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**Exploiting Differential Endogenous MicroRNA Expression to Enhance Oncolytic Vesicular  
Stomatitis Virus Tumour Tropism**

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**Exploiting differential endogenous microRNA expression to enhance  
oncolytic vesicular stomatitis virus tumour tropism.**

Robert E. Edge

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology  
*in partial fulfillment of the requirements for the degree of Doctor of Philosophy*

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Ottawa, Ontario, Canada  
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## **Abstract**

The creation of potent oncolytic viruses (OVs) suitable for the clinic may require new strategies in virus design. Replication-competent viruses facilitate a variety of approaches to achieving tumor specificity. Altered expression of microRNAs is a common hallmark of cancer that this thesis demonstrates can be used to alter expression of a potent wild-type viral gene to achieve tumor-specific replication of an engineered vesicular stomatitis virus (VSV). Incorporation of microRNA complementary sequences (miRTs) within VSV causes reduced accumulation of miRT containing mRNAs. Let-7 miRTs introduced into the 3' untranslated region (3'UTR) of VSV matrix protein mRNA eliminate undesirable replication and associated toxicity in normal cells but permit growth in cancer cells both *in vitro* and *in vivo*. Similarly the incorporation of mir34a miRTs results in reduced *in vitro* VSV replication and cytotoxicity in the presence of activated p53. This thesis provides proof of concept that viruses designed to exploit the differential microRNA expression in cancer cells is a viable approach, potentially useful in optimizing oncolytic viral gene expression for maximal antitumor activity and safety.

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## **List of abbreviations**

dsRNA	double stranded ribonucleic acid
eGFP	enhanced green fluorescent protein
eIF2	alpha eukaryotic initiation factor 2 alpha
ELISA	enzyme-linked immunosorbent assay
ES	embryonic stem
HCV	hepatitis C virus
HMGA2	high mobility group AT-hook 2
HPV	human papillomavirus
HSV-1	herpes simplex virus 1
IFN	interferon
IN	intranasal
IRES	internal ribosome entry site
IV	intravenous
microRNAs	microribonucleic acid
miRTs	microRNA target sequence
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
OV	oncolytic virus
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming units

PFV	primate foamy virus
PKR	protein kinase R
qRT-PCR	quantitative reverse-transcription polymerase chain reaction
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNP	ribonucleoprotein
RT-PCR	reverse-transcription polymerase chain reaction
SD	standard deviation
shRNA	short-hairpin ribonucleic acid
siRNA	short interfering ribonucleic acid
ssRNA	single stranded ribonucleic acid
TK	thymidine kinase
UTR	untranslated region
VEGF	vascular endothelial growth factor
VGF	vaccinia growth factor
VSV	vesicular stomatitis virus

## **1 Introduction.**

This thesis describes a novel engineering strategy to target oncolytic viruses to cancer cells by exploiting the differential expression of microRNAs between cancerous cells and normal cells.

### **1.1 Oncolytic viruses.**

The use of replicating viruses as a treatment for cancer has a long and interesting history (for a review see Kelly et al., 2008)<sup>1</sup>. The origin of oncolytic virotherapy stems from infrequent anecdotal cases of cancer patients who experience transient remission after contraction of a viral infection. Typically these cases involve patients with hematological malignancies, an associated immune suppression and a subsequent naturally acquired infection such as influenza, chickenpox or measles. The obvious hypothesis followed; that optimized intentional viral inoculation could be a cure for cancer.

Early work in the field suffered greatly from a lack of standardization and available means to propagate viruses<sup>2</sup>. For example, material for clinical trials was often straight serum from viremic blood donors<sup>3,4</sup>. Additionally, there was almost nothing known about the mechanism of virus cytotoxicity and some early clinical studies resulted in patients experiencing adverse events from viral infection of normal tissues<sup>5,6</sup>. Despite these challenges, some of these early clinical trials demonstrated transient responses with only a few mild side effects and suggested that oncolytic virotherapy held huge potential.

With successive revolutions in tissue culture, *in vivo* cancer models, virology, molecular biology, immunology and genetics, the preclinical development of oncolytic viruses has experienced accompanying advances. Development of tissue culture methods has made it possible to propagate virus *ex vivo* and quantify infectious virions by standardized assay. *In vivo* animal models of cancer have made it possible to examine many aspects of oncolytic viral therapy including dosing and delivery methods without compromising safety (for examples see Andreansky et al., 1996 and Wang et al., 2003)<sup>7,8</sup>. Perhaps the greatest impact on the field so far has been the ability to manipulate viral genomes using reverse genetics. Reverse genetic systems have enabled the rational design of many oncolytics, including influenza<sup>9</sup>, herpes simplex virus 1 (HSV-1)<sup>10,11</sup>, Semliki Forest Virus<sup>12</sup>, vesicular stomatitis virus (VSV)<sup>13</sup>, adenovirus<sup>14</sup>, vaccinia virus<sup>15</sup>, coxsackie virus<sup>16</sup>, measles virus<sup>17</sup> and poliovirus<sup>18</sup>. The potential of oncolytic viruses is no longer limited by the properties of viruses found in nature or evolved in the lab. The ability to edit viral genomes has facilitated the application of the accumulated knowledge of virology, cancer and immunology to produce novel oncolytic viruses that are much more effective and safer.

