
</tr>
<tr>
<td><strong>Serotype 3</strong></td>
<td>MDV-3/Meleagrid herpesvirus</td>
<td>Non-pathogenic to chickens.</td>
</tr>
<tr>
<td></td>
<td>Herpesvirus of turkeys</td>
<td>Strains include FC126</td>
</tr>
</tbody>
</table>

MDV-1 was originally characterized as a gammaherpesvirus because, like other gammaherpesviruses, MDV targets lymphocytes and causes lymphomas. In fact, MDV is often compared to two human gammaherpesviruses EBV and KSHV. In 1991 the MDV genome was fully sequenced and it was found that the genome shared striking similarities to alphaherpesviruses, which caused a change in classification. Alphaherpesviruses do not cause tumours and, unlike MDV, their pathogenesis is associated with their lytic infection. For instance in HSV-1 the blister or cold sore is caused by rapidly replicating virus in lytically infected cells. Despite these obvious differences, their genomes all harbour similar structures, which will be detailed below in section 1.1.3.

1.1.3. Genome

MDV’s genome size is about 177kb [13] and, as previously stated, it shares the most homology with species in alphaherpesvirinae. The genomes of alphaherpesviruses are composed of a long (L) and short (S) component. Each component consists of a unique region (UL or US) that is surrounded by a terminal (TL or TS) and internal (IRL or IRS) repeat regions (Figure 1-1). MDV genome shares the closest homology with VZV and HSV-1 genomes (Figure 1-1). VZV has the closest homology to MDV especially in the unique regions. Overall, only five VZV genes are not found on the MDV genome.
The largest difference is that VZV’s TR_L and IR_L are significantly shorter than MDV’s [9]. Interestingly, MDV’s oncogene *meq*, which is considered the key viral gene for MDV oncogenesis, is encoded within the TR_L and IR_L regions [9] (Figure 1-1 C). Meq protein is translated from one of the few RNAs transcribed during latency, and is involved in the transcriptional regulations that lead to cellular transformation (see section 1.2.3). Despite differences in the genome, VZV and HSV-1 are often used to uncover unknown mechanisms involved in lytic and latent MDV infection, which will be examined below in section 1.2.
Figure 1-1  Schematic representation of VZV, HSV-1 and MDV genome

Schematic representation of A) VZV genomes [14], B) HSV-1 [15] and C) MDV [9]. Each of these alphaherpesvirus genomes are comprised of a long (U$_L$) and a short (U$_S$) segments. Each segment has a unique sequence and these unique regions are flanked by inverted repeat sequences; terminal and internal repeat long (TR$_L$, IR$_L$) or terminal and internal repeat short regions (TR$_S$, IR$_S$). The LAT of HSV-1 is encoded in the TR$_L$ and IR$_L$ regions, whereas the LAT in MDV is in the TR$_S$, IR$_S$. Interestingly the LAT in MDV runs antisense to immediate early (IE) gene ICP4 whereas the LAT in HSV-1 runs antisense to IE gene ICP0. MDV oncogene meq is located in the TR$_L$ and IR$_L$ repeat regions.
1.2. How does MDV infect its host?

Typical of herpesviruses, MDV infects cells in two states: latent and lytic. Each infection state has different characteristics, which will be discussed in detail in sections 1.2.2 and 1.2.3. Initially, the infection dynamics of MDV and the cell types where different stages of infection occur will be described.

In the 1970-1980s a hypothesis on MDV infection was finally developed (Figure 1-2) [16]. It was found that MDV spreads to uninfected chickens by inhalation of the dust and dander shed from infected birds [17]. The virus first targets phagocytic cells in the respiratory tract (e.g. macrophages or dendritic cells), which become infected and subsequently carry the virus to the spleen, thymus and bursa of Fabricius (bursa). Bursa is unique to avian species and a primary lymphoid organ in chickens where B cells mature. It is in these lymphoid organs that MDV infects B and T cells. Interestingly, virus is detectable in the spleen, thymus and bursa within 24 hours after infection [18]. Infected B and T cells then undergo a different pattern of infection which will be introduced below [9] [19] (Table 1-3);

**B-cell infection:** When MDV infects B cells, lytic infection is established where viral progeny are produced and cell death occurs (Table 1-3).

**T-cell infection:** T cells are where the virus can enter a state of latency at approximately 7 days post infection (dpi). During latency, viral gene expression is limited and no progeny are produced (Table 1-3). The latency associated transcript (LAT) and Meq oncogene (a jun/fos family oncogene encoded by MDV genome, see Section 1.2.3) are two transcripts that are present during this state. Some latently infected T cells may be reactivated to form a second lytic infection while a few others become transformed into lymphomas (see below).

**T-cell transformation:** Latently infected T cells can become transformed and lymphomas can be seen as soon as 14 dpi, which ultimately cause death. Mechanisms required for T-cell transformation are not known but studies have shown the oligoclonal nature of MD lymphomas [20, 21]. This suggests that only a few latently infected T cells are transformed in an infected bird. However, these studies analyzed whole tumour
nodules, which are not a homogenous population of MD transformed cells. Many cells in the nodules are reacting cells or have become reactivated. Obtaining a homogenous population of transformed T-cells from infected birds for characterization is difficult.

**Virus transmission**: Latently infected T-cells can become reactivated to form a second lytic infection. Reactivated T cells are able to infect feather follicle epithelial cells, which are subsequently shed and inhaled by uninfected chickens. Interestingly, late viral proteins could be detected in feather follicle epithelial cells as early as 8 dpi; however, only material collected after 15 dpi could produce disease in uninfected chickens [22]. It is important to note that infectious MDV can only be shed naturally from feather follicle epithelial cells of infected birds [23].

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**Figure 1-2** Schematic representation of the current MDV pathogenesis model

MDV infection model. Dander and dust harboring MDV virions is inhaled into the respiratory tract of uninfected chickens. Phagocytic cells become infected and carry the virus to lymphoid organs where it infects B cells and T cells. Lytic infection occurs in these lymphocytes but latency and transformation occur only in T cells. T cells can infect feather follicle epithelial cells where MDV can mature. Infected cells are shed into the environment and inhaled by uninfected chickens and the cycle continues.
1.2.1. **Gaps in knowledge**

 Mostly due to the strict cell-associated nature of the virus, it is not yet known how the virus spreads from cell to cell in the host. It has been hypothesised that viral spread requires direct B to T cell contact through CD40 (B-cell) - CD40 ligand (T-cell) and B cell major histocompatibility complex (MHC) - T-cell receptor (TCR) mediated interactions [24]. Another large knowledge gap is that we do not know how many cells are latently vs. lytically infected or in fact what triggers transformation in limited latently infected T-cells. It has been suggested that transformed T-cells all have specific cell markers including regulatory T-cell phenotype (T\textsubscript{reg}) as well as CD30, CD25 [8, 24, 25]. However, these studies analyzed whole tumours caused by MDV, which are not a homogenous population of MD transformed cells. Currently we do not have any tools to separate latently infected and transformed cell populations.

1.2.2. **Marek’s Disease Virus Lytic Infection**

 MDV enters a lytic state *in vivo* upon initial infection and when reactivated from latent state (Table 1-3). In the lytic state viral proteins are expressed, progeny are produced and ultimately the infection causes cell death. Similar to other herpesviruses, MDV lytic gene expression occurs via a cascade of transcription. Firstly, a group of genes called the immediate early (IE) genes are induced. IE gene products then trigger the transcription of a group of early (E) genes. E genes in turn induce late (L) gene transcription. As we saw in section 1.1.3, VZV and HSV-1 have similar genome structure to MDV and as such are often used as a comparative backbone when identifying MDV protein function. By comparing the gene maps of MDV with VZV and HSV-1, several homologs were identified even before the entire MDV genome was sequenced. For instance HSV-1 IE gene ICP4 transactivates E and L genes and has been shown to be essential for viral replication [26]. Without ICP4 transcription does not proceed after IE genes [27]. Interestingly, the MDV homologue of ICP4 is antisense to LAT (Figure 1-1) and has been shown to transactivate the E genes such as pp38 (often used as a marker for early viral gene expression for MDV) [28, 29]. Therefore, it has been suggested that suppression of ICP4 is important to maintain latency [30]. Antisense to HSV-1 LAT is an
IE gene, ICP0 (Figure 1-1). ICP0 has been shown to be extremely important in HSV-1 reactivation from latency and viral lytic infection [27].

Despite the analogy to VZV and HSV-1, many MDV lytic protein functions have yet to be fully understood. It has been especially difficult to uncover MDV specific protein functions such as that of MDV oncogene Meq. This is in part due to the strict cell associated nature of MDV as well as the lack of research tools such as a susceptible avian cell line and available antibodies for the chicken system. So far chicken embryo primary fibroblasts are the sole tissue culture cells that fully support the MDV replication, which are not the natural target of MDV lytic infection (see section 1.4.1). However, the recent development of tools that allow for the manipulation of the MDV genome has vastly improved our knowledge of viral protein function (see section1.4.2).

Table 1-3  
**MDV lytic and Latent infection dynamics**

![Diagram of MDV lytic and Latent infection dynamics](image)

1.2.3. **Marek’s Disease Virus Latency and Transformation**

Unfortunately the mechanisms of MDV T-cell latency and transformation remain largely unknown. As previously stated, MDV latency is established only in CD4+ T cells approximately 7 dpi. Contrary to lytic infection, no viral progeny are produced during
latency and the majority of the viral genome is silenced. The few sections of genome that are transcribed during latency include an aforementioned LAT and meq oncogene, which encodes the 339-amino acid Meq protein. In our current knowledge Meq protein is the most consistently expressed gene during latency [31]. Unlike Meq, LAT is not translated but it does have a complicated splicing pattern (see Chapter 2 section 2.1.2) [32]. In order to establish and maintain latency, a controlled shutdown of the lytic genes must therefore occur.

Around 14 dpi, a few latently infected cells are potentially transformed causing T-cell lymphomas in infected birds. If we uncover mechanism needed for MDV latency and transformation, our understanding of MDV oncogenesis will significantly improve. Additionally, as MDV vaccines prevent the transformation of lymphocytes but do not eliminate MDV from the host (see section 1.3.2), this information is critically important for understanding vaccine efficacy. Discussed below are a few mechanisms that were found to be important in latency and transformation. This demonstrates the wide range of ideas currently being discussed in the literature and illustrates just how complex these viral processes are.

**Role of MDV integration into host genome**

MDV, EBV and HHV-6 (detected in human lymphoproliferative disorders) are three herpesviruses that illustrate exclusive viral integration into the host genome during latency [33]. MDV harbours telomeric repeats at both ends of their genome which are identical to the host telomere sequence [33]. Therefore, MDV favours integration into the host telomeric regions. However they integrate into any chromosome with no apparent preference [34]. It has been suggested that MDV is able to escape integration and exist as non integrated DNA when the virus reactivates to form a secondary lytic infection [35]. The specific mechanism by which MDV integrates into and escapes from the host genome remains unknown.

Other alphaherpesviruses, such as HSV-1 maintain their genome as a circular episome during latency in neuronal cells [36]. As neuronal cells do not divide, HSV-1 does not need to replicate its genome along with cell division. Contrary to HSV-1, MDV establishes latency in T cells that occasionally divide even when they are not activated.
To maintain latency in the host cell, the MDV genome must replicate when the host cell divides. Integration to the host chromosome may be a convenient strategy for the virus to overcome this difficulty since the virus genome can be faithfully replicated, even without the need of viral proteins, when the cell divides [33].

Studies on MDV viral integration use either MDV transformed cell line (MSB-1) or in vivo MDV tumour nodule samples, but both are problematic. For instance, the virus may have accidently integrated into the chromosomes of the transformed cell line during cell division. Tumour samples consist of a heterogeneous cell population (latently infected, reactivated, transformed and reacting cells), so results suggesting viral integration may not be accurate. Due to the lack of reliable in vitro system to establish latent infection and transformation, it is difficult to conclude if integration is a key component for transformation and MDV life cycle. One of the potential solutions to this problem is to establish a method to label latently infected/transformed cells in vivo so that a homogeneous cell population can be generated.

**Latency Associated Transcript**

The LAT is situated in the IR$_S$ and TR$_S$ regions of the MDV genome (Figure 1-1C) and is one of the few transcripts present in latently infected and transformed cells [37]. The transcription of the 10kb LAT RNA occurs from one promoter site and the resulting transcript is spliced in a variety of complex ways [32]. There is no known protein product translated from any of these LAT transcripts. LAT spliced transcripts have been shown to interfere with the transcription of IE gene ICP4 that is encoded antisense to LAT (Figure 1-1 and 1-3) [36, 37]. As previously stated, ICP4 is an essential transactivator of the virus protein expression cascade therefore LAT transcripts have an important role in the suppression of lytic infection [32, 38]. A major finding in LAT research was that within the transcript a number of microRNAs (miRNAs) were identified that are highly expressed in tumours and MDV transformed cell line (MSB-1), which will be further discussed below.

**Role of miRNA**

MicroRNAs (miRNAs) are small RNA molecules of 21-25 nucleotides in length that are capable of regulating protein expression at the post-transcriptional level. Their
targets are extremely variable and include regulation of many physiological processes including proliferation, apoptosis and cell survival [39]. After transcription, miRNA are in a precursor state, pre-miRNA, with a hairpin structure. Pre-miRNAs are exported from the nucleus and cleaved to form its functional mature length miRNA [40]. MiRNAs have been identified in viruses and most belong to the herpesvirus family.

MDV miRNAs were first discovered in 2006 [41] and there are currently 26 mature miRNAs identified. Most of these 26 miRNAs are encoded in the repeat regions of the MDV genome, R_S and R_L (Figure 1-3). Two major clusters of miRNAs have been found: one at the 5’ end of the meq oncogene (in R_L) and one at the 5’ end of the LAT (in R_S). As meq and LAT transcripts are expressed during latency, it has been suggested that these miRNAs are associated with latency and transformation [32]. Many of these miRNAs can actually be found in viral induced tumours [42]. For instance, MDV miRNA miR-M4 (encoded in the meq cluster) is the most abundantly expressed miRNA in viral tumours [43]. Interestingly, this miRNA is an ortholog of host miR-155 and overexpression of miR-155 leads to B cell lymphomas in mice [41, 44, 45]. Another example, viral miRNA miR-M7-5p (encoded in the LAT cluster) is highly expressed in MSB-1 cells as well as in MD tumours. It has been shown that MDV IE transactivator proteins ICP4 and ICP27 contain binding sites for miR-M7-5p. During reactivation miR-M7-5p levels have been shown to decrease and ICP27 levels increase suggesting that miR-M7-5p may have a role in maintenance or initiation of latency [32].
Two MDV miRNA clusters have been identified. One is located in the TR_L and IR_L repeat regions and defined as the Meq miRNA cluster. The second is located within the TR_S and IR_S repeat regions and defined as the LAT miRNA cluster. MDV miRNA miR-M4 within the Meq cluster is abundantly expressed in MDV induced tumours. MDV miRNA miR-M7 within the LAT cluster has also been found expressed in tumours as well as MD tumour-derived MSB-1 cell line. miRNA miR-M7 has a target site within the IE genes ICP4 and ICP27 suggesting its role in latency and tumorigenesis [32, 43, 44].

**Role of Meq**

A fundamental difference of MDV compared to other herpesviruses is that it encodes a Jun/Fos-like oncoprotein called Meq. Meq is expressed during latency and has been shown to play an essential role in the transformation of latently infected T-cells into cancerous cells [46]. Meq is a 339-amino acid protein and has a basic leucine zipper (bZIP) structure at its N-terminal end similar to other Jun/Fos family members [47]. Meq is able to form heterodimers with other bZIP proteins (e.g. c-Jun) or bind to another Meq protein to form a homodimer. Due its oncogenic properties, it has become the most investigated MDV protein and as such a few roles have emerged.

Meq has been shown to increase the transcription of genes involved in growth and anti-apoptotic pathways during MDV lymphomagenesis [48]. However, the transactivation properties of Meq depend on its dimerization partner. A Jun/Meq heterodimer for example activates ICP4 expression whereas a Meq/Meq homodimer represses MDV E protein, pp38, expression [49]. Interestingly, in a study using recombinant MDVs that encode modified meq genes, viruses with Meq that are able to
bind c-Jun but unable to form a homodimer lose their oncogenicity [49]. This suggests the importance of Meq homodimerization for lymphomagenesis.

Meq has also been shown to bind and transactivate the promoter of the host miRNA gga-miR-21. MiR-21 is overexpressed in human cancers and recent studies have illustrated that gga-miR-21 expression increased with MDV infection [50].

Despite Meq being arguably the most studied MDV protein, we still do not fully know the expression levels and kinetics of Meq during lytic infection, latent infection, and T-cell transformation. As only a few latently infected T-cells seem to undergo transformation, various expression levels of Meq in different subsets of CD4+ T cells could be an important trigger for MDV oncogenesis.