## **1.2 Targeting Oncolytic viruses to cancer.**

The previously mentioned anecdotal cases of remission upon naturally acquired viral infection suggest that at least some viruses inherently replicate in and destroy cancer cells. This has been repeatedly confirmed in tissue culture with a

wide variety of viruses and indeed innate tropism for cancer cells is perhaps common<sup>19-21</sup>. Natural strains of virus however, often have associated pathogenic effects *in vivo*. For example, the naturally occurring strains of coxsackie A21, poliovirus, HSV-1 and VSV display oncolytic potential *in vitro* but all have associated pathologies as a result of viral replication in normal cells of the host. Various genetic engineering strategies to restrict viral replication to cancer cells have therefore been employed to reduce the possibility of these pathologies occurring in a therapeutic setting.

### 1.2.1 Viral Surface Protein Engineering.

One of the most common tumour targeting strategies is to engineer viral surface proteins such that they bind to tumour specific cell surface proteins. For example, the fibre knob of adenoviruses, which normally binds the cell surface coxsackie-adenoviral receptor, has been modified to bind to various tumour associated cell surface antigens. This includes the melanoma-associated cancer-testis antigen and the epidermal growth factor receptor and results in expanded tumour specificity of oncolytic adenoviruses<sup>22-25</sup>. In another excellent example of this strategy, the surface glycoprotein of VSV was replaced with a Sindbis glycoprotein that had been conjugated to a single chain antibody directed against the breast cancer tumour antigen Her2/neu, resulting in an oncolytic VSV which had a 100-fold replication advantage on Her2/neu expressing cells<sup>26</sup>. This strategy unfortunately has the disadvantage that the modified viral surface protein may not function as well

as the native surface protein<sup>26,27</sup>. Also, for each individual cancer a tumour specific surface antigen must be known and a modified viral surface protein must be developed.

Modification of oncolytic viral surface proteins is not limited to increasing binding affinity for tumour specific surface proteins. Measles virus requires a specific proteolytic cleavage, by the intracellular protease furin, of the viral envelope fusion protein for activation and subsequent infection. The fusion surface protein of an oncolytic measles virus has been engineered to be activated specifically in the presence of matrix metalloproteases that are highly enriched in the tumour microenvironment. This proteolytic cleavage site alteration therefore results in a virus that is activated preferentially in the tumour microenvironment<sup>28</sup>. Unfortunately this strategy has a limited applicability as most viruses do not require proteolytic cleavage in order to be infectious.

### *1.2.2 Transcriptional Tumour Targeting.*

The lifecycles of both adenovirus and HSV-1 have a component occurring within the nucleus of mammalian cells enabling tumour specific transcriptional targeting of viral virulence. There are many examples of engineering the inclusion of tumour specific or tissue specific promoters to drive transcription of essential adenoviral genes, E1A and/or E1B and/or E2, to create a tumour/tissue specific oncolytic adenovirus. Promoters that have been used for this strategy include the prostate specific antigen promoter<sup>29</sup>, the tyrosinase promoter<sup>30</sup>, the alpha-fetoprotein

promoter<sup>31</sup>, Tcf4 responsive promoter elements<sup>32</sup>, carcinoembryonic antigen promoter and enhancer elements<sup>33</sup>, the E2F promoter<sup>34</sup>, the survivin promoter<sup>35</sup>, the human telomerase reverse transcriptase promoter<sup>36</sup>, hypoxia responsive promoter elements<sup>37</sup>, and the regulatory elements of Flk-1 and endoglin genes<sup>38</sup>. One additional report uses this same strategy of employing cellular transcriptional elements in HSV-1<sup>39</sup>. In this case the B-myb promoter was used to drive expression of the gamma 34.5 gene, which serves to counteract the cell's antiviral response<sup>39,40</sup>. Transcriptional targeting strategies demonstrate promising tumour specific viral replication however these strategies are not compatible with most preclinical oncolytic viruses which do not employ cellular transcriptional machinery for viral gene expression.

### *1.2.3 Translational Tumour Targeting.*

One unique strategy to target poliovirus to cancer cells eliminates the associated pathological neurotropism by swapping the internal ribosome entry site (IRES) of poliovirus for that of human rhinovirus type 2<sup>18,41</sup>. The resulting chimeric virus retains the capability to infect malignant glioma cells but removes the neurovirulence and associated pathologies of poliovirus. The neuropathogenicity of poliovirus relies on the cell-type specific function of its internal ribosomal entry site. This strategy is unique and has produced an effective preclinical oncolytic, however, it has not successfully been applied to any other oncolytic platform<sup>42</sup>. It is possible that this strategy is limited to viruses that employ IRES-activity for translation.

#### *1.2.4 Genetic Complementation Targeting.*

Conceptually, genetic complementation targeting is similar to classical genetic complementation, from which the term is borrowed, to categorize this oncolytic targeting strategy. The complementation referred to here, in contrast, is occurring between a mutant oncolytic virus and a cancer cell, as opposed to between two different mutant individuals of the same species and the complementation is accomplished by a loss of function in the cancer cell.

The only oncolytic virus that is approved for clinical use in human cancer patients is an oncolytic adenovirus that was designed using this strategy. Originally designated dl1520, later renamed ONYX-015, this virus was thought to replicate in cells deficient in tumour suppressor p53 function, a common hallmark of many cancers<sup>43,44</sup>. It was hypothesized that the deletion of E1B, known to counteract p53 function<sup>45,46</sup>, attenuated the virus in wild-type p53 cells while still permitting replication in cancer cells defective in p53 function. It was this mechanism that formed the basis for clinical trials and a patent by ONYX pharmaceuticals<sup>47</sup>. Later, during clinical trials, the tumour specificity of this virus was determined to be due to an RNA export phenotype of cancer cells that complements the E1B deletion<sup>48</sup>. ONYX pharmaceuticals has sold the worldwide patent rights and the virus is approved for clinical use in China<sup>49</sup>.

Other oncolytic viruses that use genetic complementation targeting are in various stages of preclinical testing and trials. As mentioned previously, the gamma

34.5 protein of HSV-1 serves to counteract the host cell antiviral response. Through activation of the cellular protein phosphatase 1 $\alpha$ , gamma 34.5 reverses inactivation of the translation factor eIF2 $\alpha$ . The inactivation is initiated by the RNA-dependent protein kinase, PKR in response to viral infection<sup>50-53</sup>. The phosphorylation of eIF2 $\alpha$  by PKR is an antiviral response that shuts down all host cell translation to inhibit viral replication. The functions of PKR in translational regulation and/or innate immune response are often defective in cancer cells<sup>54</sup>. Therefore oncolytic strains of HSV-1 contain deletions in one or both copies of gamma 34.5 in order to genetically complement the PKR deficient phenotype of some cancers<sup>11</sup>. The resulting viruses have none of the undesirable pathogenic replication in normal neurons of the parental strains, yet replicate well in transformed cells<sup>11,55,56</sup>.

Functionally related to gamma 34.5 knockout oncolytic herpes viruses is an oncolytic strain of the influenza A virus in which the NS1 protein has been deleted<sup>9,57</sup>. The NS1 protein of influenza, like gamma 34.5 of HSV-1, inhibits the antiviral response initiated by PKR<sup>58,59</sup>. Cancer cells are often deficient in the relevant PKR antiviral activities and genetically complement the NS1 deficient phenotype of the oncolytic influenza A virus. Normal cells remain nonpermissive to replication of this oncolytic because they possess a fully functional antiviral response mediated by PKR<sup>58</sup>.

Oncolytic vaccinia viruses also have had genetic deletions to improve tumour tropism. For example JX-594, JX-963 and JX-929 are oncolytic vaccinia viruses in clinical trials and preclinical research, have deletions in one or both of the vaccinia

growth factor (VGF) and thymidine kinase (TK) genes<sup>60,61</sup>. TK catalyzes a key step in the biosynthesis of deoxynucleotides and therefore vaccinia virus lacking this gene is forced to rely on the pool of nucleotides in the host cell, which are more abundant in rapidly dividing cancer cells<sup>62</sup>. The viral protein VGF is secreted by infected cells and stimulates nearby cells to replicate, thus increasing their available nucleotide pool and priming them for infection by vaccinia<sup>63</sup>. Thus in JX-963 and JX-929 this deletion complements the TK deletion and serves to enhance the tumour specificity of the TK deleted oncolytic vaccinia<sup>61</sup>.

An oncolytic VSV has also been targeted to cancer cells using this strategy and is introduced in section 1.32 VSV oncolysis.