1.3. What is Marek’s Disease and how is it controlled?

The first report of MD dates backs to 1907 when a clinician of a veterinary school in Budapest, Jozef Marek, found inflamed nerves of four chickens. He also noted that the sciatic nerves and areas of the spinal cord were infiltrated with mononuclear cells. It wasn’t until the 1960s when the disease had spread to countries with successful poultry industries that it was finally named Marek’s Disease. It was in this era that a plethora of research on the disease began [9]. Due to the growing agriculture industry at this time, MD became increasingly problematic. As such, effort was placed on identifying MDV pathogenesis and potential MD treatments. In fact MD was such a large problem that the agriculture industry was starting to create poultry lines that were resistant to MD, but that meant losing genetic qualities favourable for the consumer market. In the 1960s, research into MD vaccines emerged and finally in the 1970s a breakthrough vaccine was created. The MD vaccine became the first ubiquitously used vaccine to combat oncogenesis occurring from a natural agent. Unfortunately, MDV became more virulent so vaccine breaks and sporadic outbreaks occurred which will be discussed in section 1.3.2.
1.3.1. Signs of Marek’s Disease

MDV infection causes bursa and thymus atrophy, which are associated with the lytic infection. As previously stated, MDV latently infected T-cells can become transformed and cause tumour nodules in visceral organs such as the spleen, heart and kidney. Bursa and thymus atrophy can be seen as early as 3 dpi, and tumours develop at approximately 14 dpi. It is important to note that the presence of tumours is a more reliable indication of MD incidence as bursa and thymus atrophy can be caused by other reasons such as stress. Another prominent clinical feature of MD is enlargement of brachial, sciatic and vagus nerves [9].

1.3.2. Vaccines

During the 1960s the aetiology of MD was established and the development of vaccines began. The success of the MD vaccine meant it was the first vaccine produced to combat cancer from an infectious agent [9]. The current MDV vaccines are live attenuated vaccines that are administered in ovo to all battery chicken eggs costing the US agriculture industry billions of dollars each year [1, 51].

In 1970, the first vaccine that was used against vMDV was HVT. However, in the late 1970s strains emerged that caused disease in HVT vaccinated birds. This vaccine break prompted the production of a new generation of vaccine against vvMDV strains. This second generation vaccine utilized a naturally avirulent MDV, gallid herpesvirus 3 virus, called SB-1 (Table 1-4, section 1.1.2). In the early 1980s vv+ MDV strains emerged in the field that were resistant to HVT-SB-1 vaccination. VV+ strain of MDV is currently controlled with an attenuated MDV-1 virus strain called Rispens. The vaccine is made by in vitro serial passage of the virus causing loss of virulence [9].

It is important to note that vaccination protects against the disease (i.e. tumourigenesis) but not infection. It has been suggested therefore that vaccination may play a role in driving the evolution of MDV virulence. This is because emerging virulent pathotypes can be linked to the introduction of a new generation of vaccine [52] (section 1.1.2). There have been other notable problems caused by administering the vaccine including contamination and misuse. The vaccine is administered in ovo to an embryo
that does not have a fully mature immune system. So if the vaccine is contaminated by other pathogens it can result in lethal complications. The MD vaccines are commonly prepared as infected cells rather than lyophilized cell-free virions due to the strict cell-associated nature of the virus, which requires liquid nitrogen storage. Therefore, they are extremely sensitive to mishandling. Unfortunately, in many developing countries they are often used in suboptimum conditions due to hot climates, transportation difficulties and lack of trained personnel. All of these factors can contribute to sporadic outbreaks of the disease in the field [1]. Despite these problems the MD vaccine is still extremely important to not only the industry but also to vaccine and cancer research. The only other tumour vaccine in existence is the human papillomavirus (HPV) vaccine. However, the HPV vaccine is made up of virus like particles that trigger specific immune responses and eliminate HPV from the host like many other viral vaccines. As previously mentioned, MD vaccine effect is completely unique in preventing tumourigenesis but not sterilizing.

**Vaccine Effect**

We are still unsure how the MD vaccine works because there are currently no tools to study MDV and vaccine infection patterns *in vivo*. However, we do know that MD vaccine effect is quite different from conventional viral vaccinations. Firstly, it does not require a mature immune system to provide protection [53]. The vaccines are inoculated *in ovo* when the embryonic immune system is not completely mature. Secondly, birds are protected as early as 5 days post vaccination [54] but it takes approximately 1 week for specific antibodies to be produced. It has been shown that MD vaccine virus decreases CD4+ T cell count in the host which suggests that the vaccine virus infects cells similarly to MDV [55].

There are noticeable similarities and differences between virulent MDV and the current MD vaccine virus. For example, MD vaccine virus still harbours *meq* oncogene even though it does not transform cells [56]. Interestingly, recent research has shown that a widely used vaccine strain, Rispens strain, has a variation in the LAT region compared to vv+ MDV strain [57].
In order to understand the mechanisms behind MDV vaccine effect, the \textit{in vivo} infection kinetics of both the vaccine and virulent virus must be uncovered. Unfortunately without appropriate tools or methodologies at hand this cannot yet be achieved.

<table>
<thead>
<tr>
<th>Table 1-4</th>
<th>MDV Vaccine Generation</th>
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<tr>
<td><strong>Generation</strong></td>
<td><strong>When it came to market</strong></td>
</tr>
<tr>
<td>1</td>
<td>Early 70s</td>
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<tr>
<td>2</td>
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<tr>
<td>3</td>
<td>Late 80s</td>
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* Current vaccine controlling MDV very virulent + strain that is circulating in the field.

1.4. **Marek’s Disease Virus Research Tools**

1.4.1. **Cells**

Despite a general lack of chicken-derived immortalized cell lines there are many MD tumour-derived lymphocytic cell lines available. The most common cell line that is used in MDV research is the aforementioned MSB-1 [58]. MSB-1 is an established cell line derived from MD lymphomas that developed in chickens. It contains MDV in the latent state and upon certain conditions virus replication can be reactivated.

To amplify MDV \textit{in vitro}, primary cells such as chicken embryo fibroblasts (CEF) or chicken kidney cells are used. Duck embryo fibroblasts are also susceptible to MDV lytic infection and also often used. MDV is able to replicate in these primary cells and causes cytopathic effect (CPE), which are visualized as plaques. However, no cell-free virions are shed in the culture supernatants. There are a couple of known chicken fibroblast-derived cell lines, DF-1 [59] and CHCC OU2 [60], but they are not commonly used in MDV amplification. This is because there are difficulties associated with either the handling these cells (OU2), or MDV replicates poorly within these cells and does not produce CPE (DF-1).
1.4.2. **Recombinant Viruses**

In the past 10 years, the chicken genome has been completely sequenced and several MDV strains have been cloned into bacterial artificial chromosomes (BAC). BAC is a cloning vector based on *Escherichia coli* F-plasmid [61]. In 1992, Shizuya *et al* took essential components of the F plasmid and established the first BAC cloning system [61]. The BAC plasmid system was ideal for cloning the entire MDV genome for several reasons: 1) It is able to maintain cloned DNA fragments of up to 300 kilobase pairs (kb), which can therefore easily accommodate the 177kb genome of MDV 2) BAC replication is highly controlled and it retains a low copy number of only one to two copies per cell [61]. This reduces the chance of recombination between DNA fragments. 3) MDV forms a circular genome as a replication intermediate. Thus the circular BAC plasmid that contains the entire MDV genome can be infectious upon introduction to susceptible cells.

In fact, MDV-BAC clones are infectious [62] and able to be genetically manipulated in *E.coli*. By using genetic recombineering methodology [63, 64], which utilizes the homologous recombination machinery encoded in lambda phage, many recombinant MDVs have been generated. A popular modification is to fuse a fluorescent protein to an MDV protein. This allows the viral protein to be visualized *in situ* [65]. For example green fluorescent protein (EGFP) was fused to the C-terminus of the MDV late gene UL47. This recombinant virus was used to illustrate that UL47 protein is abundantly expressed in feather follicle epithelia cells [66]. Unfortunately, a recombinant virus that had EGFP fused to viral UL49 lost its pathogenicity *in vivo*, so this technique is not 100% reliable [66]

1.4.3. **In Vitro Model of MDV infection**

Recently a new model to visualize MDV lytic infection, latent infection and transformation *in vitro* has been established [25]. A large obstacle for *in vitro* MDV research was that either a) the natural B cell target of MDV does not survive for an extended period of time *in vitro* b) cell lines that are available are not true to *in vivo* infection model i.e. fibroblasts are used to study lytic infection but these are not the natural target of MDV. By using a sequence of cytokines and monoclonal antibodies, Schermuly *et al.* (2015) [25] were able to promote the survival of infected B-cells *in vitro*. 
This means that MDV lytic infection in B-cells can be mimicked in vitro. Interestingly, these infected B-cells were able to infect isolated primary T-cells in vitro, which went on to show characteristics of latent infection. This in vitro system is a valuable tool to MDV research as it could be used to examine molecular events required for lytic infection, latency and T-cell transformation. However it cannot be used to investigate more complicated events such as the infection kinetics in a host or vaccine mechanisms that occur in vivo.

1.5. Concluding Comments

There is a large knowledge gap in the mechanisms of MDV latency and transformation. We do not know exactly how many cells are latently infected and how the virus transforms cells or even what types of T-cells are transformed. This is partly because of the strict cell associated nature of MDV. Due to this strict cell association, infecting isolated cells with MDV in vitro is very inefficient thus investigating molecular mechanisms in tissue cell culture is very difficult. Additionally, the only fully susceptible tissue culture cells are primary cells and they are not the natural targets of MDV. Even MDV tumour nodules consist of not only of transformed cells but reactivated infected cells and uninfected responding T lymphocytes. Recently, Schermuly et al (2015) [25] may have solved a part of these problems but the developed in vitro system requires a battery of reagents that are not commercially available. For the analysis of virus replication dynamics in vivo there is still currently no tool to easily and precisely identify MDV latently or transformed cells. My study will aim to construct recombinant viruses that express fluorescent proteins during latent infection. This will allow us to isolate latently infected populations to further analyse this complex and vital state of MDV infection.
Chapter 2.

Construction of Recombinant Marek’s Disease Virus to Visualize Latently Infected Lymphocytes \textit{in situ}

2.1. Introduction

Mechanisms involving MDV latency, T-cell transformation, and vaccine effect are three major areas in MDV research that have yet to be well defined. In order to reveal these mechanisms, a tool must be constructed that will allow us to identify and separate cells in different states of infection. Fortunately, recombinant MDVs have been constructed to specifically identify lytically infected cells for analysis. For example, using homologous recombination methodology \cite{67}, EGFP was fused to the 3’ end of an MDV late gene UL47 \cite{66}. This resulted in an infectious recombinant MDV that could be used to visualize lytically infected cells, and therefore further characterize lytic infection. Unfortunately, we currently do not have a tool that can identify latently infected cells \textit{in vivo}. This makes it extremely difficult to fully characterize key events required for latency and transformation. This chapter will demonstrate two recombinant viruses, rMd5-MiR7mCh and rMd5-LATmCh, which were constructed in an attempt to identify cells that were latently infected with MDV.

2.1.1. Fluorescent Proteins

One potential approach to identify cells latently infected with MDV is to construct a recombinant virus that expresses a fluorescent protein during latent infection. Fluorescent proteins are bioluminescent proteins derived from a variety of different organisms. The first fluorescent protein to be cloned was a green fluorescent protein (GFP), which was derived from jellyfish in 1992 \cite{68}. Since then more have been identified from a variety of species or mutated to produce a vast number of colors...
ranging from green, blue, yellow, and red. Because of their natural fluorescent properties, fluorescent proteins can be expressed in a cell of interest and used for live-cell or whole-animal imaging [69]. Fluorescent proteins can be fused to protein targets and expressed as a part of the target protein. This makes them very useful not only because external reagents are not required for visualizing protein location, they can also be used to quantitate protein expression. As fluorescent proteins come in many colours, the most appropriate must be carefully selected especially if multiple labelling is desired. Each fluorescent protein has a specific emission and excitation wavelength that is required for fluorophore visualization. If two fluorescent proteins are required the emission and excitation wavelengths of each protein must have minimal overlap so that they can be distinguished from one another. For this reason, mCherry was chosen for visualizing MDV latently infected cell. mCherry is a derivative of Red Fluorescent Protein (mRFP), which was isolated from Discosoma sp and has an excitation of 587nm and emission of 610nm [70]. As previously stated EGFP has already been successfully fused to MDV lytic protein UL47 [71]. As EGFP has an excitation peak of 395nm and emission peak at 509nm, the overlap with mCherry fluorophore is minimal. If the constructed recombinant MDV virus can identify MDV latently infected lymphocytes by mCherry expression, it will be useful to use this fluorescent protein and target site in combination with UL47-EGFP recombinant virus.

2.1.2. Identifying Targets for Fluorescent Protein Insertion

The paramount characteristic of latency is the expression of a limited set of viral transcripts, so the fluorescent protein insertion target choice is very restricted. In fact, there were three targets to choose from; Meq, viral microRNAs, LAT transcript. I chose two target sites for fluorescent protein gene insertion both of which are expressed during latency: the 3’ end of MDV mature miRNA miR-M7 and the 3’ end of the LAT (Chapter 1 Figure 1-3). Meq was not chosen as a potential target because I did not want to disrupt the function of the oncogene (see section 1.2.3). Removal of meq gene caused the complete attenuation of MDV without significant impact on the replication [72]. In order for the recombinant viruses to be used as a successful tool, their ability to cause disease must be comparable to parental virus.
**Figure 2-1  Schematic representation of LAT splicing and miRNA location**

LAT transcript, located in the IR$_S$ and TR$_3$ regions of MDV genome, has been shown to have diverse splicing patterns. The green boxes indicate the 15 LAT exons that have been suggested, the orange box indicates suggested LAT introns. The blue boxes indicate the locations of miRNA M8, M6, M7, and M10. The purple box to the right hand side of LAT exon 15 indicates the location of the LAT PolyA sequence. The red arrows indicate the proposed location for mCherry fluorescent protein insertion.

**MDV miR-M7 Target Site**

Two clusters of viral miRNAs have been identified within the MDV genome [37]: one mapping to *meq oncogene*, the other to the LAT region. Several of these miRNAs were found to be highly expressed in MSB-1 cell line and spleen lymphomas indicating their expression in latent infection [73]. The most abundant miRNA found in MSB-1 cells was miR-M7, which is located in the intron of the LAT (Figure 2-1). Northern blot hybridization confirmed the expression of miR-M7 in MSB-1 cells as well as in MDV splenic tumour cells but with weaker signal in lytically infected CEF cells [74]. MiR-M7 has been shown to decrease IE gene ICP27 by 70% [32]. ICP27 is a multifunctional virus protein and one of its roles is in post translational regulation by inhibiting splicing of both host and viral genes [32]. Another target of miR-M7 is ICP4. Although ICP4 function is not fully characterized, it has been shown to transactivate transcription of E genes, and in HSV-1 it is a crucial transactivator of early and late viral genes [27].

As miR-M7 is abundantly expressed in latent and transformed cells with minimal expression during lytic infection, this makes it an excellent target for fluorescent protein insertion. mCherry fluorescent protein gene was inserted to the 3’ end of the pre-miRNA
strand of miR-M7 (Figure 2-2). The mature microRNA structure is therefore not disrupted and minimal interruption of miRNA function is expected. As the miRNA transcript is not translated, for mCherry translation to occur a 5’ internal ribosome entry site (IRES) sequence and a 3’ polyadenylation (PolyA) signal were also inserted. Typically translation initiation is restricted to the 5’ end of mRNA transcript where the translation initiation complex binds to a 5’ cap nucleotide sequence. IRES is a nucleotide sequence that allows for the direct recruitment and binding of ribosomes to the mRNA transcript. Therefore the IRES sequence allows for translation initiation to occur at any site along an mRNA transcript, which in my case is at the 3’ end of pre-miRNA miR-M7 structure [75]. The resulting insertion construct was therefore IRES-mCherry-PolyA (Figure 2-2). When the resulting recombinant virus, described as rMd5-MiRM7-mCh-MDV, was used in vivo, we expected latently infected cells to emit mCherry fluorescence.
Figure 2-2  Schematic representation of mCherry gene insertion to the MDV genome at the 3' end of mirR-M7.