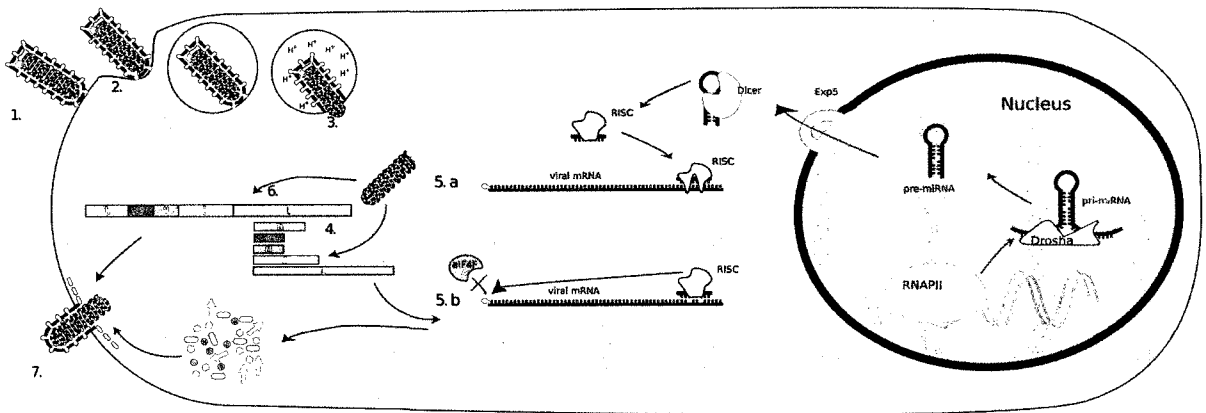
### **1.3 Vesicular Stomatitis Virus.**

Another virus that has been successfully targeted to cancer cell using genetic complementation targeting (see Section 1.24) is vesicular stomatitis virus (VSV). This thesis describes work using VSV as a prototype for a novel oncolytic virus targeting strategy, and thus a more detailed introduction to the viral biology and history as an oncolytic is relevant. An excellent review of VSV biology and VSV oncolysis is available for further reading and from which Figure 2 is adapted to schematically illustrate the hypothesis of this work (Litchy et al., 2004)<sup>13</sup>.

#### *1.3.1 VSV biology.*

Despite being a subject of scientific inquiry for over 70 years, the ecology of

**Figure 1: Vesicular stomatitis virus lifecycle and hypothesized interaction with cellular endogenous microRNAs of the host cell.** The VSV lifecycle can be divided into the following steps as diagramed schematically: attachment (1), endocytosis (2), membrane fusion and nucleocapsid entry (3), gene transcription (4), viral protein translation (5a and 5b), genome replication (6) and virus assembly and budding (7). Endogenous microRNA biogenesis starts with transcription by RNA polymerase II (RNAPII), as depicted. MiRT incorporation into viral transcripts will cause an interactions between endogenous microRNAs and viral transcripts, as depicted in 5a and 5b. In the case of perfectly complementary miRTs to an endogenous microRNA this will result in transcript instability (5a), whereas imperfectly complementary miRTs will instigate translational repression of the miRT containing viral transcript. Abbreviations: RNAPII, RNA polymerase II; Exp5, Exportin 5; RISC, RNA induced silencing complex.



VSV is not fully understood. VSV has very broad tropism and negatively impacts agriculture by infecting cattle, swine and horses, presumably through insect vectors<sup>64</sup>. Clinical manifestation of disease in these animals is low, even in areas where seropositivity is high<sup>65</sup>. The dominant serotypes in the Americas are New Jersey and Indiana. Infection is relatively harmless with symptoms consisting of vesiculation and ulceration of the oral cavity, feet and teats<sup>66</sup>. While infections in humans have been reported in individuals exposed to VSV in a laboratory or enzootic environment, it is thought to be largely asymptomatic<sup>67-69</sup>. The low VSV seropositivity in the general population and the lack of pathogenicity in humans are advantageous features of a potential oncolytic virus<sup>70</sup>.

VSV is a negative-sense ssRNA virus and is a typical member of the viral family *Rhabdoviridae*, belonging to the order *Mononegavirales*. VSV is a bullet-shaped particle about 180nm in length and 75nm in diameter. It has a genome of approximately 11kb that encodes for 5 distinct polypeptides.

The nucleocapsid protein, N, binds the genomic RNA and plays a role in transcription, replication and encapsulation of the genome into newly assembled progeny virus. The nucleocapsid protein and the genome form a nuclease resistant helical structure in the ribonucleoprotein (RNP), which also contains P and L<sup>71</sup>.

The phosphoprotein, P, interacts with L to form the functional viral polymerase and influences the polymerase activities between transcription and replication<sup>72</sup>.

The glycoprotein, G, is on the surface of the virion, and functions in attachment to host cell and fusing with late endosomal membranes allowing the

RNP access to the cytoplasm<sup>73,74</sup>.

The matrix protein, M, is multifunctional. It serves a structural role as an integral component of the virion, is essential for viral progeny assembly and budding<sup>75,76</sup>, induces host cell apoptosis<sup>77-79</sup> and counteracts the host cell antiviral response by blocking the nucleocytoplasmic transport of newly transcribed RNA<sup>80-82</sup>. The block of newly transcribed mRNAs from the nucleus to the cytoplasm prevents expression of antiviral proteins such as type I interferons (IFNs). In this way, M protein in the infected cell prevents type I interferon production from signaling nearby uninfected cells to adapt an antiviral state<sup>80</sup>. Normally a cell exposed to type I interferons is highly resistant to VSV infection thus the M protein is essential for spread of the virus<sup>80,83</sup>.

Each protein encoded by the VSV genome is transcribed by the viral RNA-dependent RNA polymerase, L, encoded by the virus. The L protein is over 240kDa, and serves many viral functions including ribonucleotide polymerization, mRNA 5'capping, cap structure methylation, and 3'mRNA polyadenylation. Viral gene transcription catalyzed by L occurs in association with P and directly utilizes the negative stranded genome as a template. The mRNA abundance of each viral gene reflects the position of these genes within the genome. This is a result of the sequential transcription of each gene with a 29-33% attenuation of transcription at each successive gene junction<sup>71</sup>. This mechanism results in the transcript abundance N>P>M>G>L<sup>84,85</sup>. Viral genome replication, also catalyzed by L in association with P, begins after transcription and translation of viral genes.

The noncoding features of the genome include transcriptional stop and start signals, and a short, 47bp, viral leader sequence transcribed from the extreme 3' end of the genome. The VSV leader RNA may play an important role *in vivo* in the synthesis of the full-length positive-strand genome RNA by providing the necessary *cis*-regulatory sequences for signaling encapsidation with the N protein.

Interestingly, the leader RNA also accumulates in the nucleus shortly after infection, the significance of which is unclear<sup>86,87</sup>. Viral untranslated regions (UTRs) of the five VSV mRNAs, both 5' and 3'UTRs, are less than 25bp long with the exception of the 3'UTR of VSV M mRNA which is 55bp, and the 5'UTR of VSV L mRNA which is 49bp long. There is no known function of these sequences in VSV, although the length of the intergenic region that includes UTRs can affect the efficiency of which these genes are transcribed, suggesting the length of the UTRs may be significant for maintaining optimal gene expression<sup>88</sup>.

### 1.3.2 VSV oncolysis.

VSV is exquisitely sensitive to interferon, whereas many tumours are insensitive to interferon. These observations suggested that VSV might have useful oncolytic properties<sup>83,89</sup>. There are laboratory isolates and engineered mutants of VSV in which the matrix protein demonstrates defective inhibition of nucleocytoplasmic transport. These viruses are less pathogenic *in vivo*<sup>80</sup>. Infection with such mutant viruses results in the production of type 1 interferons from the infected cell protecting nearby neighbouring cells<sup>80</sup>. These interferon inducing

mutant VSVs make excellent genetic complementation targeted oncolytic viruses because cancer cells often acquire deficits in interferon responsiveness and/or production<sup>80,89</sup>. A normal cell that has adopted an antiviral state after exposure to type 1 interferons is highly resistant to subsequent infection with VSV, whereas interferon insensitive cancer cells remain permissive. The mutant viruses originally isolated contain point mutations in the matrix gene resulting in an amino acid change. The phenotype of defective inhibition of nucleocytoplasmic transport has been engineered into the VSV genome by deletion of a single amino acid at position 51 of the M protein<sup>80</sup>. This engineered virus, VSV  $\Delta$ 51, has proven itself to be an excellent oncolytic virus in many preclinical studies<sup>90-98</sup>.

Despite the preclinical success of VSV  $\Delta$ 51, improvements to this oncolytic platform are desirable. The interferon inducing mutants of VSV have reduced oncolytic capacity as compared to a wt VSV<sup>99,100</sup>. Unfortunately, VSV containing a wt M protein causes lethal CNS infection in mice making it an unsuitable oncolytic<sup>79,80,101</sup>. Recent reports have cited increased VSV oncolytic efficacy by the incorporation of various immunomodulatory genes<sup>102,103</sup>. While increasing potency, this approach does not increase safety of the virus. Novel engineering strategies are therefore clearly needed to improve targeting VSV replication to tumours, ensuring safety, while maintaining oncolytic potency. This thesis describes a novel strategy of using differential expression of microRNAs as means to target oncolytic viruses using VSV as a prototype.

## 1.4 MicroRNA.

MicroRNAs are a class of recently discovered small (20-24nt) single-stranded RNAs (ssRNAs) that play an important role in the post-transcriptional regulation of eukaryotic gene expression. Much like other previous revolutions in our understanding of biology and cancer, microRNAs offer new strategies for oncolytic virus design.

### 1.4.1 Biogenesis.

Our understanding of the cytoplasmic steps of microRNA biogenesis arose from studies of RNA interference (RNAi). RNAi is a phenomenon commonly exploited to specifically inhibit gene expression through a sequence specific mechanism. In 1998, Fire et al. originally observed that RNAi is triggered efficiently by exogenous double-stranded RNA (dsRNA)<sup>104</sup>. This contrasted the view that RNAi was a result of single stranded antisense sequences binding mRNA transcripts to inhibit translation<sup>105</sup>. It was subsequently shown that RNAi is the result of dsRNA cleavage by the RNAase III-like endonuclease, Dicer, into 21-24bp fragments. These RNA fragments then direct the endoribonuclease activity of the RNA-induced silencing complex (RISC) to complementary RNAs<sup>106-109</sup>. These pathways, it was later determined, are the cytoplasmic component of the biogenesis of endogenous microRNAs<sup>110-112</sup> (see Figure 1).