IRES and PolyA were also inserted at the 5' and 3' end of mCherry respectively for efficient mCherry translation. A) This schematic shows the insertion of IRES-mCherry-PolyA into the IR_s copy of miR-M7 in relation to the LAT transcript. As miR-M7 is situated in the repeat short region, it is present in both the IR_s and TR_s. IRES-mCherry-PolyA was inserted to both copies of miR-M7 regions B) This schematic illustrates the location of IRES-mCherry PolyA insertion into the 3' end pre-miR-M7. The top figure illustrates the insert location in terms of the MDV genome. The bottom figure illustrates the location in terms of the miR-M7 hairpin loop structure. The mature miR-M7 strand is depicted in yellow. The orange arrows indicate the location of IRES-mCherry-PolyA insertion.
**LAT Target Site**

The LAT is one of the few transcripts present in latency infected and transformed cells [37] and is therefore an obvious insertion target for fluorescent protein expression during latency. The 10kb LAT transcript has been found to have alternative splicing patterns resulting in a total of 22 transcript types [32]. It was found that all 22 transcripts share the 3’ end of exon 15 [32]. This made exon 15 situated at the 3’ end of LAT an ideal target for mCherry gene insertion (Figure 2-3). As before, IRES was also inserted to aid with translation at the 5’ of mCherry gene. PolyA signal was not required this time as the 3’ end of the LAT already contains a PolyA sequence. The resulting recombinant virus, described as rMd5-LATmCh, therefore has IRES followed by mCherry gene at the 3’ end of the LAT, 5’ to the PolyA sequence (Figure 2-3). When this virus infects T-cells *in vivo*, we expected latently infected cells to emit mCherry fluorescence.

![Schematic representation of mCherry gene insertion to the MDV genome at the 3’ end of LAT.](image)

**Figure 2-3**  Schematic representation of mCherry gene insertion to the MDV genome at the 3’ end of LAT.

IRES was inserted at the 5’ of mCherry for efficient protein translation. PolyA signal is not needed in this case as there is an endogenous signal at the 3’ end of the LAT sequence identified in purple. This schematic shows the insertion of IRES-mCherry into the IR\textsubscript{s} LAT. As the LAT is situated in the repeat short region, it is also present in the TR\textsubscript{s} in an inverted orientation. IRES-mCherry was inserted to both copies of LAT located in the IR\textsubscript{s} and TR\textsubscript{s} regions.
2.1.3. **Construction of recombinant viruses rMd5-MiR7mCh and rMd5-LATmCh**

An infectious BAC clone of very virulent MDV strain Md5 (Md5-BAC) was used to construct rMD5-MiR7mCh and rMd5-LATmCh recombinant viruses [61]. Inserting the fluorescent protein gene intoMd5-BAC relied on the markerless two-step Red mediated homologous recombination protocol established by Tischer *et al* (2006) [67]. Briefly a kanamycin resistance gene fragment, containing an *I*-sceI restriction site at the 5’ end, was cloned into the inserting sequence, IRES-mCherry-PolyA or IRES-mCherry (for rMD5-MiR7mCh and rMd5-LATmCh, respectively) (Figure 2-4 in materials and methods). *E. coli* strain SW105, which carries Md5-BAC plasmid, was then utilized for construct insertion. SW105 contains the λ prophage recombination proteins that are repressed by a temperature sensitive repressor [73]. The recombination machinery is only activated following an induction at 42°C [76]. By utilizing this machinery, the inserting sequence containing kanamycin resistance was inserted into the target site by homologous recombination (Figure 2-5 in materials and methods). Kanamycin was then utilized as a positive selection marker for clones that successfully received the inserting sequence. The kanamycin resistance gene was then removed so that the inserting sequence became intact. For kanamycin removal, SW105 with the MDV-BAC was transformed with a second plasmid, pBAD- *I*-sceI. pBAD- *I*-SceI encodes arabinose inducible *I*-sceI endonuclease. When *I*-sceI is induced, it cleaves the 5’ end of kanamycin resistance gene at the *I*-sceI restriction site. Homologous recombination was induced for a second time and the kanamycin resistant gene was cleaved out of the construct (Figure 2-6 in materials and methods).

2.1.4. **In vivo study of rMd5-MiR7mCh and rMd5-LATmCh MDV**

There is no reliable *in vitro* system to establish latently infected cells by MDV. Therefore, infecting chickens with the constructed recombinant MDV was essential to test the expression of inserted fluorescent gene in latently infected cells. Additionally, in previous studies the insertion of fluorescent protein gene to certain areas of the MDV genome severely attenuated the virus. For example, Jarosinski *et al.* inserted EGFP into the c-terminus of either UL47 or UL49 gene [66]. Only the recombinant virus that contained UL47-EGFP insertion remained pathogenic. The recombinant virus UL49-
EGFP became attenuated and challenged birds did not show signs of MD. The addition of EGFP to UL49 could have affected the structure of the encoded protein, VP22. VP22 is a major tegument protein in alphaherpesvirus and, although the exact role in MDV is not known, HSV-1 VP22 is essential for viral egress and cell-to-cell spread. By disrupting the structure of VP22 by the fusion of EGFP, viral spread could have been obstructed in vivo. Therefore, in order to examine whether virulence was maintained or not it was important to characterize constructed rMd5-MiR7mCh or rMd5-LATmCh in vivo by infecting specific pathogen free (SPF) chicks with either recombinant virus.

2.2. Materials and Methods

2.2.1. Cells and viruses

Primary chicken embryo fibroblasts (CEF) were prepared from 10 or 11 day old specific pathogen free chicken embryos (Canadian Food Inspection Agency). They were prepared by removing the head and trypsinizing the body. CEF cells were maintained at 37 °C in 5% CO₂ atmosphere in Eagle’s minimum essential medium (EMEM) supplemented (Sigma or Life Technologies) with 10 % Tryptose phosphate broth (TPB, Difco), antibiotics (penicillin and streptomycin, 100 units and 100 µg/ml, respectively, Life Technologies), and 4 or 10% fetal bovine serum (FBS, Fisher). Marek’s disease virus (MDV) strain Md5, a very virulent pathotype strain [13] was used as the positive infection control virus. MDV Md5 strain was obtained from USDA-ARS Avian Disease and Oncology laboratory (East Lansing, MI) and used at passage level 14.

Infectious bacterial artificial chromosome (BAC) containing the entire Md5 genome (Md5SN5 BAC) was used to construct recombinant viruses [62]. Md5SN5 BAC-derived virus remains pathogenic and causes extensive tumours in infected chickens.

2.2.2. PCR amplification

PCR amplifications throughout the construction used High Fidelity PCR System Kit (Roche). The following master mixes were prepared for each reaction: 2.5µl buffer with MgCl₂ (10x), 2µl dNTP (2.5mM stock), 1µl forward primer (5pmol), 1µl reverse
primer (5pmol), 1µl DNA template (1ng/µl), 0.5µl enzyme, made up to 25µl with reagent quality water (MilliQ system, Millipore, d.w.). All primers were purchased from Integrated DNA Technologies. PCR reactions used the following amplification conditions: 1) 94°C for 2 minutes, 2) 94°C for 1 minute, 3) 55°C for 1 minute, 4) 72°C for 1 minute, 5) steps 2-4 cycled 34 times, 6) 72°C for 4 minutes.

2.2.3. **Construction of IRES-mCherry-PolyA insertion structure**

*Insertion of mCherry gene into pBluescript–IRES*

IRES was first cloned to pBluescript. Briefly pBabe-puro-IRES-EGFP (Plasmid #14430, Addgene) was digested with Ncol enzyme. The plasmid was made blunt end using T4 polymerase and further digested with *Bam*HI enzyme, which excised the IRES. *Bam*HI-blunt end IRES fragment was then cloned into *Bam*HI and *Sma*I site of pBluescript II SK(+) resulting in pBluescript-IRES.

The *Pst*I restriction site in pBluescript-IRES was removed by digesting the plasmid with *Pst*I restriction enzyme followed by T4 DNA polymerase treatment (Promega) and self-ligation (Rapid DNA ligation kit, Roche). Ligated plasmid was recovered into DH5alpha bacteria (Life Technologies). mCherry gene (a plasmid containing mCherry was a gift from G. Prefontaine, Simon Fraser University) was PCR amplified with primers mc/o-RI-ATG-F and mc/o-TER-Xho-R (Table 2-1) so the mCherry gene was flanked with an *Eco*RI and *Xho*I restriction sites at 5’ and 3’ ends respectively. The PCR amplicon was cloned into pGEM-T Easy Vector (Promega) using Rapid DNA ligation kit. mCherry insertion was confirmed by *Eco*RI and *Xho*I digestion followed by agarose gel electrophoresis. The excised mCherry fragment was gel purified using QIAquick Gel Extraction Kit (Qiagen) and ligated to *Eco*RI and *Xho*I digested pBluescript-IRES with Rapid DNA ligation kit. Insertion of mCherry to the 3’ end of IRES was confirmed by *Eco*RI and *Xho*I digestion and gel electrophoresis. Successfully constructed plasmids were recovered into DH5alpha bacteria and designated as pBluescript-IRES-mCherry (Figure 2-4 A).
**Insertion of PolyA into pBluescript-IRES-mCherry**

The SV40 PolyA signal sequence from pEGFP-N1 (Clontech) was PCR amplified with primers PolyA-Xhol F and PolyA-Xhol R (Table 2-1) so the PolyA signal sequence was flanked with Xhol restriction sites at 3’ and 5’ ends. The PCR amplicon was cloned into pGEM-T Easy Vector using Rapid DNA ligation kit. PolyA signal sequence insertion was confirmed by Xhol digestion followed by agarose gel electrophoresis. The excised PolyA fragment was gel purified using QIAquick Gel Extraction Kit and ligated to Xhol digested pBluescript-IRES-mCherry with Rapid DNA ligation kit. Insertion and orientation of PolyA signal sequence to the 3’ end of mCherry was confirmed by sequencing (Eurofins MWG Operon). Successfully constructed plasmids were recovered into DH5alpha bacteria and designated as pBluescript-IRES-mCherry-PolyA (Figure 2-4 B).

**Insertion of mCherry partial overlap sequence into pBluescript-IRES-mCherry-PolyA**

A partial overlap sequence of mCherry must be present at the 5’ and 3’ end of a kanamycin resistance gene in order for its removal by a second recombination. A section of mCherry gene was therefore PCR amplified using primers m/co RI-ATG-F and mc 1-472-R-BglII-NSI (Table 2-1). The resulting PCR amplicon, designated 1-472-mCh, contained a 5’ portion of mCherry gene with a 5’ EcoRI and 3’ BgII and NsiI restriction sites. The PCR amplicon was cloned into pGEM-T Easy Vector using Rapid DNA ligation kit. 1-472-mCh insertion was confirmed by EcoRI and NsiI digestion followed by agarose gel electrophoresis. The excised 1-472-mCh fragment was gel purified using QIAquick Gel Extraction Kit and ligated to EcoRI and NsiI-compatible PstI digested pBluescript-IRES-mCherry-PolyA with Rapid DNA ligation kit. Successfully constructed plasmids were recovered into DH5alpha bacteria and designated as pBluescript-IRES-1-472mCh-mCherry-PolyA (Figure 2-4 C).

**Insertion of kanamycin resistance gene into pBluescript-IRES-1-472mCh-mCherry-PolyA**

Kanamycin resistance gene, containing an I-sceI restriction site at the 5’ end was obtained from pEPKan-S plasmid (pEPKan-S provided by K. Osterrieder, Cornell University). Kanamycin resistance gene was PCR amplified with primers BgIII-KnF and BgIII-KnR (Table 2-1) so the gene was flanked with BgIII restriction sites at 3’ and 5’
ends. The PCR amplicon was cloned into pGEM-T Easy Vector using Rapid DNA ligation kit. Kanamycin insertion was confirmed by *Bgl*II digestion followed by agarose gel electrophoresis. The excised kanamycin resistance gene fragment was gel purified using QIAquick Gel Extraction Kit and ligated to *Bgl*II digested pBluescript- IRES-1-472mCh-mCherry-PolyA with Rapid DNA ligation kit. The insertion and orientation of kanamycin resistance gene was confirmed by sequencing (Eurofins MWG Operon). Successfully constructed plasmids were recovered into DH5alpha bacteria and designated as pBluescript- IRES-1-472mCh-Kanamycin-mCherry-PolyA (Figure 2-4D).

2.2.4. **Insertion of IRES-mCherry-PolyA to the 3’ end of MDV miR-M7**

**Amplification of inserting fragment**

An inserting fragment was PCR amplified from pBluescript-IRES-1-472mCh–kanamycin-mCherry-PolyA with primers 3’MiR7-mChF and 3’MiR7-mChR (Table 2-1). The resulting PCR amplicon contained IRES-1-472mCh–kanamycin-mCherry-PolyA flanked by 50 base pair arms at the 5’ and 3’ end. The 50 base pair arms were homologous to the MDV genome insertion site (3’ end of the MDV miRNA MiRM7). The reaction mixture was *Dpn*I digested to destroy the template DNA and the PCR product was purified using QIAquick Gel Extraction Kit (Figure 2-5A).

**Preparing electrocompetent SW105 cells that contain MD5SN5 BAC plasmid**

The recombineering method, which utilizes a modified E. coli strain SW105 (SW105 obtained from Dr. D. Court Lab at National Cancer Institute, MA), was used to insert IRES-1-472mCh–kanamycin-mCherry-PolyA fragment into an infectious MDV-BAC clone, Md5SN5BAC. SW105 containing Md5SN5BAC (lab stock) was made electrocompetent and the recombination machinery induced following an established protocol [67]. Briefly, 500µl of an SW105-Md5SN5 BAC overnight culture was inoculated to 25ml of fresh lysogeny broth (LB) containing 25µg/ml of chloramphenicol. The culture was grown at 32°C until absorbance at 600 nm was between 0.5 and 0.7 Absorbance Units (AU). The recombination machinery was induced by incubating the culture at 42°C for 15 minutes. The bacteria was centrifuged at 0°C for 5 minutes at 5,000 rpm and the
pellet was re-suspended with 10ml of ice cold d.w. The bacteria was washed two additional times with d.w. and the final pellet was re-suspended with d.w. at twice the pellet volume.

**Homologous recombination of inserting fragment into MD5SN5 BAC plasmid**

Twenty-five µl of electrocompetent cells were electroporated with 2µl of gel purified inserting fragment (see 2.2.4 “Amplification of inserting fragment”) using Gene Pulser (BioRad) in a cuvette with 0.1 cm gap at 1.6kV. Bacteria was recovered with 1ml of LB and incubated for 2 hours at 32°C. Bacteria was spread onto LB agar plates containing 25µg/ml chloramphenicol and 50µg/ml of kanamycin and incubated at 32°C for 48 hours (Figure 2-5A). Clones were screened using PCR primers 3’MiR7-Mch-insert-1F and 3’MiR7-Mch-insert-1R (Table 2-1) that amplify the insertion junction. DNA from positive clones was purified using Qiagen Midiprep kit. BamHI restriction digestion was used to analyze banding pattern of modified verses parental Md5SN5BAC plasmids. Positive clones were carried forward for kanamycin resistance gene removal.

**Removal of kanamycin resistance gene from inserted fragment**

Positive clones of SW105 containing IRES-1-142mCh-kanamycin-mCherry-PolyA at the 3’end of MiR-M7 in MD5SN5BAC (Figure 2-5B) were made electrocompetent as above but without heat induction. One hundred ng of ampicillin resistant pBAD-I-SceI plasmid containing an arabinose-inducible expression of I-SceI (pBAD-I-SceI provided by K. Osterrieder) was transfected into 25µl of electrocompetent cells [67]. Bacteria was recovered with 1ml of LB and incubated for 1 hour at 32°C. Bacteria was spread onto LB agar plates containing 25µg/ml chloramphenicol, 50µg/ml of kanamycin and 50µg/ml ampicillin and incubated at 32°C for 48 hours.

An overnight culture of SW105 containing pBAD-I-SceI and the modified Md5SN5BAC was made in LB containing 25µg/ml chloramphenicol, 50µg/ml kanamycin and 50µg/ml ampicillin. One hundred µl of overnight culture was inoculated to 2ml of fresh LB containing 25µg/ml chloramphenicol, and 50µg/ml ampicillin and incubated for 2 hours at 32°C. An equal volume of LB containing 25µg/ml chloramphenicol, 50µg/ml ampicillin, and 1% arabinose was then added and incubated for an additional 2 hours at
32°C. The culture was then transferred to 42°C and incubated for 15 minutes to induce the recombination machinery. The bacteria was recovered for 1 hour at 32°C, plated on LB agar plates containing 25µg/ml chloramphenicol, 50µg/ml ampicillin and 1% arabinose, and incubated for 2 days at 32°C. Colonies were screened for their lack of kanamycin resistance by inoculating the bacteria onto replica LB plates with or without kanamycin. Modified Md5SN5BAC plasmids from kanamycin sensitive clones were purified using Qiagen Midiprep kit. BamHI restriction digestion was used to analyze banding pattern of modified verses parental Md5SN5 BAC plasmids (Figure 2-6A-B).

**Insertion of second IRES-mCherry-PolyA**

Homologous recombination of IRES-1-472mCh–kanamycin-mCherry-PolyA was repeated in order to modify the second copy of miRNA MiR-M7 that is present in the MDV genome. The primers and conditions were identical to the first insertion. However, an additional PCR screening step was required, before kanamycin resistance gene removal, to ensure the second copy did not homologously recombine with the existing IRES-mCherry-PolyA signal insert. Primers used for screening were 3'MiR7-Mch-insert-2F and 3'MiR7-Mch-insert-2R (Table 2-1). 5’ and 3’ junction was analyzed using PCR and sequencing (Eurofins MWG Operon). Modified Md5SN5BAC plasmids with IRES-mCherry-PolyA signal insert at the 3’ end of both miRNA MiR-M7 copies was designated as rMd5-MiR7mCh BAC.

**Removal of pBAD-Iscel plasmid**

SW105 containing rMd5-MiR7mCh BAC and pBAD-I-SceI was grown overnight in 5ml of LB containing 25mg/ml chloramphenicol at 32°C. The plasmids were purified using BAC PSI kit (Princeton Separations) and the final purified plasmids was re-suspended in 20µl of TE buffer (10 mM Tris, pH 7.5 containing 1 mM EDTA). The DNA was then diluted 1:100 with TE buffer. Two µl of diluted DNA was electroporated to 25µl of DH10B electrocompetent cells (Life Technologies) at 1.8kV in Gene Pulser. Bacteria was recovered for 1 hour at 37°C in 1ml LB. After recovery, 600µl was centrifuged at 5,000rpm for 3 minutes, re-suspended in 100µl of LB, and spread onto LB agar plates containing at 25mg/ml chloramphenicol. Plates were incubated at 37°C overnight. To test the removal of pBAD-I-SceI plasmid, colonies were screened for their lack of
ampicillin resistance by inoculating the bacteria onto replica LB plates with or without 50µg/ml ampicillin.

Figure 2-4  Schematic representation of the construction of IRES-mCherry-PolyA inserting fragment that contains kanamycin resistance gene
Each box represents a gene and the gene name is found below the box. The vertical blue lines above each box indicate restriction enzyme sites used. A. Insertion of mCherry gene into pBluescript-IRES plasmid. B. Insertion of PolyA signal into pBluescript-IRES-mCherry. C. Insertion of a part of mCherry gene (1-472mCh) into pBluescript-IRES-mCherry-PolyA. D. Insertion of Kanamycin resistance gene into pBluescript-IRES-1-472mCh-mCherry-PolyA.
Figure 2-5  Schematic representation of the homologous recombination of inserting sequence to target site

Each box represents a gene with the gene name found below the box. A. Homologous recombination of the inserting sequence into the MDV genome. MDV genome is represented by grey. The MDV sequence that was used for homologous recombination (depicted by “x”) is shown in orange (5’) and pink (3’). B. MD5SN5BAC plasmid after homologous recombination. IRES-1-472mCh-Kanamycin-mCherry-PolyA sequence is now inserted to the 3’ end of MDV miRNA MiRM7 following homologous recombination.
Figure 2-6  Schematic representation of the removal of kanamycin resistance gene from the inserted sequence

A. I-sceI endonuclease encoded on pBAD-I-sceI plasmid was induced by incubation with 1% arabinose which cleaved the I-sceI restriction site at the 5’ end of kanamycin resistance gene. B. Homologous recombination machinery was induced by incubation at 42°C for 15 minutes. This promoted a second recombination between two homologous regions of mCherry gene resulting in the removal of kanamycin resistance gene. “V” illustrates I-sceI cleavage at the 5’ end of Kanamycin resistance gene. “X” illustrates homologous recombination which cleaved out Kanamycin resistance gene.
2.2.5. **In vitro analysis of rMd5-MiR7mCh**

*Restriction digest of rMd5-MiR7mCh with BamHI of PstI enzyme*

rMd5-MiR7mCh BAC and parental Md5SN5 BAC were purified from DH10B cells by Plasmid Midiprep kit (Qiagen). Approximately 400ng of DNA was digested with BamHI or PstI enzyme. After 1 hour incubation at 37°C, DNA was ethanol precipitated and re-suspended in 10µl of TE. Samples were run on a 0.6% agarose gel for 3 hours at 135 volts. The gel was stained after electrophoresis with SYBER safe DNA gel stain (Life Technologies) and bands were visualized with UV light.

*Southern blot analysis of rMd5-MiR7mCh*

Agarose gel, that contained electrophoretically separated BamHI or PstI digested rMd5-MiR7mCh BAC and Md5SN5BAC fragments, was submerged in 0.2N HCl for 10 minutes with gentle agitation. The DNA was denatured by placing the gel in a solution containing 1.5M NaCl and 0.5N NaOH for 45minutes with agitation. The gel was then rinsed with d.w. and neutralized by soaking with 1M Tris (pH7.4) containing 1.5M NaCl for 30 minutes with agitation. Solution was replaced and gel was washed for an additional 15 minutes. By capillary action of 10x Saline Sodium Citrate buffer (20x stock made with 0.3M sodium citrate, 3M sodium chloride adjusted to pH7.0 with 1M HCl), DNA fragments separated by the electrophoresis were transferred overnight from the agarose gel to a nylon membrane (Biodyne, Pall). DNA was cross-linked to the membrane by 30 second exposure to UV light. mCherry probe that targets the entire open reading frame was prepared by PCR amplifying mCherry gene using primers mc/o-RI-ATG-F and mc/o-TER-Xho-R (Table 2-1). PCR fragment was gel purified using QIAquick Gel Extraction Kit and recovered DNA was denatured by boiling for 10 minutes. The purified and denatured fragment was labeled with DIG by DIG-High Prime labeling and detection starter kit I (Roche) according to the manufacturer instruction. Probe hybridization and detection was performed using the same kit as instructed. After detection of mCherry gene, the membrane was stripped as per kit instructions and re-probed with Md5SN5BAC probe. Md5SN5 BAC probe was prepared by DpnI-digesting Md5SN5BAC plasmid and labelling resulting fragments with DIG using DIG-High Prime labeling and detection starter kit I.
**Generation of rMd5-MiR7mCh-MDV**

rMd5-MiR7mCh BAC DNA was transfected to a CEF monolayer in order to generate recombinant MDV. Transfected CEF was designated rMd5-MiR7mCh-MDV. Briefly, 1µg of rMd5-MiR7mCh BAC DNA was transfected to 3x10^6 CEF using Primary Fibroblast Nucleofection kit and program O-008 on Nucleofector (Lonza). Following nucleofection, cells were retrieved by 37°C EMEM containing 10% TPB, 10% FBS with 100U/ml penicillin and 100µg/ml streptomycin and placed into a 35mm dish. When the cells formed a confluent monolayer, FBS concentration in the medium was reduced to 4 %. After 7 days, transfected CEF were dislodged by 0.025% trypsin with 0.002% EDTA and passed to a 10cm dish containing fresh CEF monolayer. After 7 days, the monolayer became infected and was passed as above but this time into two 15cm dishes containing fresh CEF monolayers. Passages were repeated until the cytopathic effect (CPE) spread over the entire monolayer. In this case a total of 4 passages was carried out. The infected monolayers with extensive CPE (passage 4) were trypsinized as above and aliquots were frozen in 45% EMEM, 45% FBS and 10% DMSO. The recombinant MDV infected CEF, rMd5-MiR7mCh-MDV, aliquots were stored in liquid nitrogen.

**Titration of rMd5-MiR7mCh-MDV and mCherry visualization**

Titration of passage 4 rMd5-MiR7mCh-MDV was carried out in 6-well plates that contained fresh CEF monolayers. rMd5-MiR7mCh-MDV was diluted in EMEM containing 10% TPB and 10% FBS to various concentrations. Each dilution was tested in triplicate by placing 200µl of each onto fresh CEF monolayers. After 7 days, viral plaques were counted under an inverted microscope and plaque forming units/ml (pfu/ml) calculated. Plaques were also analyzed under a fluorescent inverted microscope with Texas Red filter to visualize mCherry expression.

**Growth curve of rMd5-MiR7mCh-MDV and MDV strain Md5**

To test if the replication of rMd5-MiR7mCh-MDV was comparable to the parental Md5, 100 pfu of each was dispensed onto 4 fresh CEF monolayers in a 6 well plate. Monolayers at 1, 3, 5, and 7 dpi were trypsinized and frozen at -80°C in 45% EMEM, 45% FBS and 10% DMSO. Collected monolayers were then titrated by infecting CEF
monolayers with various dilutions in duplicate. After 7 days, plaques were counted using inverted microscope.

2.2.6. **In vivo analysis of rMd5-MiR7mCh-MDV**

**Chicken experiments**

One-day-old specific pathogen free chicks (purchased from the Canadian Food Inspection Agency) were challenged intra-abdominally with either 500 pfu of very virulent strain Md5 at passage 14 (22 birds, positive infection control) or 500 pfu of rMd5-MiR7mCh-MDV at passage 4 (21 birds). Twelve birds were inoculated similarly with the EMEM diluting medium as mock infected controls. Three birds from each group were euthanized and necropsied on days 3, 13, 24, 41 post infection. Birds were also euthanized and necropsied when they reached experimental endpoint, which was defined by visual symptoms of sickness such as lethargy, emaciation, and down-turned wings. Pathological changes such as thymus and bursa atrophies as well as any noticeable tumours were recorded. Total MD signs were statistically analysed with Fisher’s test using GraphPad software.

At necropsy the spleen, thymus, bursa and blood were collected for further analysis. Peripheral blood mononuclear cells (PBMC) were separated from whole blood using Histopaque 1077 (Sigma-Aldrich) as recommended by the manufacturer and stored at -80°C. Thymus, spleen and bursa tissues were submerged in Tissue-Tek O.C.T compound (Sakura), snap frozen in liquid nitrogen and stored at -80°C.

All the experiments using animals were performed according to the guidelines set by Canadian Council on Animal Care and under prior approval of Animal Care Committee of Simon Fraser University (Permit numbers 932HS-09 and 1101HS-09).

**Viral genome quantification by quantitative PCR (qPCR)**

DNA was purified from collected PBMC using DNeasy Blood and Tissue kit (Qiagen). Concentration was quantified using NanoDrop instrument and all samples were adjusted to 50ng/µl, so that 100ng can be used per qPCR reaction. A standard curve of MDV genome copy number was prepared. To do this, the copy number of
purified Md5SN5BAC was calculated based on DNA concentration and plasmid size. The Md5SN5BAC was then diluted to achieve a standard curve ranging from $5 \times 10^7$ to 50 copies. To standardize the reactions, the diluted BAC plasmids were mixed with 100ng of chicken genomic DNA. Chicken genome standard curves were also made using CEF DNA of known copy number from $5 \times 10^4$ to 50 copies based on the cell counts before DNA extraction.

Quantifying MDV genome copy number by qPCR analysis was performed using primers Meq qPCR F and Meq qPCR R (which amplify a part of MDV meq gene) along with Meq qPCR probe (Table 2-1). Meq qPCR probe contains an internal quencher “Zen” and a 3’ NFQ-MGB quencher “31ABkFQ” with Fam reporter. The final concentration of Meq forward and reverse primers was 0.5µM and 0.05µM for the Meq qPCR probe.

Quantifying genomic DNA copy number by qPCR analysis was performed using primers iNOS- qPCR F and iNOS- qPCR R (that amplify a part of chicken inducible nitric oxide synthase (iNOS) gene) [77] along with iNOS qPCR probe (Table 2-1). iNOS qPCR probe contains a 3’ NFQ-MGB quencher “31AbRQS” and Tamra reporter. The final concentration of iNOS forward and reverse primers was 0.4µM and 0.2µM for the iNOS qPCR probe.

All reactions were done in a total volume of 12µl containing 6µl Kapa probe fast qPCR master mix (Kapa Biosystems) and in triplicate on ABI step-one plus instrument (Life Technologies). The PCR cycles used was as follows: holding 95°C for 1 minute, cycling 95°C for 2 seconds, 60°C for 20 seconds and repeated for 40 cycles.

Standard curves were constructed in Excel using average Ct value of the triplicate reactions vs. known copy number. This was used to interpolate copy number of samples tested. Values obtained from mock infected PBMC DNA were subtracted from Md5 and rMd5-MiR7mCh-MDV. Unpaired Student T-test was performed by Prism 6 (Graphpad Software Inc.) to analyze significant differences between samples.
**Flow cytometry and Immunohistochemistry**

PBMC from rMd5-MiR7mCh-MDV, parental Md5 or mock infected birds were analyzed with a flow cytometry using 561nm laser for mCherry expression, and blue laser 530/30nm for CD4. FloJo software was used for analysis.

Frozen tissue sections were made using a cryotome at 5µm thickness. The sections were placed onto glass slides and fixed with 4% paraformaldehyde for 15 minutes. To block endogenous peroxidase activity, slides were incubated in 0.3% H₂O₂ solution for 10 minutes at room temperature. Slides were incubated in a blocking solution (10% FBS in PBS) for 1 hour. A polyclonal antiserum specific to Meq oncoprotein [78] (a gift from J. Dunn, USDA) was diluted to 1:3200 in PBS. Each tissue section was incubated with the diluted antiserum at room temperature for 30 minutes followed by 5 PBS washes. Anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch) was applied at 1:500 dilution in PBS at room temperature for 30 minutes. After 5 PBS washes, DAB (3, 3'-diaminobenzidine) HRP substrate kit was used as per manufacture’s instruction (Vector laboratories). After HRP staining, a drop of ProLong Gold Antifade with Dapi (Life Technologies) was placed onto the tissue section before a cover glass was positioned. Slides were visualized for Meq staining using an upright microscope at 40x magnification.
Table 2-1  Primer and probe sequences used for construction and analysis of recombinant Md5SN5BAC plasmid

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mc/o-RI-ATG-F</td>
<td>5’-GAATTCACCATGGTGAGCAAGGGCGAGGAG-3’</td>
</tr>
<tr>
<td>mc/o-TER-Xho-R</td>
<td>5’-CTCGAGTCATTACTTTGACTGCTTGAGCGCCCTGGCAGCC-3’</td>
</tr>
<tr>
<td>PolyA-XhoI F</td>
<td>5’-CTCGAGGGCCCGGACTCTAGATCATAATC-3’</td>
</tr>
<tr>
<td>PolyA-XhoI R</td>
<td>5’-CTCGAGGATAGATGATGTTGGAACAAGAC-3’</td>
</tr>
<tr>
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<td>5’-ATGCATAGATCTCGGGGTACATCC-3’</td>
</tr>
<tr>
<td>BglIII-KnF</td>
<td>5’-AGATCTAGGATAACAGGGATCGATT-3’</td>
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<tr>
<td>BglIII-KnR</td>
<td>5’-AGATCTCAACAAATTAACCATCTGATT-3’</td>
</tr>
<tr>
<td>3’MiR7-mChF</td>
<td>5’-GAGATCCCGATCTCTACAGCAAACGATCTCTACGAGATTACAGTTGACCCCTTCCTCCCCTC-3’</td>
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<tr>
<td>3’MiR7-mChR</td>
<td>5’-GAGATGGAGCGTTCTAGGATAGGCAAGGACACATTTCCTCCCTCCCTC-3’</td>
</tr>
<tr>
<td>3’MiR7-Mch-insert-1F</td>
<td>5’-CGAGCTGTACAAGTAAT-3’</td>
</tr>
<tr>
<td>3’MiR7-Mch-insert-1R</td>
<td>5’-TGCAAAGCCATCC-3’</td>
</tr>
<tr>
<td>3’MiR7-Mch-insert-2F</td>
<td>5’-ATGTTGTCGTTGATGATC-3’</td>
</tr>
<tr>
<td>3’MiR7-Mch-insert-2R</td>
<td>5’-TACATGTTGTTGAGC-3’</td>
</tr>
<tr>
<td>iNOS-qPCR F</td>
<td>5’-GAGTGGTAAAGGAGTTGATGCT-3’</td>
</tr>
<tr>
<td>iNOS-qPCR R</td>
<td>5’-TTCCAGACCTCCACCTCAA-3’</td>
</tr>
<tr>
<td>iNOS-qPCR probe</td>
<td>5’-/56-TAMN/CTCTGCTGCTGTTGCAACATGC/31AbRQSp/-3’</td>
</tr>
<tr>
<td>Meq qPCR F</td>
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</tr>
<tr>
<td>Meq qPCR R</td>
<td>5’-TGAGGGAGGAGTGAGTGCAAAT-3’</td>
</tr>
<tr>
<td>Meq qPCR probe</td>
<td>5’-/56-FAM/TAACGGATGA/ZEN/CCCCTGACTGCTTAACACCC/31ABkFQ/-3’</td>
</tr>
</tbody>
</table>

Underlined sequence indicates homology to MDV genome
2.2.7. Insertion of IRES-mCherry to the 3’ end of MDV LAT

Amplification of inserting fragment

An inserting fragment was PCR amplified from pBluescript-IRES-1-472mCh–kanamycin-mCherry-PolyA (from section 2.2.3) with primers 3’LAT-mChF and 3’LAT-mChR (Table 2-2). The resulting PCR amplicon contained IRES-1-472mCh–kanamycin-mCherry flanked by 50 base pair arms at the 5’ and 3’ end. The 50 base pair arms were homologous to the insertion site in the MDV genome (3’ end of the MDV LAT). The reaction mixture was DpnI digested to destroy the template DNA and the PCR product was purified using QIAquick Gel Extraction Kit.