In the nucleus, microRNAs originate as a variety of capped and polyadenylated RNA-polymerase II transcripts, referred to as pri-miRNAs. In

animals, short hairpin structures within these transcripts are substrates for the microprocessor complex. This complex, composed of Drosha and DGCR8 (humans) or Pasha (in *Caenorhabditis elegans* and *Drosophila*), contains RNase III-like enzymatic activity which cleaves these short hairpin structures at their stem. This releases a smaller hairpin composed of a dsRNA with a terminal loop at one end and ~2 unpaired nucleotides at the 3' end termed a pre-miRNA<sup>110-112</sup>.

Following nuclear processing, the resulting pre-miRNA is exported from the nucleus into the cytoplasm. The specific length and 3' overhang of the pre-miRNA is recognized by exportin 5 and is subsequently transported through the nuclear pore complex.<sup>110-113</sup>

Pre-miRNAs are then substrates for Dicer and the products are loaded onto the RISC complex. MicroRNAs originate exclusively in the nucleus, as described, however, after Dicer cleavage in the cytoplasm, the composition of microRNAs is indistinguishable from short interfering RNAs<sup>110,114</sup>.

Short interfering RNA (siRNA) refers to short dsRNA products of Dicer activity of an exogenous or endogenous source. Exogenous siRNAs that mimic Dicer products are commonly used for sequence specific gene expression inhibition<sup>110,114</sup>. Perfect sequence complementarity between siRNA and substrate mRNA induces potent gene repression by endoribonucleolytic cleavage catalyzed by a component of the RISC complex. The distinction between siRNA and microRNA, apart from their origin, is that the latter rarely share perfect sequence complementarity with their target mRNAs. This results in a different mechanism of gene expression

regulation between siRNA and microRNA<sup>110</sup>.

#### *1.4.2 Regulation of gene expression by microRNA/siRNA.*

Small RNAs generated by Dicer activity determine the sequence specificity of RISC mediated translational repression by binding complementary sequences on mRNAs. As mentioned previously, it is partially complementary target sequences that facilitate microRNA mediated translation repression. The precise sequence requirements of a functional microRNA target sequence are not clear and are likely context dependent. Perfect complementarity is required in the 'seed' sequence, which comprises ribonucleotides 2-7 of the microRNA, although the 'seed' concept has exceptions<sup>115,116</sup>. Computational genomic analyses of putative target sequences suggest that microRNAs affect the translation of more than 10% of human mRNAs<sup>117</sup>. The redundancy of microRNA target sequences enables a single microRNA to affect the expression of possibly hundreds of different mRNAs to regulate complex cellular programs<sup>118</sup>.

An mRNA recruits a microRNA loaded RISC complex (miRISC) indirectly through a target sequence located in its 3'UTR. The precise mechanism by which microRNAs inhibit translation of endogenous mRNAs is a subject of ongoing debate<sup>118</sup>.

Also, it is not clear whether miRISC mediated inhibition occurs at the level of translation initiation or elongation<sup>118</sup>. Translation initiation of the vast majority of cellular mRNAs is dependent on the 5'cap structure. Internal ribosome entry sites

initiate translation in a cap-independent mechanism and have been used to probe the role of translation initiation in microRNA mediated repression. Unfortunately, conflicting reports suggest mRNAs containing viral IRES sequences are subject to microRNA mediated translational repression while others demonstrate these transgenes are refractory to microRNA repression<sup>116,117,119</sup>. Adding to the complexity is the fact that different cellular IRES isoforms of the VEGF gene are differentially susceptible to microRNA mediated translational repression<sup>120</sup>. Interestingly, an mRNA cap-binding domain has been found in one of the components of the RISC complex, suggesting that translation initiation is inhibited by miRISC<sup>121</sup>. Indeed the bulk of the evidence favours translation initiation as the step at which miRISC exerts its effect but it is possible that there are exceptions<sup>118</sup>.

In contrast to partially complementary sequences, perfectly complementary target sequences, rarely found on endogenous mRNAs in animals, results in miRISC mediated cleavage of the target mRNA. The products are two highly unstable mRNAs, resulting in more potent gene expression inhibition than occurs in the case of partially complementary target sequences<sup>122</sup>. This mechanism, RNAi, is identical to that which occurs when siRNAs are loaded onto the RISC complex. The determinant of the mechanism of translation inhibition, therefore, is the degree of target sequence complementarity to the small RNA loaded on the RISC complex<sup>123,124</sup>. The RNAi mechanism remains functional in mammalian cells, however, there is only one known example of an endogenous mammalian microRNA instigating RNAi of a perfectly complementary endogenous mRNA<sup>125</sup>.