Homologous recombination of inserting fragment into MD5SN5BAC plasmid

Homologous recombination of the IRES-1-472mCh-kanamycin-mCherry inserting sequence to MD5SN5BAC in SW105 bacteria was done as described in section 2.2.4. Positive clones were screened using PCR primers 3’LAT-mCh-insert-1F and 3’LAT-mCh-insert-1R (Table 2-2) that amplify the insertion junction. DNA from positive clones was purified using Qiagen Midiprep kit. BamHI restriction digestion was used to analyze banding pattern of modified verses parental Md5SN5BAC plasmids. Positive clones were carried forward for kanamycin resistance gene removal.

Removal of kanamycin resistance gene from inserted fragment

The kanamycin resistance gene was removed from a positive clone as described in section 2.3.4.

Insertion of second IRES-mCherry-PolyA

Like miRNA MiR-M7, the LAT region is repeated in the MDV genome therefore a second insertion of the mCherry construct was necessary. Homologous recombination of IRES-1-472mCh–kanamycin-mCherry was repeated in order to modify the second copy of LAT that is present in the MDV genome. The primers and conditions used were identical to the first insertion. However, an additional PCR screening step was required before kanamycin removal, to ensure that the second copy did not homologously recombinize to the existing IRES-mCherry insert. Primers used for screening were
3’MiR7-Mch-insert-2F and 3’LAT-mCh-insert-1R (Table 2-2). 5’ and 3’ junction was analyzed using PCR and sequencing (Eurofins MWG Operon). Modified Md5SN5 BAC plasmids with IRES-mCherry insert at the 3’ end of both LAT copies was designated as rMd5-LATmCh BAC.

**Removal of pBAD-Iscel plasmid**

After successfully modified BAC clones had been identified the co-existing pBAD-I-scel was removed by transferring it to DH10B bacteria as described in section 2.2.4. The BAC clone was designated as rMd5-LATmCh BAC.

**2.2.8. In vitro analysis of rMd5-LATmCh**

**Restriction digest analysis of rMd5-LATmCh BAC with BamHi or KpnI enzyme**

rMd5-LATmCh BAC and parental Md5SN5BAC were purified from DH10B cells by Plasmid Midiprep kit. Approximately 400ng of DNA was digested with BamHI or KpnI enzyme. After 1 hour incubation at 37°C, DNA was ethanol precipitated and re-suspended in 10µl of TE. Samples were run on a 0.6% agarose gel for 3 hours at 135 volts. The gel was stained after electrophoresis with SYBER safe DNA gel stain and bands were visualized with UV light.

**Southern blot analysis of rMd5-LATmCh BAC**

Agarose gel that contained electrophoretically separated BamHI or KpnI digested rMd5-LATmCh BAC and Md5SN5BAC fragments were subject to Southern blot analyses as described in section 2.2.5.

**Generation of rMd5-LATmCh-MDV**

As in section 2.2.5, rMd5-LATmCh BAC DNA was transfected to CEF cells in order to create working recombinant MDV stock, which was designated rMd5-LATmCh-MDV. rMd5-LATmCh-MDV was used in all experiments at passages 5.
**Titration of rMd5-LATmCh-MDV and mCherry visualization**

Titration of rMd5-LATmch-MDV (passage 5) was performed as described in section 2.2.5.

**Growth curve of rMd5-LATmCh-MDV and MDV strain Md5**

To test if the replication of rMd5-LATmCh-MDV was comparable to parental Md5, 100 pfu of each was dispensed onto 4 fresh CEF monolayers in a 6 well plate. Monolayers at 1, 3, 5, and 7 dpi were trypsinized and frozen at -80°C in 45% EMEM, 45% FBS and 10% DMSO. Collected cells were then titrated by infecting CEF monolayers with various dilutions in duplicate. After 5 days, viral plaques were observed by staining monolayers with a monoclonal antibody against MDV glycoprotein B [79]. Briefly, monolayers were fixed with methanol-acetone (1:1) for 5 minutes at room temperature and washed with PBS. Monolayers were incubated at room temperature for 1 hour with anti-MDV glycoprotein B (1 in 4 dilution of a hybridoma culture supernatant). Monolayers were washed three times with PBS. Anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch) was applied at 1:250 dilution (in PBS) and monolayers were incubated at room temperature for 1 hour. Monolayers were wash three times with PBS. DAB (3, 3’-diaminobenzidine) HRP substrate kit was used as per manufacture’s instruction to visualize antibody staining (Vector laboratories).

**2.2.9. In vivo analysis of rMd5-LATmCh-MDV**

**Chicken experiments**

Three-day-old specific pathogen free chicks (Canadian Food Inspection Agency) were challenged intra-abdominally with 500pfu of either of parental Md5 at passage 14 (6 birds, positive infection control) or 500pfu of rMd5-LATmChMDV at passage 5 (11 birds). Five birds were inoculated similarly with the EMEM diluting medium as mock infected controls. Three birds from experimental group and 1 from each control group were euthanized on 3, 13, and 41 dpi. Birds were also euthanized and necropsied when they reached experimental endpoint, which was defined by visual symptoms of sickness.
such as lethargy, emaciation, and down-turned wings. Pathological changes such as thymus and bursa atrophies as well as any noticeable tumours were recorded.

At necropsy the spleen, thymus, bursa and blood were collected for further analysis. PBMC were separated from whole blood using Histopaque 1077 (Sigma-Aldrich) as recommended by the manufacturer and stored at -80°C. Thymus, spleen and bursa tissues were submerged in Tissue-Tek O.C.T compound (Sakura), snap frozen in liquid nitrogen and stored at -80°C.

**Viral genome quantification by qPCR**

All qPCR analysis was performed as described in section 2.2.6.

**Flow cytometry and Immunohistochemistry**

PBMC from rMd5-LATmCh-MDV, parental Md5 or mock infected birds were stained with anti-chicken CD4 monoclonal antibody [80] and FITC-conjugated Goat-anti mouse antibody (Jackson ImmunoResearch). Fluorescence was analyzed with a flow cytometry using 561nm laser for mCherry expression, and blue laser 530/30nm for CD4. FloJo software was used for analysis.

Immunohistochemistry analyses were performed as described in section 2.2.6.

**Table 2-2 Primer and probe sequences used for construction and analysis of recombinant Md5SN5 BAC plasmid**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’LAT-mChF</td>
<td>5’TTCCCTGACTTGAACAGGGGAAGGGGAGGGGAGTGGTTATCTTGTGATCCGCCCCCTCTCCCCCCCCCCCC -3’</td>
</tr>
<tr>
<td>3’LAT-mChR</td>
<td>5’TATAAAAAAGAGAATCTCGTATAGGCCAATAATTATTTATGGGTTCTGATTACTGTACAGCTCGTCCATGCAGC -3’</td>
</tr>
<tr>
<td>3’MiR7-Mch-insert-2F</td>
<td>5’-ATGTGGGTATGGCTATTATGATC -3’</td>
</tr>
<tr>
<td>3’LAT-mCh-insert-1F</td>
<td>5’-ACGAGGACTACACCACCTCGTGGAAAC -3’</td>
</tr>
<tr>
<td>3’LAT-mCh-insert-1R</td>
<td>5’-AGACACCGTGTTAGCTCGTGCG -3’</td>
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</tbody>
</table>

Underlined sequence indicates homology to MDV genome
2.3. Results

2.3.1. In Vitro analysis of rMd5-MiR7mCh

*Restriction fragment and Southern blot analyses of recombinant rMd5-MiR7mCh BAC*

With *Bam*HI digestion the inserted sequences in rMd5-MiR7mCh BAC increased the sizes of two very large 20836bp and 14743bp fragments of Md5SN5BAC to 22399bp and 16306bp, respectively. Due to the large sizes of these fragments no apparent difference in the banding patterns between rMd5-MiR7mCh and parental Md5SN5BAC was noticeable (Figure 2-7A).

With *Pst*I digestion, Md5-MiR7mCh BAC should have lost two bands at 6863 and 5813bp and acquired three bands at 6158bp, 5108bp and a duplet at 2268bp compared to the parental Md5SN5BAC. The restriction enzyme cut the Md5SN5BAC plasmid within the highly repetitive *α*-like sequences that exist at the junctions of TRS/TRL and IRS/IRL [81], so the accuracy of the sequence was not known. Thus, bands migrated to different positions from the predicted sizes (Figure 2-7B). The two extra 5108bp and 6158bp bands in rMd5-MiR7mCh BAC migrated to unpredicted positions: 5108bp fragment likely migrated to a slightly larger position of 5258bp fragment; the 6158bp fragment likely co-migrated with a 7500bp fragment which is common to parental and recombinant BAC. Finally the 2268bp extra duplet band for rMd5-MiR7mCh BAC likely co-migrated with common 2280 and 2282bp bands. Therefore the restriction pattern for rMd5-MiR7mCh BAC lacked 2 bands at 5108bp and 6158bp and contained one additional band above 5258bp band compared to that of Md5SN5BAC.

To visualize the insertion of mCherry gene, the agarose gel containing *Bam*HI or *Pst*I digested rMd5-MiR7mCh-BAC or Md5SN5BAC was further analysed by Southern blot using mCherry gene and Md5SN5BAC probes (Figure 2-8 A and B). mCherry gene probe bound to a band at approximately 22kb on *Bam*HI digested rMd5-MiR7mCh. This corresponded to the predicted 22,399bp mCherry position (Figure 2-8A). mCherry probe bound to two bands at approximately 2kb and 5kb on *Pst*I digested rMd5-MiR7mCh. This corresponded to the predicted mCherry gene-containing band positions of 2268bp
and 5108bp (Figure 2-8B). Both restriction digestion and Southern blot analysis confirmed the integrity of rMd5-MiR7mCh BAC DNA.

Figure 2-7  Analysis of rMd5-MiR7mCh BAC vs. parental MD5SN5 BAC by restriction enzyme

DNA of rMd5-MiR7mCh BAC and parental MD5SN5BAC were digested with BamHI (panel A) or PstI (panel B) restriction enzymes and ran on a 0.6% agarose gel to analyse banding pattern. A. Two rMd5-LATmCh BAC clones vs. parental MD5SN5BAC look comparable which is expected. B. rMd5-MiR7mCh BAC clones digested with PstI. Red arrows indicate the two bands at approximately 6863 and 5813bp, which are expected to be missing. The green arrows illustrate expected additional bands; the first number indicates the likely position of the band due to the unknown repetitive a-like sequence and the number in the bracket indicates the predicted band size.
Figure 2-8  Analysis of rMd5-MiR7mCh BAC vs. parental Md5SN5 BAC by Southern blot.

Following gel electrophoresis, BamHI or PstI digested rMd5-MiR7mCh BAC and Md5SN5BAC DNA were subjected to Southern blot analyses. DIG labeled probes were used to detect mCherry gene and entire MDV genome. A. A band containing mCherry gene after BamHI digestion is located at 22,399bp and is indicated by white arrow. B. Bands containing mCherry gene after PstI digestion are located at 5108 and 2268bp and are indicated by green arrows. In the merged images, mCherry gene-containing fragments are indicated as red bands.
**In vitro replication analysis of rMd5-MiR7mCh-MDV**

Replication of rMd5-MiR7mCh-MDV at a passage level 4 was compared to the parental MDV strain Md5 at a passage level 14 (Figure 2-9). Both viruses replicated to approximately 860 plaques by 7 dpi. The result indicates that the replication of rMd5-MiR7mCh-MDV and the MDV Md5 strain are comparable *in vitro*.

![Graph showing replication of rMd5-MiR7mCh-MDV compared to Md5 MDV strain](image)

**Figure 2-9** Replication of rMd5-MiR7mCh-MDV compared to Md5 MDV strain *in vitro*

Confluent CEF monolayer was infected with 100pfu of rMd5-MiR7mCh-MDV (red line) or Md5 MDV strain (green line). Monolayers were collected at 1, 3, 5, and 7 dpi and titrated on fresh monolayers. Recombinant virus replication was comparable to MDV Md5 strain as they both reached approximately 860 plaques by 7 dpi.
In vitro expression of mCherry fluorescence in rMd5-MiR7mch-MDV

rMd5-MiR7mch-MDV infected CEF monolayer was analyzed for mCherry expression with fluorescence microscopy (Figure 2-10). mCherry fluorescence could be visualized only within the cells of viral plaques (Figure 2-10 A) but not within viral plaques of Md5 positive infection control (Figure 2-10 B). This confirmed that mCherry from constructed recombinant virus, rMd5-MiR7mch-MDV, is expressed in vitro.

Figure 2-10 Fluorescent microscopy of CEF cells infected with either Md5 or rMd5-MiR7mCh-MDV virus
A. Viral plaque caused by rMd5-MiR7mCh-MDV is illustrated with black circle. mCherry fluorescence was detected within the viral plaque using a fluorescent microscope. B. Viral plaque caused by Md5 parental virus is illustrated with black circle. mCherry fluorescence was not detected in the viral plaque.
2.3.2. *In Vivo* analysis of rMd5-MiR7mCh-MDV

**Pathogenicity of rMd5-MiR7mCh-MDV**

One-day old SPF chicks were infected with rMd5-MiR7mCh-MDV or parental MDV strain Md5, or mock infected (diluting media). At 3 dpi, no pathological signs of MD could be seen in any infection group (0 out of 3 necropsied birds). At 6 dpi the first signs of pathological change, thymus atrophy, could be seen in 1 out of 3 MDV Md5-infected birds (Table 2-3). At 13 dpi, 2 out of 3 Md5-infected birds developed splenic tumours as expected. However, only 1 out of 3 birds in the rMd5-MiR7mCh MDV recombinant virus group developed a splenic tumour. From 23 dpi until termination all birds examined in the Md5-infected positive control group had pathological signs of MD and tumours in spleen (1 out of 1, 3 out of 3, 6 out of 6, and 3 out of 3 for 23, 24, 26 and 41 dpi, respectively) (Table 2-4). Tumour nodules in the kidney, stomach, and heart could also be seen in 70% of birds (data not shown). In contrast, rMd5-MiR7mCh-MDV infected birds did not have any tumour nodule development at day 24-post infection and 41 dpi (0 out of 3, and 0 out of 8 respectively). However, at day 26 post infection 1 bird developed a splenic tumour. As expected, birds in the negative group did not show signs of MD throughout the study.

Only 3 our of 21 birds in the rMd5-MiR7mCh-MDV test group showed MD associated pathological signs (nerve enlargement, thymus, or bursa atrophy) compared to 17 out of 22 in the Md5 infected positive control (P=<0.001). Additionally, only 2 out of 21 of birds in the rMd5-MiR7mCh-MDV test group developed splenic tumours compared to 15 out of 22 in the Md5 infected positive control (P=<0.001). Thus, the virulence of rMd5-MiRmCh-MDV was significantly attenuated and not comparable to the parental Md5 strain.
Table 2-3  Number of birds that showed MD associated pathological signs (nerve enlargement, thymus or bursa atrophy) at necropsy

<table>
<thead>
<tr>
<th>Group</th>
<th>Numbers of birds showing MD associated pathological signs (nerve enlargement, thymus or bursa atrophy)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>3dpi*</td>
</tr>
<tr>
<td>Md5 (n=22)</td>
<td>0/3</td>
</tr>
<tr>
<td>rMd5-MiR7mCh (n=21)</td>
<td>0/3</td>
</tr>
<tr>
<td>Negative (n=12)</td>
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</tr>
</tbody>
</table>

n= Total number of birds in that group  
* Days post infection  
** No animals at this time point  
*** Total animals with signs of MD (nerve enlargement, thymus or bursa atrophy)

Table 2-4  Number of birds that developed tumours associated with MD

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of birds that developed MD associated tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3dpi*</td>
</tr>
<tr>
<td>Md5 (n=22)</td>
<td>0/3</td>
</tr>
<tr>
<td>rMd5-MiR7mCh (n=21)</td>
<td>0/3</td>
</tr>
<tr>
<td>Negative (n=12)</td>
<td>0/3</td>
</tr>
</tbody>
</table>

n= Total number of birds in that group  
* Days post infection  
** No animals at this time point  
*** Total animals with tumours from 13dpi until termination of experiment

**In vivo viral replication of rMd5-MiR7mCh-MDV in PBMC**

In order to investigate virus replication in vivo, MDV genome copy number was estimated by qPCR in PBMC. The MDV copy numbers were estimated in three birds at each time point and the qPCR was run in triplicate. Each genome copy number was calculated per 100ng. Figure 2-11A shows MDV genome copy number/100ng for each bird at 3, 6, 13, and 41 dpi. Averages from each time point group were calculated and statistically analysed (Figure 2-11B).