Mammalian RNAi can also occur through endogenous siRNAs (endo-siRNAs) produced by pseudogene-derived dsRNA, natural antisense transcripts and transposons<sup>126</sup>. A transgene containing a perfectly complementary target sequence to an endogenous microRNA, also known as a miRT sequence, is also subject to RNAi<sup>122,127</sup>. Conversely, a synthetic microRNA, or shRNA, with perfect complementarity to an endogenous mRNA can silence expression through RNAi<sup>128</sup>. A component of the hypothesis of this work is that oncolytic viral gene expression will also be subject to the translational inhibition of endogenous microRNA. The novel oncolytic targeting strategy demonstrated in this work also relies on the aberrant endogenous microRNA expression in cancer.

#### *1.4.3 MicroRNAs and Cancer.*

MicroRNAs are normally expressed within cells in a precise time and tissue specific manner, allowing them to be involved in a broad range of biological processes including development, differentiation, proliferation and apoptosis. As deregulation of these same processes is the hallmark of cancer, microRNAs have been implicated in cell transformation and tumour progression (see review Ventura and Jacks 2009)<sup>129</sup>.

Global microRNA expression analysis studies suggest that the expression of most microRNAs, although not all, is decreased in tumours as compared to the tissue of origin<sup>130-132</sup>. It is possible this is due to the less differentiated state of tumour cells, as onset of expression of many microRNAs correlates with

differentiation. This is also supported by observations of undifferentiated embryonic stem cells (ES cells) with introduced deficits in microRNA biogenesis that become refractory to differentiation<sup>133,134</sup>. Deficits in microRNA biogenesis are also found in cancers with poor prognosis and these deficits enhance cellular transformation and tumourgenesis<sup>135-138</sup>. Together these observations have led to some speculation that the observed abnormalities in cancer microRNA expression support the 'cancer stem cell hypothesis'<sup>130,139</sup>. Cancer stem cells have indefinite proliferative potential that can drive tumour growth and can share global microRNA expression profiles with related stem cells<sup>140-142</sup>. It has been argued that cancer stem cells are an important therapeutic target<sup>139</sup>. Global microRNA defects are a relatively recently identified property of some cancers and cancer stem cells that may help identify new possibilities for cancer diagnosis and treatment.

Aberrant expression of specific microRNAs is also implicated in metastasis and tumour progression<sup>143</sup>. Some microRNAs that are downregulated in many different cancers are putative tumour suppressors. The concept that noncoding RNAs could have tumour suppressive properties is supported by the fact that many microRNAs are found at loci commonly deleted in human cancer<sup>144</sup>. This thesis aims to demonstrate that the aberrant expression of putative tumour suppressor microRNA species is a therapeutically relevant target using oncolytic viruses.

#### 1.4.3.1 *let-7*.

Some of the most actively studied putative tumour suppressor microRNAs

are the let-7 family of microRNAs<sup>145</sup>. The let-7 microRNAs repress cell proliferation pathways<sup>146</sup> and have repeatedly been observed to have decreased expression in cancer<sup>130,147-151</sup>. Decreased expression of the mature let-7 microRNA is also associated with less differentiated cancer<sup>137,149,152</sup>. The mechanism of decreased let-7 expression, in at least some cancers, is at the post-transcriptional level, controlled by Lin28 and Lin28B<sup>153-157</sup>. These RNA-binding proteins are expressed specifically in undifferentiated cells<sup>153,158,159</sup>, overexpressed in hepatocellular carcinoma and induce cancer cell proliferation *in vitro*<sup>160</sup>. Lin28B is also a transcriptional target of c-Myc, contributing significantly to its oncogenic activity<sup>161</sup>. The association of Lin28 with the induction of the stem cell phenotype<sup>162</sup>, cancer and let-7 maturation have led to the suggestion that low let-7 expression is associated with the maintenance of the 'cancer stem cell'<sup>151</sup>.

The functional significance of low microRNA expression in cancer is upregulation of target mRNAs. Let-7 is one of only a few microRNAs with well established target mRNAs. Experimentally validated mRNAs translationally repressed by mature let-7 microRNA are members of the Ras family of proto-oncogenes<sup>163</sup>, Myc<sup>164,165</sup> and HMGA2<sup>166,167</sup>. These genes have well established roles in oncogenesis and their coordinated regulation by let-7 microRNA strongly support its role as a tumour suppressor.

A major hypothesis of this work is that low let-7 expression exhibited by cancer cells represents a therapeutically relevant target for engineered oncolytic viruses.

#### 1.4.3.2 *mir-34a*.

Another family of microRNAs with putative tumour suppressor activity is the *mir-34* family of microRNAs. It is well established that *mir-34* is a direct p53 transcriptional target and is a central mediator of this well known tumour suppressor gene's function<sup>168,169</sup>. Increased *mir-34a* expression is associated with p53 activation. Small molecules stabilizing p53 and DNA damaging agents known to activate p53 transcriptional programs also induce *mir-34* through a p53 dependent mechanism<sup>170-173</sup>.