Viral genome copy number increased significantly from 6 dpi to 13 dpi (p=<0.0001***), and from 13 to 41 dpi (P=0.0134*) in the Md5 infection control. MDV genome copy number also increased significantly in the rMd5-MiR7mCh-MDV group from 6 dpi to 41 dpi (p= 0.0026*). As only 2 birds were tested at 13 dpi, statistical
analysis was not done on this group (Figure 2-11A). Interestingly, at both 3 dpi and 6 dpi, there is a significantly higher MDV genome copy number in the rMd5-MiR7mCh-MDV group compared to the Md5 infection control, which was unexpected (p=0.0010*** and P=<0.0001***, respectively). However, this changed at 41dpi where rMd5-MiR7mCh-MDV group had about 10 times fewer MDV genome copies than Md5 infection control (P=0.0188*). Overall, it is clear that the rMd5-MiR7mCh-MDV recombinant virus is able to replicate in vivo despite demonstrating a lower pathogenicity than the parental Md5 strain. However, rMd5-MiR7mCh-MDV PBMC virus genome copy numbers were higher earlier in the infection and lower later in the infection compared to the parental Md5.
Figure 2-11  MDV genome copy number in PBMC from MDV Md5 or rMd5-MiR7mCh-MDV infected chicks

MDV genome copy number was estimated by a qPCR amplifying a part of meq gene sequence. Resulting Ct values were interpolated using a standard curve of known copy number. Ct value from negative infection control were subtracted from all values. Results from individual birds were plotted in panel A and their average for each time point group shown in panel B. P values in Student T-test between two groups are found above bar columns in panel B. Only two PBMC samples were available for rMd5-MiR7mCh infected group at 13 dpi, therefore statistical analysis could not be performed.
Establishment of infection in lymphoid tissue

To investigate the establishment of infection in lymphoid organs early after infection, thymus tissue sections from rMd5-MiR7mCh-MDV or Md5 infected birds at 3 dpi were made and stained with antibody against Meq oncoprotein. Specific staining signals could be seen in the thymus from all rMd5-MiR7mCh-MDV and Md5 infected birds but not in the thymus from the negative control bird (Figure 2-12).

These results demonstrate that rMd5-MiR7mCh-MDV can establish infection in the thymus (lymphoid organ where T-cell is the predominant lymphocyte population) as efficiently as the parental Md5. These results verify the efficient replication of rMd5-MiR7mCh-MDV early after the infection that was suggested by the qPCR results at 3 dpi.

Figure 2-12  Immunohistochemistry of rMd5-MiR7mCh-MDV, Md5 or mock infected chicken thymus at 3 dpi
Frozen sections of the thymus from rMd5-MiR7mCh-MDV, Md5 or mock infected chickens were stained with antibody against Meq oncoprotein. HRP-conjugated secondary antibody along with DAB substrate was used for visualization. Red arrows indicate examples of stained cells. Sections in the first row are from three individual rMd5-MiR7-mCh MDV infected birds. Sections in the second row are from three individual Md5 infected birds. The bottom panel is a section from a mock infected bird.
Expression of mCherry fluorescent protein in PBMC and thymus tissue

PBMC from rMd5-MiR7mCh-MDV, parental Md5, and mock infected chickens were analyzed using flow cytometry for mCherry expression. No samples showed expression of mCherry in rMd5-MiR7mCh-MDV when compared with Md5 PBMC. Figure 2.13A shows an example dot plot of un-gated PBMC from rMd5-MiR7mCh-MDV infection group bird at 13 dpi.

Frozen thymus tissues sections from rMd5-MiR7mCh-MDV, parental Md5, and mock infected birds at 3 dpi were examined under fluorescent microscope for mCherry expression (Figure 2.13B). Although fluorescence was observed in the tissues sections from rMd5-MiR7mCh-MDV infected birds, similar positive signals were seen in the sections from parental Md5 infected birds (in the Texas Red channel). These signals were also seen in additional channels used to detect different excitation and emission wavelengths. Therefore, I consider the fluorescence observed in all samples to be autofluorescence. Thus, mCherry expression could not be confirmed in rMd5-MiR7mCh-MDV samples.

Figure 2-13  mCherry expression in rMd5-MiR7mCh-MDV infected birds
A. PBMC from either Md5 (blue) or rMd5-MiR7-MDV (red) infected birds at 13dpi. PBMC was visualized for mCherry expression. No mCherry was detected in either sample. B. Tissue section of collected thymus from Md5 infection control group at 3 dpi. Tissue section was mounted onto a slide glass and observed under fluorescent microscope through Texas Red filter. Autofluorescence was observed in Md5 infected thymus therefore mCherry expression could not be determined in rMd5-MiR7mCh-MDV samples.
2.3.3. *In Vitro* analysis of rMd5-LATmch

**Restriction fragment and Southern blot analyses of recombinant rMd5-LATmCh BAC**

With *Bam*HI digestion the inserted sequences in rMd5-LATmCh BAC increased the sizes of two very large 20836bp and 14743bp fragments of Md5SN5BAC to 22170bp and 16197bp, respectively. Due to the large sizes of these fragments no apparent difference in the banding patterns between rMd5-LATmch BAC and parental Md5SN5BAC was noticeable (Figure 2-7A).

With *Kpn*I digestion, rMd5-LATmCh BAC should have three extra bands at 5884bp, 4603bp, and 4187bp compared to parental Md5SN5 BAC, which was confirmed by gel electrophoresis (Figure 2-14B). rMD5-LATmCh BAC should have lost 8737bp and 7336bp fragments. However this was not apparent due to co-migration with common 8630bp and 8553bp bands, and 7264bp band, respectively.

To visualize the insertion of mCherry gene, the agarose gel containing *Bam*HI or *Kpn*I digested rMd5-LATmCh-BAC or Md5SN5BAC was further analysed by Southern blot using mCherry gene and Md5SN5 BAC probes (Figure 2-15 A and B). mCherry probe bound to a band at approximately 22kb on *Bam*HI digested rMd5-LATmCh. This corresponded to the predicted 22,170bp mCherry position (Figure 2-15 A). mCherry probe bound to a band at approximately 5kb on *Kpn*I digested rMd5-LATmCh. This corresponded to the predicted 5884bp mCherry position (Figure 2-15 B).

Both restriction digestion and Southern blot analysis confirmed the integrity of rMd5-LATmCh BAC DNA.
Figure 2-14  Analysis of rMd5-LATmCh BAC vs. parental MD5SN5 BAC by restriction enzyme.
DNA of rMd5-LATmCh BAC and parental MD5SN5BAC were digested BamHI (panel A) or KpnI (panel B) restriction enzymes and ran on a 0.6% agarose gel to analyze banding pattern. A. rMd5-LATmCh BAC vs parental MD5SN5 BAC look comparable which is expected. B. rMd5-LATmCh BAC have the additional expected bands at 5886, 4603, and 4187bp which are indicated by red arrows.
Figure 2-15  Analysis of rMd5-LATmCh BAC vs. parental Md5SN5 BAC by Southern blot.

Following gel electrophoresis, BamHI or KpnI digested rMd5-LATmCh BAC and Md5SN5BAC DNA were subjected to Southern blot analyses. DIG labeled probes were used to detect mCherry gene and entire MDV genome. A. A Band corresponding to mCherry gene after BamHI digestion is located at 22,170bp and is indicated by white arrow. B. Band containing mCherry gene after KpnI digestion is located 5884bp and is indicated by a green arrow. In the merged images, mCherry gene-containing fragments are indicated as red bands.
**In vitro replication analysis of rMd5-LATmCh-MDV**

Replication of rMd5-LATmCh-MDV at a passage level of 5 was compared to the parental MDV strain Md5 at a passage level 14 (Figure 2-16). Both viruses replicated to approximately 25 plaques by 3 dpi. By 7 dpi the Md5 strain replicated to approximately 380 plaques whereas the recombinant virus replicated to only approximately 210 plaques. The result indicates that rMd5-LATmCh-MDV does replicate *in vitro*; however, it is potentially not as efficient as parental Md5 strain.

![Graph showing replication of rMd5-LATmCh-MDV compared to Md5 MDV strain in vitro](image)

**Figure 2-16 Replication of rMd5-LATmCh-MDV compared to Md5 MDV strain *in vitro***

Confluent CEF monolayer was infected with 100pfu of rMd5-LATmCh-MDV (red line) or Md5 MDV strain (green line). Monolayers were collected at 1, 3, 5, and 7 dpi and titrated on fresh monolayers. The recombinant virus replicated like parental up to 3 dpi. At 7 dpi recombinant virus had approximately 210 plaques whereas MDV Md5 strain had approximately 380 plaques.

**In vitro expression of mCherry fluorescence in rMd5-LATmch-MDV**

rMd5-LATmch-MDV infected CEF monolayer was analyzed for mCherry expression with florescent microscopy (Figure 2-17). mCherry fluorescence could be visualized only within the cells of viral plaques (Figure 2-17 A) but not within viral
plaques of parental Md5 infection control (Figure 2-17 B). This confirmed that mCherry from constructed recombinant virus, rMd5-LATmCh-MDV, is expressed in vitro.

Figure 2-17  Fluorescent microscopy of CEF cells infected with either Md5 or rMd5-LATmCh-MDV virus.
A. Viral plaque caused by rMd5-LATmCh-MDV is illustrated with black circle. mCherry fluorescence was detected within viral plaques using a fluorescent microscope. B. Viral plaque caused by Md5 parental virus is illustrated with black circle. mCherry fluorescence was not detected in viral plaques.
### 2.3.4. *In Vivo* analysis of rMd5-LATmCh-MDV

#### Pathogenicity of rMd5-LATmCh-MDV

Three-day old SPF chicks were infected with rMd5-LATmCh-MDV or parental MDV strain Md5, or mock infected (diluting media). At 3 dpi 1 of 2 euthanized birds infected with the parental Md5 showed pathological symptoms, bursa atrophy. At 13 dpi both 2 birds in the Md5 infected group examined at necropsy showed both bursa and thymus atrophy but no signs of tumour development. At 26 dpi 1 necropsied bird in the Md5 infected group had symptoms conducive of splenic tumour; however, at 42 dpi the necropsied bird only had thymus and bursa atrophy (1 out of 1). No birds showed pathological symptoms in rMd5-LATmCh-MDV infected group in the study as well as negative control group (Table 2-5 and 2-6). Thus, rMd5-LATmCh-MDV most likely lost virulence as it did not cause atrophy in lymphoid organs or tumours in infected birds. However, this was not conclusive since the birds in Md5 infection group did not develop severe MD symptoms as frequently as expected.

### Table 2-5 Number of birds that showed MD associated pathological signs (nerve enlargement, thymus or bursa atrophy) at necropsy

<table>
<thead>
<tr>
<th>Group</th>
<th>Numbers of birds showing MD associated pathological signs (nerve enlargement, thymus or bursa atrophy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3dpi*</td>
</tr>
<tr>
<td>Md5 (n=6)</td>
<td>1/2</td>
</tr>
<tr>
<td>rMd5-LATmCh(n=11)</td>
<td>0/3</td>
</tr>
<tr>
<td>Negative (n=5)</td>
<td>0/1</td>
</tr>
</tbody>
</table>

n = total number of birds in group  
* Days post infection  
** No animals at this time point  
*** Total animals with signs of MD (nerve enlargement, thymus or bursa atrophy)
Table 2-6  Number of birds that developed tumours associated with MD

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of birds that developed MD associated tumours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3dpi*</td>
</tr>
<tr>
<td>Md5 (n=6)</td>
<td>0/2</td>
</tr>
<tr>
<td>rMd5-LATmCh (n=11)</td>
<td>0/3</td>
</tr>
<tr>
<td>Negative (n=5)</td>
<td>0/1</td>
</tr>
</tbody>
</table>

n = total number of birds in group
* Days post infection
** No animals at this time point
*** Total animals with tumours from 13dpi until termination of experiment

**In vivo viral replication of rMd5-LATmCh-MDV in PBMC**

As in the rMd5-MiR7mCh-MDV study, rMd5-LATmCh-MDV replication was estimated by qPCR with DNA from PBMC. Due to unexpected loss of animals before the experiment, only 2 birds and 1 bird were used for 6 and 13 dpi analysis, respectively (Figure 2-18A and B). In Md5 infected group and negative control group only 1 bird was used for each time point (Figure 2-18A). Figure 2-18A shows MDV genome copy number/100ng in each bird at 3, 6, 13, and 41 dpi. Averages from each time point group were calculated and statistically analysed (Figure 2-18B). Statistical analysis could only be performed in the rMd5-LATmCh-MDV infected birds between at 3 and 41 dpi. MDV genome copy number increased significantly (p=0.0071**) from 3 to 41 dpi in rMd5-LATmCh-MDV infected birds. There was an overall increasing trend of MDV genome copy numbers in the birds infected with Md5 from 3 to 41 dpi (approximately 10 fold). MDV genome copy numbers in parental Md5 infected birds and rMd5-LATmCh-MDV infected birds were very similar at 3, 6, and 13 dpi. Interestingly, MDV genome copy number in the Md5-infected bird at 41 dpi was approximately 7 fold higher than the average of rMd5-LATmCh-MDV. Despite the few number of birds used in the experiment, it is clear that the rMd5-LATmCh-MDV was able to replicate in vivo similarly to the parental Md5 strain early in the infection.
Figure 2-18  MDV genome copy number in PBMC from MDV Md5 or rMd5-LATmCh-MDV infected chicks

MDV genome copy number was estimated by a qPCR amplifying a part of meq gene sequence. Resulting Ct values were interpolated using a standard curve of known copy number. Ct value from negative infection control were subtracted from all values. Result of individual bird was plotted in panel A and the average for each group at each time point where applicable shown in panel B. Significant difference in MDV genome copy numbers was seen in rMd5-LATmCh-MDV infected birds between 3 and 41 dpi by Student T-test (p=0.0071). All other time points did not have sufficient data therefore the same statistical test could not be performed. There is an increasing trend of MDV genome in parental Md5 infected birds from 3 to 41 dpi. At 41 dpi, MDV genome copy number in the Md5 infected bird was approximately 7 fold higher than the average of rMd5-LATmCh-MDV infected birds.
Establishment of infection in lymphoid tissue

The establishment of infection in lymphoid organs by rMd5-LATmCh-MDV was investigated as in rMd5-MiR7mCh-MDV. Tissue sections of thymus from rMd5-LATmCh-MDV or Md5 infected birds at 3 dpi were made and stained with antibody against Meq oncoprotein. Specific staining could be seen in the thymus from all three birds in rMd5-LATmCh-MDV infected group, though one of the birds showed slightly less staining. The thymus from Md5 infected bird also showed specific staining but no staining was seen in the thymus from negative control bird (Figure 2-19). These results demonstrate that rMD5-LATmCh-MDV can establish infection in thymus as efficiently as the parental Md5. These results corroborate the efficient replication of rMd5-LATmCh-MDV early after the infection that was suggested by the qPCR results at 3 dpi.

Figure 2-19  Immunohistochemistry of rMd5-LATmCh-MDV, Md5 MDV or mock infected chicken thymus at 3 dpi

Frozen sections of the thymus from rMd5-LATmCh-MDV Md5 or mock infected chickens were stained with antibody against Meq oncoprotein. HRP -conjugated secondary antibody along with DAB substrate was used for visualization. Red arrows indicate examples of stained cells. Sections in the first row are from three individual rMd5-LATmCh MDV infected birds. The second row is a tissue section from a Md5 MDV infected birds, and the bottom panel is a section from mock infected bird.
Expression of mCherry fluorescent protein in PBMC and thymus tissue

PBMC from rMd5-LATmCh-MDV, parental Md5, and mock infected chickens were stained with chicken CD4 specific antibody and analyzed using flow cytometry for mCherry expression. No samples showed expression of mCherry while CD4 positive cells were confirmed. Figure 2.20A shows an example dot plot of un-gated PBMC from either Md5 or rMd5-LATmCh-MDV infection group bird at 6 dpi. Although CD4 staining was seen, no mCherry expression can be observed in rMd5-LATmCh-MDV PBMC.

Frozen thymus tissues sections from rMd5-LATmCh-MDV, parental Md5, and mock infected birds at 3 dpi were examined under fluorescent microscope for mCherry. Figure 2.20B shows a tissue section from parental Md5 infected bird. As in the case of rMd5-MiR7-mCh-MDV, significant autofluorescence existed in the control sections and thus, mCherry expression could not be confirmed in rMd5-LATmCh-MDV infected samples.

Figure 2-20  mCherry expression in rMd5-LATmCh-MDV infected bird
A. PBMC of either Md5 or rMd5-LATmCh-MDV infected bird at 6 dpi. PBMC was stained with CD4 antibody and visualized using flow cytometry. CD4 specific staining was seen in 19.7% of analyzed cells. No mCherry was detected in either Md5 or rMd5-LATmCh. B. A frozen section of the thymus from Md5 infected control group at 3 dpi. Tissue section was observed under fluorescent microscope through Texas Red filter. Autofluorescence was observed in Md5 infected thymus therefore mCherry expression could not be determined.
2.4. Discussion

In order to reveal infection dynamics and uncover MDV's unique vaccine effect, tools are required to trace the MDV infection in its natural avian host. The aim of this study was to generate a recombinant MDV in order to attempt in situ visualization of lymphocytes latently infected with MDV. This was attempted by inserting a fluorescent protein into the MDV genome at sites where transcription is active during latency. Using the markerless two step red mediated recombination system [67], two recombinant viruses were generated: rMd5-MiR7mCh-MDV that contained IRES-mCherry-PolyA construct at the 3' end of MDV microRNA miR-M7, and rMd5-LATmCh-MDV that contained IRES-mCherry construct at the 3' end of MDV LAT transcript. Unfortunately the modification caused a loss in their oncogenicity even though they both replicated in vitro and in vivo. Additionally, although mCherry expression was observed in vitro, I could not obtain evidence that these recombinant MDVs illuminated lymphocytes latently infected with MDV in vivo.

2.4.1. In vitro analysis of rMd5-MiR7mCh and rMd5-LATmCh MDV

The insertion of the mCherry construct into the desired target sites of the MDV BAC was successful (IRES-mCherry-PolyA to the 3' end of MiR-M7, IRES-mCherry to the 3' end of LAT). Both gel electrophoresis and Southern blot analysis illustrated the integrity of the recombinant BAC clones and mCherry structure insertion.

Recombinant virus replication

Finding a suitable target site on the MDV BAC plasmid is troublesome. Virus genomes including those in herpesviridae are very compact and concise, so modifications such as insertion or deletion (even in non-coding regions of the genome) can easily interfere with viral replication. This study illustrated that inserting the mCherry structure to the 3' end of MiR-M7 did not affect virus replication. Upon infection to CEF cells, replication of rMd5-MiR7mCh-MDV was comparable to parental Md5 MDV. Conversely, the insertion of mCherry structure to the 3' end of LAT may have affected replication but further analysis is required. It is important to note that at the 7 dpi, the parental Md5 MDV strain showed higher plaque counts during the experiments with
rMd5-MiR7mCh-MDV compared to the experiments with Md5-LATmCh-MDV (Md5 plaque counts of 860 compared to 260 respectively). Different batches of CEF cells were used for each experiment which could account for this fluctuation. The difference in parental Md5 plaque counts may suggest that the integrity of the CEF monolayer was not as reliable when studying rMd5-LATmCh-MDV replication compared to rMd5-MiR7mCh-MDV. In fact, the titration of the collected monolayers during rMd5-LATmCh-MDV analysis had to be terminated at 5 days post initiation compared to 7 days for rMd5-MiR7mCh-MDV. Perhaps this early termination did not allow some viral plaques to grow to a detectable size. It can be suggested that the 3’ end of MiR-M7 may be a more efficient target, but both recombinant viruses replicated in vitro illustrating that the insertion sites were appropriate for the aim of this study.

**mCherry translation from recombinant viruses**

Neither MiR-M7 nor LAT transcripts are translated during virus replication. IRES was therefore included in the insertion construct at the 5’ end of mCherry gene so that mCherry protein is translated. Upon infection of CEF cells, mCherry expression was seen in both rMd5-MiR7-MDV and rMd5-LATmCh-MDV infected cells. These results demonstrate that IRES was functional in these transcripts. Recombinant viruses have been constructed to visualize infected cells by expressing fluorescent proteins. For instance, EGFP was inserted to the c-terminus of MDV UL47 [71], and EGFP was fused with viral HSV-1 glycoprotein B [82]. However, alphaherpesviruses do not produce viral proteins during the latency except for MDV’s oncoprotein Meq. If the function of Meq is compromised the virus will be severely attenuated. Therefore, to label latently infected cells by the expression of marker proteins, untranslated RNAs are preferably utilized. This study suggests that IRES may be useful for driving marker protein expression from other untranslated transcripts encoded on the MDV genome.

2.4.2. **In vivo analysis of rMd5-MiR7mCh and rMd5-LATmCh derived MDVs**

**rMd5-MiR7-MDV analysis**

Successful replication of rMd5-MiR7mCh-MDV in vivo was confirmed by immunohistochemistry and qPCR. Immunohistochemistry results with day 3 tissue
sections demonstrated rMd5-MiR7mCh-MDV established early stage of infection in the thymus similarly to parental MDV. In qPCR, MDV genome copy number in PBMC increased from day 3 towards day 41 post infection. This likely means rMd5-MiR7mCh-MDV went through the normal infection cycle in the body.

At 3 and 6 dpi, there was significantly higher MDV genome copy number in rMd5-MiR7mCh-MDV infected bird group compared to parental Md5 infected control (P=0.0010 and P=0.0001 for days 3 and 6 post infection, respectively). This illustrates that the recombinant virus might actually be replicating more efficiently than parental virus. At 3 and 6 dpi the virus is likely in lytic phase, which ultimately causes cell death. Therefore more viral replication during lytic infection could cause more severe bursa and thymus atrophy [83, 84]. However, at 3 and 6 dpi no necropsied birds in the rMd5-MiR7mCh-MDV group showed atrophy in bursa and thymus even though the immunohistochemistry results showed similar MDV staining in the thymus between rMd5-MiR7mCh-MDV and Md5 infected birds on day 3.

At 13 dpi, the rMd5-MiR7mCh-MDV infected birds continued to show approximately 10x higher MDV genome copy number than MD5 infected control birds. But again, atrophy in lymphoid organs was limited to only 1 bird out of 3 in the rMd5-MiR7mCh-MDV infected group compared to all 3 in the Md5 infected control birds. It is possible that the genetic modification may have prompted the virus to replicate more efficiently in PBMC rather than in lymphocytes within the lymphoid organs. Inefficient replication has been associated to both lack of atrophy in lymphoid organs and oncogenesis in former studies [84]. Thus, the higher virus genome copy numbers seen in rMd5-MiR7mCh-MDV infected birds without apparent atrophy in lymphoid organs is exceptional and needs further investigation.

At 41 dpi a shift is observed. The genome copy number in rMd5-MiR7mCh-MDV infected birds was only ~10^6 copies/100ng DNA, which is 10 times less than that seen with the parental virus infected birds (~10^7 copies/100ng DNA). The lower copy number seen in rMd5-MiR7mCh-MDV group is probably due to the lack of oncogenicity in this recombinant virus. MD lymphomas develop at approximately 14 dpi and all lymphoma cells harbour MDV genome in the latent form. Therefore, when the percentage of
lymphoma cells in PBMC population increase due to uncontrolled cellular replication, the virus genome copy number should also increase. As expected, from 13 days to study termination at 41 days, the Md5 infection control group showed an increase of viral genome copies from \( \sim 10^5 \) to \( \sim 10^7 \) copies/100ng; and all but one bird developed lymphomas. On the contrary, the rMd5-MiR7mCh-MDV group did not show an increase in MDV genome copy number and only 2 out of the 15 birds developed MD tumour lesions during the study period. This suggests that the insertion of IRES-mCherry-PolyA to the 3’ end of MDV microRNA MiR7 interfered with viral oncogenicity as well as its disruptive effect on lymphoid organs.

Overall, rMd5-MiR7-mCh-MDV was able to replicate \textit{in vivo} but did not show significant signs of MD compared to parental Md5. There are a few hypotheses that could explain these results:

**Hypothesis 1: rMd5-MiR7-mCh-MDV does not establish efficient latent infection**

It can be suggested that rMd5-MiR7-mCh-MDV might not be able to establish latency as efficiently as the parental Md5. As previously explained, MDV ICP4 is an important viral gene transactivator and has been shown to have a binding site for miR-M7. It has been suggested that ICP4 suppression, potentially by miR-M7, is an important factor in the establishment and maintenance of latency [32]. If the insertion of IRES-mCherry-PolyA somehow disrupted the miR-M7 function, ICP4 suppression may not occur and lytic infection will continue. It would be interesting to test if the modification made to rMd5-MiR7mCh-MDV genome affected the expression level of miR-M7. This could be done by quantifying miR-M7 using RT-qPCR. Although quantitation of such small RNA transcripts is problematic, a number of methodologies have been reported [85]. As only latently infected cells can become transformed, inefficiency in the establishment of latency can explain the reduced incidence of lymphomas observed in this study.

On the other hand, if MiR-M7 function was disturbed in rMd5-MiR7-mCh-MDV it could result in a persistent lytic infection; but significant bursa or thymus atrophy was not observed. Eventually the lytic infection should be cleared by immune system, which was also not observed as MDV genome copy number persisted. For example, it has been
shown that macrophage and cytokine activity are increased after MDV infection [86, 87]. Conversely, it has been shown that during lytic infection host major histocompatibility complex (MHC) class 1 is downregulated by MDV early genes. This downregulation is maintained throughout lytic infection thus evading CD8+ T cell immune responses [9]. The clearance of lytic infection is therefore on a subtle balance between virus function, cell death caused by the virus, and the immune response. Therefore, to reveal if the disruption of miR-M7 function resulted in the observation described in this thesis, significant amount of further analysis may be required.

**Hypothesis 2: rMd5-MiR7-mCh-MDV establishes latent infection but does not efficiently transform cells**

Another hypothesis is that rMd5-MiR7mCh-MDV is able to establish latency as efficiently as the parental Md5 but is unable to transform cells. This would certainly explain reduced tumour incidence but again further analysis is needed to confirm this hypothesis. For example, tissue sections at later time points in the infection could be stained with an antibody against MDV late proteins (such as MDV glycoprotein B) and combined with in situ hybridization analysis to detect MDV genome. If glycoprotein B staining is not observed but MDV is present this suggests that latency has been established. This type of analysis could therefore reveal whether or not this virus is able to establish latency as efficiently as the parental virus.

**rMd5-LATmCh MDV**

Similar to rMd5-MiRM7 MDV the replication of another recombinant MDV, rMd5-LAT-MDV was confirmed by immunohistochemistry and qPCR. Immunohistochemistry results with day 3 tissue sections demonstrated rMd5-LATmCh-MDV established early stage of infection in the thymus similarly to parental MDV. In qPCR analysis, rMd5-LATmCh-MDV also demonstrated successful replication in vivo as MDV genome copy number in PBMC increased from $\sim 10^4$ copies/100ng at 3 dpi to $\sim 10^5$ copies/100ng at 41 dpi. This increase of virus genome copy numbers was similar to that observed in rMd5-MiR7mCh-MDV infected birds.

According to the qPCR results, rMd5-LATmCh-MDV and the parental Md5 replicated similarly in PBMC at 3 and 6 dpi. If the viruses replicated similarly in
lymphocytes in the lymphoid organs, then bursa and thymus atrophy should also be comparable (due to lytic infection expected at this time point). However this was not the case. No bursa or thymus atrophy was seen in birds from the rMd5-LATmCh-MDV group at both 3 and 13 dpi. Contrary, 3 out of 4 birds in the Md5 control group developed bursa and thymus atrophy: 1 out of 2 on day 3, and 2 out of 2 on day 13. This suggests that insertion of IRES-mCherry into the 3’ end of LAT did not interfere with viral replication in PBMC, but may have disturbed efficient replication in lymphoid organs. This could have prevented the virus from causing the typically observed cell death at early stages of infection.

**Differences in Md5 infection control between the two in vivo studies**

Contrary to rMd5-MiR7mCh-MDV, the difference in viral genome copy numbers at 41 dpi between rMd5-LATmCh-MDV and Md5 infection control was not as substantial. In this experiment the Md5 infection control genome copy number increased only up to \( \sim 10^{5.75} \) copies/100ng compared to \( \sim 10^7 \) copies/100ng seen in the first study with same virus. Additionally, the Md5 infection did not cause extensive tumours in this study. Only 1 out of the 6 birds in the Md5 infected control group developed tumours, which corresponds with the lack of MDV viral genome increase. Unfortunately, birds challenged with rMd5-LATmCh-MDV also lacked tumour development. However, in this experiment the control Md5 did not cause MD tumour nodules as frequently as expected; therefore, I cannot conclude for certain whether rMd5-LATmCh-MDV was non-oncogenic or the experimental conditions were not conducive for the development of MD.

The reason for the observed fluctuation between the Md5 parental control groups is not clear, but host factors might have played a role. It is known that some chickens are genetically resistant to MD [88]. Additionally, the availability of chickens for the second study was limited and fewer birds were used than planned due to poor hatching rate. This meant that statistical analysis on some data sets was not possible. Unfortunately, in order to analyze the behaviour of rMd5-LATmCh-MDV *in vivo*, the experiment should be repeated with genetically susceptible chickens.
2.5. *In vivo* expression of mCherry from recombinant viruses

mCherry positive cells could not be detected in any PBMC samples from rMd5-MiR7mCh-MDV or rMd5-LATmCh-MDV infected birds when analysed by flow cytometry. A potential reason could be either the lack of mCherry expression *in vivo* PBMC, or the numbers of infected cells were too low to detect expression. When looking at HRP-stained thymus at 3 dpi, only a few cells were infected with MDV and not all may express high levels of mCherry.

It would have been interesting to analyse cells from tumour nodules in rMd5-MiR7mCh-MDV infected chickens with immunofluorescent assay as the number of infected cells would be substantial; thus increasing the chance for mCherry visualization. However, when tissue sections from multiple time points were examined under a fluorescent microscope, severe autofluorescence interfered with mCherry observation in both *in vivo* studies. This meant that mCherry expression could not be confirmed by this method. Autofluorescence was also observed when tissue sections were stained with an antibody against MDV Meq protein and FITC-conjugated secondary antibody. To overcome this problem, a non-fluorescent colorimetric method that used peroxidase-labeled antibody was employed to detect the MDV protein in tissue sections. Using the colorimetric method, infected cells were observed without major background. mCherry expression may be tested using a similar method in future experiments. Otherwise, the presence of mCherry mRNA could be examined by using RT-qPCR.

To rule out if mCherry was mutated in some way during *in vivo* studies (thus inhibiting visualization), PBMC from rMd5-MiR7mCh or Md5-LATmCh MDV infected birds could be collected and used to infect a fresh CEF monolayer. If viral plaques are established they could be observed under fluorescent microscope to visualize mCherry expression. This method was utilized by Jarosinski *et al* (2015) who infected SPF chicks with recombinant MDV that contained red fluorescent protein (mRFP) fused to the 3' end of MDV RLOF5 gene. Virus was collected from feather follicle cells and used to infect chicken embryo cell cultures. RFP was then visualized using fluorescent microscopy [89].
2.6. Conclusion and Further research

The aim of this study was to construct a recombinant MDV that allowed for the visualization of latently infected cells in situ. Two recombinant viruses were successfully constructed, rMd5-MiR7mCh-MDV and rMd5-LATmCh-MDV, which contained mCherry expression constructs. Both viruses were able to replicate in vitro and in vivo, but mCherry expression was only seen in vitro lytic infection. SPF chickens were challenged with the recombinant viruses but tumour development was not extensively seen. This suggests that these recombinant viruses were either a) unable to efficiently establish latency or b) able to establish latency efficiently but were not highly oncogenic.

The results imply that the insertion structure inhibited the function of miR-M7 and LAT, which have both been suggested to be essential for the establishment and maintenance of latency [32, 38]. However, this cannot be concluded without further analysis. The in vivo experiment should be repeated in order to identify how the infection dynamics of the virus changed. Additionally, IRES was able to translate mCherry in vitro from MDV transcripts that are typically not translated. The expression level by this system was high enough for mCherry detection in CEF cells. With this information, additional target sites on MDV genome may be utilized towards the visualization of latently infected cells. For example, MDV miR-M4, which is encoded in the meq cluster, is the most abundantly expressed miRNA in viral tumours and has been shown to be essential for lymphomagenesis [43]. If a fluorescent protein gene with a 5'IRES and 3'PolyA signal is inserted to the 3’ end of miR-M4, the visualization of MDV latently infected lymphocytes may be achieved.
References


Appendix A.

Using SmartFlare RNA detection probes to visualize Marek’s Disease Virus latency

When a fluorescent protein structure is placed into the MDV genome there is always a risk of the virus losing its ability to replicate or becoming avirulent. To avoid this problem, a new approach was considered. By using RNA detection probes that target transcripts expressed during latency, we may be able to identify latently infected cells ex vivo. RNA detection probes by SmartFlare consist of gold nanoparticles that have a quenched reporter sequence. When the target sequence comes into contact with the SmartFlare probe, it anneals to the quencher sequence and disperses the reporter probe (Figure A1).

In order to visualize MDV latency, a SmartFlare probe must be designed which will report the expression of transcripts present during latency. As previously stated, the LAT transcript is highly expressed during latency but has different splicing patterns (Chapter 1 Figure 2-1). By using the splicing map reported by Strassheim et al. [36], two potential candidate sequences were chosen. These sequences map to exon 9 and 15 (Chapter 1 Figure 2-1) and are present in multiple splice variants. The SmartFlare probe was therefore designed to target these two exons and to emit cyan5 (Cy5) fluorophore when the quencher was displaced (excitation of 650nm and emission peak of 670nm).

![Schematic representation of SmartFlare probe.](image)

Figure A1  Schematic representation of SmartFlare probe.
SmartFlare probe (left hand side) is made of a gold nanoparticle with a quenched reporter sequence (green line with red circle). When specific transcript comes into contact with the quenched SmartFlare probe, in my case exon 9 and 15 from LAT transcript (purple line), it binds to the quencher sequence (black line). This displaces the reporter probe and Cy5 can be visualized (right diagram).

Along with the designed SmartFlare probes, two controls were used: uptake SmartFlare control which did not have a quencher, and a negative control probe that had a scrambled sequence. To make sure that background was not seen, the SmartFlare negative control scrambled sequence was compared to MDV genome and chicken
genome. Also the two designed SmartFlare detection probes were compared to chicken genome to make sure no cross reactivity will occur.

Each probe was incubated with MSB-1 cells line and MDV infected PBMC (Figure A2). Cells were also stained with CD4+ primary antibody and visualized using FITC-conjugated secondary antibody (Figure A2). FITC was chosen as it has an excitation peak at 495nm, and emission peak at 519nm therefore staining is distinguishable from Cy5.

![Cells incubated with SmartFlare probe](image)

**Figure A2.** Schematic representation of expected fluorescence in cells latently infected with MDV.
Either MSB-1 cells or PBMC from MDV infected chickens (left hand side) will be incubated overnight with SmartFlare probe designed to target LAT transcript containing exon 9 or exon 15. Cells will also be stained with CD4 antibody which will be visualized using FITC-conjugated secondary antibody. Latently infected cells are expected to be positive for Cy5 and CD4 antibody staining.

I hypothesized that if the SmartFlare probes specifically detect the latently infected cells the Cy5+/FITC+ cell population should have significantly more MDV genome than the Cy5-/FITC+ cell population (Figure A3). However, I found that the probes did not distinguish MDV infected cells very well and does not offer us a suitable alternative to constructing recombinant MDV the expresses fluorescent protein during latency.
Figure A3  Schematic representation of expect results when PBMC from MDV infected birds are stained with CD4 or CD4 and SmartFlare detection probe 15

PBMC from MDV infected birds will be stained with SmartFlare detection probe 15 and CD4 antibody. Two cell population, CD4+/SmartFlare+ and CD4+/SmartFlare-, are sorted. MDV genome copy numbers in these sorted PBMC are analyzed by qPCR. If the SmartFlare probes specifically detect the latently infected cells the CD4+/SmartFlare+ cell population (cell on right hand side) should have significantly more MDV genome than the CD4+/SmartFlare- cell population (cell on left hand side).
Materials and Methods

Cells, virus and SmartFlare probes

An MDV-transformed cell line, MSB-1, was used to test the SmartFlare probes specific to MDV transcripts. MSB-1 cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin (Life Technologies). MDV very virulent strain RB1B expressing EGFP fused to UL47 late gene product [71] (gift from B. Kaufer, Freie Universitat Berlin) was used in in vivo experiment.

Two SmartFlare probes that contained a quenched Cy5 fluorophore were designed to specifically bind to MDV LAT at exons 9a (Probe 9) and 15b (Probe 15) [32], respectively (Millipore).

The sequences of SmartFlare probes were:

Probe 9 5’-AAGATGATGACGGTGAGGATGATGAAG-3’

Probe 15 5’-CGGTGCATATCTGCAAGTAAACAAAAC-3’

Additionally, SmartFlare uptake control (Cy5 fluorescence) and a SmartFlare scramble control were purchased from Millipore and used as positive and negative controls. All controls and probes were reconstituted in nuclease-free water according to manufacture guidelines.

In vitro analysis of SmartFlare probe with MSB-1 cell line

A total of 5X10^5 MSB-1 cells were used per reaction. Probes were diluted at 1:20 with sterile PBS and 16µl was incubated with 5X10^5 MSB-1 cells in a 96-well plate in a total volume of 200µl of supplemented RPMI media (supplemented with 10% FBS, 100U/ml penicillin and 100µg/ml streptomycin). Cells were incubated with probe for 16 hours at 37°C in 5% CO_2 atmosphere. Cells were washed with PBS and fluorescence was analyzed with a flow cytometry using red laser 670/30nm for SmartFlare probe, and blue laser 530/30nm for CD4. BD sortware was used for acquisition and FloJo for analysis.

Analysis of SmartFlare probe with PBMC from RB1B infected chicks

One-day-old specific pathogen free chicks (Canadian Food Inspection Agency) were challenged intra-abdominally with 500pfu of the recombinant RB1B expressing EGFP [71]. Blood samples were collected at 3, 9, 18, 25, 31, 36 dpi and PBMC was separated using Histopaque 1077 according to manufacture protocol. Separated PBMC (2.5x10^5 cells) was incubated overnight with 16µl of reconstituted and diluted (1:20 with PBS) SmartFlare probe 15 or SmartFlare scramble negative control according to manufacture’s protocol. PBMC was then stained with anti-chicken CD4 monoclonal antibody [80] and FITC-conjugated Goat-anti mouse antibody (Jackson ImmunoResearch). Fluorescence was analyzed with a flow cytometry using red laser 670/30nm for SmartFlare probe, and blue laser 530/30nm for CD4. BD sortware was used for acquisition and FloJo for analysis. Birds were euthanized and necropsied when
they reached experimental endpoint, which was defined by visual symptoms of sickness such as lethargy, emaciation, and down-turned wings

**Viral genome quantification using qPCR**

PBMCs from two birds on day 26 post infection were stained with SmartFlare probe 15 and anti-CD4 and sorted using Jazz flow cytometer. Two populations were sorted: population stained only for CD4 and population stained for both CD4 and SmartFlare probe 15. DNA was extracted from the sorted cells using Qiagen DNeasy Blood and Tissue kit. qPCR analysis on purified DNA was carried out as described in Chapter 2 Materials and Methods section 2.2.6.

**Results and Discussion**

**SmartFlare probe enters chicken lymphocytes in vitro**

SmartFlare uptake control was incubated with $5 \times 10^6$ MSB-1 cells (Figure A4). After 16 hour incubation, cells were analysed using flow cytometry. There was a shift in fluorescence with the SmartFlare uptake control with 84.9% of analysed cells showing Cy5 staining. This indicates that SmartFlare probe can be taken up by chicken lymphocytes.

![SmartFlare Cy5](image)

**Figure A4**  MSB-1 cells stained with SmartFlare uptake control. MSB-1 cells ($5 \times 10^6$) were incubated with either 16ul of PBS (no probe, left dot plot) or 16ul reconstituted SmartFlare uptake control (right dot plot) and analyzed with flow cytometry. A shift in fluorescence can be observed in the uptake control group. Approximately 85% of MSB-1 cells analyzed were positive for Cy5 fluorescence suggesting that the probe was able to enter MSB-1 cells.
SmartFlare Scramble control or detection probes designed to target MDV LAT exon 15 or 9, (Probe 15 and Probe 9, respectively) were incubated with $5 \times 10^5$ MSB-1 cells (Figure A5). After a 16-hour incubation, cells were analysed using flow cytometry. A Cy5 cell population could be seen in Probe 9 and Probe 15. Approximately 37.4%, and 45.2% of MSB-1 cells were positive for Cy5, respectively. This indicates that SmartFlare detection Probe 9 and 15 are able to enter chicken lymphocytes and potentially bind to their specific LAT exon transcripts causing the quenched Cy5 fluorophore to become released. There is a Cy5 negative population in Probe 9 and 15 samples but this could account for reactivated MSB-1 cells. As both Probe 9 and 15 showed promising results, only one probe will be carried forward to ease sample handling.

**Figure A5**  MSB-1 cells stained with SmartFlare Probe 9 and 15. MSB-1 cells ($5 \times 10^5$) were incubated with either 16ul of reconstituted SmartFlare Probe 9 (top left), Probe 15 (top right) or Scramble Probe (bottom), and analyzed with flow cytometry. A shift in fluorescence can be observed in the Probe 9 and Probe 15. Approximately 37.4% and 45.2% of MSB-1 cells analyzed were positive for Cy5 fluorescence, respectively. This suggests that the detection probes were able to enter MSB-1 cells and potentially bind to LAT region on the MDV genome.

In order to assess if SmartFlare detection probe can be used to visualize MDV latently transformed cells, one-day-old specific pathogen free chicks were challenged with virulent MDV strain RB1B that expressed UL47-EGFP. Birds were euthanized and necropsied at study termination (36dpi) or when they reached experimental endpoint. Necropsied birds were analysed for signs of MD including bursa and thymus atrophy as well as MDV associated tumour nodules. In total 8 out of 15 birds challenged with RB1B-UL47-EGFP showed splenic tumour nodules. In total 11 out of 15 birds showed signs of
thymus and bursa atrophy (Table A1). As expected, no birds in mock infected group showed any signs of MD.

**Table A1**  
Number of birds that showed MD associated pathological signs

<table>
<thead>
<tr>
<th></th>
<th>Mock infection</th>
<th>RB1B UL47-EGFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Birds per group</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Number of birds that showed MDV induced tumour nodules</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Number of birds that showed other signs of MD *</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

*Thymus and Bursa Atrophy

**Analysis of PBMC from recombinant RB1B infected chickens**

Blood samples from recombinant RB1B infected and mock infected birds were collected on 3, 9, 18, 25, 31, 36 dpi. PBMC was separated and stained with CD4 antibody and either SmartFlare scramble or Probe 15. Both Probe 15 and 9 demonstrated similar performance in preliminary experiments with MSB-1 cells (Figure A5) therefore only one probe, probe 15, was selected for all *in vivo* analysis to ease sample handling.

A time course analysis of PBMC from whole blood of 3 birds (Figure A6, A7, A8 respectively) was used to assess SmartFlare performance. We expect the number of cells that are positive for both SmartFlare detection probe 15 and CD4 to increase. This is because as the infection progresses, the number of latently infected T-cells should increase. Additionally, transformed T-cell will rapidly divide from day 14 post infection thus increasing the number of cells stained with SmartFlare detection probe 15.

Figure A6 illustrates time course analysis of stained PBMC from bird 1 at 9, 25, and 31 dpi. The data shows that cells double positive for Probe 15 and CD4 increased by approximately 15% from day 9 to 31. However, a large population of cells stained with Probe 15 can be seen that are not positive for CD4. This population can be seen in PBMC from both RB1B and mock infected chickens. This population accounts for approximately 76%, 51%, and 41% of analysed cells from day 9, 25, and 35 post infection, respectively in the RB1B infected bird and 44% in the mock infected bird.

Figure A7 illustrates time course analysis of stained PBMC from bird 2 at 3, 18, and 25 dpi. The data shows that cells double positive for Probe 15 and CD4 increased by approximately 16% from day 2 to 25. However as in bird 1, a large population of cells stained with Probe 15 can be seen that are not positive for CD4 in both RB1B and mock infected chickens. This population accounts for approximately 74%, and 29% of analysed cells from day 18 and 25 post infection, respectively.

Figure A8 illustrates time course analysis of stained PBMC from bird 3 at 9, 25, and 31 dpi. The data shows that cells double positive for Probe 15 and CD4 increased by approximately 14% from day 3 to 31. However, as in bird 1 and 2, a large population of cells stained with SmartFlare detection probe 15 can be seen that are not positive for CD4 in both RB1B and mock infected chickens. The population accounts for
approximately 57%, 40%, and 28% of analysed cells from day 9, 25, and 31 post infection, respectively in RB1B infection.

Figure A6  Time course analysis of SmartFlare detection probe 15 using PBMC from recombinant RB1B infected bird 1
PBMC at 9, 25, and 31 dpi were incubated with Probe 15 (right column) or Scramble control (left column). PBMC was then stained with CD4 antibody and analyzed for fluorescence using flow cytometry. The x-axis on the dot plot indicate SmartFlare stain and y-axis indicates CD4 stain. The black arrow indicates the population that has both Probe 15 and CD4 while the orange arrow indicates Probe 15 positive CD4 negative population. Note the latter population existed in mock infected control.
Figure A7  Time course analysis of SmartFlare detection probe 15 using PBMC from recombinant RB1B infected bird 2

PBMC at 3, 18, and 25 dpi were incubated with Probe 15 (right column) or Scramble control (left column). PBMC was then stained with CD4 antibody and analyzed for fluorescence using flow cytometry. The x-axis on the dot plot indicate SmartFlare stain and y-axis indicates CD4 stain. The black arrow indicates the population that has both Probe 15 and CD4 while the orange arrow indicates Probe 15 positive CD4 negative population. Note the latter population existed in mock infected control.
Figure A8  Time course analysis of SmartFlare detection probe 15 using PBMC from recombinant RB1B infected bird 3
PBMC at 9, 25, and 31 dpi were incubated with Probe 15 (right column) or Scramble control (left column). PBMC was then stained with CD4 antibody and analyzed for fluorescence using flow cytometry. The x-axis on the dot plot indicate SmartFlare stain and y-axis indicates CD4 stain. The black arrow indicates the population that has both Probe 15 and CD4 while the orange arrow indicates Probe 15 positive CD4 negative population. Note the latter population existed in mock infected control.
Although the cell population that is double positive for CD4 and Probe 15 increased over the time course as expected, the observed population of Probe 15 positive but CD4 negative cells is somewhat troublesome. As LAT is also expressed during lytic infection [9], this population could be infected CD4 negative T cells or B cells. However there are two reasons why this may not be the case: 1) the recombinant virus used expresses EGFP fused to viral UL47. If this Probe 15 positive cell population was lytically infected, they should still have been within the FITC population. b) the same population was also seen in mock infected PBMC. Therefore, these results raised a serious concern that the SmartFlare Probe 15 is binding non-specifically to chicken RNA and may not be suitable for detection of MDV latently infected lymphocytes.

**MDV genome copy number in Probe 15 positive PBMC from recombinant RB1B infected birds**

In order to further investigate if Probe 15 is specifically labelling infected CD4+ T cells, qPCR analysis was carried out on sorted PBMC from two birds challenged with recombinant RB1B at 26 dpi. Two cell populations were sorted: cells that were stained positive with CD4 only, and cells that were stained positive with both CD4 and Probe 15 (Figure A9 A).

In both birds, we found that the double positive cell population had substantially higher number of MDV genome copy/100ng compared to CD4 single positive cell population. In bird 1, double positive population had approximately 25000 MDV genome copies/100ng compared to approximately 1000 MDV genome copies/100ng. In bird 2, double positive population had approximately 36,000 MDV genome copies/100ng compared to approximately 7300 MDV genome copies/100ng (Figure A9 B).
Figure A9  MDV genome copy number quantified on DNA from SmartFlare detection probe and CD4 antibody sorted PBMC

PBMC from two birds infected with recombinant RB1B at 26 dpi was stained with Probe 15 and anti-CD4 antibody. A. PBMC was analyzed on flow cytometry and two populations of cells were sorted: CD4 single positive (black circle), and CD4/Probe 15 double positive (red circle). B. DNA from sorted cells was analyzed using qPCR to quantify MDV genome copy number. Double positive cells had substantially higher MDV genome copy number than CD4 single positive cells. This result is anticipated as a high number of LAT transcripts are expected in transformed cells which would be present at 26 dpi.
In conclusion, we found that SmartFlare detection probe 15 did target a population of CD4 lymphocytes that expressed LAT. Sorted CD4 single positive PBMC at 26 dpi had substantially lower MDV genome copy number/100ng than double positive PBMC. However, significant background seen in entire PBMC from mock infected birds and suggests that the SmartFlare probe 15 is not suitable to identify infected cells in the entire PBMC population. Additionally, the amount of cells that can be run per SmartFlare reaction is very small (2x10^5 cells) and scaling up is not easy considering the cost performance. Therefore, the SmartFlare detection probe 15 may not be the most efficient system for detecting MDV latently infected lymphocytes. However, because of the encouraging qPCR results, further studies with probe 9 or perhaps another RNA target sequence may reveal more promising results.