*Mir-34* is downregulated in cancer through a variety of mechanisms. Oncogenic human papillomavirus (HPV) type 16 and 18 positive cervical cancer cell lines both have low *mir-34* expression due to a HPV protein that destabilizes p53<sup>174</sup>. Two members of the *mir-34* microRNA family are located in regions frequently deleted in neuroblastoma<sup>175,176</sup> or transcriptionally silenced by CpG methylation in many cancer cell lines<sup>177</sup>. *Mir-34* is also found transcriptionally repressed by oncogenic c-Myc<sup>132,161</sup>. Reintroduction of *mir-34* results in cell cycle arrest, senescence or apoptosis of cancer cells indicating that low expression of *mir-34* is not simply coincidental but plays a causal role in oncogenesis<sup>170,177-179</sup>.

Targets of *mir-34* translational repression include E2F3<sup>170,176</sup>, hdmX<sup>180</sup>, bcl2<sup>175</sup>, mycN<sup>175,181</sup>, cdk6<sup>177</sup> and sirT1<sup>182</sup> all of which have been found overexpressed in cancer.

While the expression of *mir-34* does not correlate with differentiation of

tumours, like the let-7 family of microRNAs, mir-34 represses the expression of oncogenes through a p53 dependent mechanism. Additionally the high frequency of functional loss of p53 activity in cancer cells and the evidence that mir-34 mediates many important functions of p53, including apoptosis and senescence, place mir-34 as a putative tumour suppressor microRNA<sup>168</sup>. A major hypothesis of this work is that the inability to induce p53-dependent mir-34 expression in cancer cells represents a therapeutically relevant target for engineered oncolytic viruses.

#### *1.4.4 Cellular microRNAs and viruses.*

The partially complementary sequence requirement for microRNA mediated translational repression enables promiscuous complementarity and possible inhibitory effects on virally derived RNAs. The interactions of endogenous mammalian microRNAs with infectious virus are currently being investigated by several groups.

There are a couple of examples that indicate microRNAs may have some antiviral effects. The replication of primate foamy virus (PFV) is inhibited in Hela and BHK21 cells, by the endogenous microRNA, mir-32. A mir-32 target site present in a poorly conserved region in the 3' portion of the PFV genome is responsible for this effect<sup>183</sup>. It has also been found that transient upregulation of several microRNAs containing partially complementary sequences to the hepatitis C virus (HCV) genome occurs after interferon treatment of hepatocytes. Transient transfection of two of these microRNAs, mir-196 and mir-448, reduces HCV RNA accumulation in a

mechanism dependent on the presence of complementary sequences in the HCV genome<sup>184</sup>. While it is unclear from this study whether endogenous levels of these microRNAs would have an effect on HCV replication, these studies raise the question of whether specific cellular microRNAs have evolved to limit the replication of particular viruses. VSV also appears to be sensitive to endogenous microRNAs expressed in an infected cell<sup>185</sup>. The microRNAs mir-24 and mir-93 account for some of the interferon independent attenuation of VSV that is not observed in Dicer deficient mouse cells. Predicted partially complementary sequences for mir-24 and mir-93 are within the open reading frame of VSV P and VSV L, respectively<sup>185</sup>. Clearly endogenous microRNAs influence a variety of viruses including VSV and it has therefore been suggested that microRNAs may have a biologically significant role in determining the tissue tropism of animal viruses<sup>186</sup>.

MicroRNAs serve a vital role in gene expression regulation through translational repression of target mRNAs, and can induce RNAi if an RNA contains a perfectly complementary sequence. The RNAi mechanism represents a vital component of the innate immune response in plants and invertebrates. In mammals, however, it has been argued that the protein based interferon mediated antiviral response has largely replaced RNAi<sup>187</sup>. As discussed in section 1.42, RNAi can be triggered in mammalian cells either by siRNA or by an endogenous microRNA sharing perfect complementarity to a target transcript.

Interestingly, many viruses that infect mammalian cells are susceptible to RNAi. Large reductions in progeny virus are observed upon transfection of siRNA

specific for two retroviruses, human immunodeficiency virus and Rous sarcoma virus; a negative-strand RNA virus, respiratory syncytial virus (RSV); and a positive-strand RNA virus, poliovirus. Exogenous siRNA can also inhibit the gene expression of a DNA virus, human papillomavirus<sup>188</sup>. This diversity suggests viral mRNAs transcribed in the nucleus or the cytoplasm are both subject to RNAi and that viruses may be universally susceptible to exogenous siRNA.

VSV is a nonsegmented negative-strand RNA virus and, similar to RSV, is also subject to RNAi<sup>189,190</sup>. Inhibition of M protein expression in VSV infected HEP-2 cells occurs in response to prior transfection of siRNA complementary to the open reading frame of the VSV M mRNA. This decreased expression affects the M protein and its associated cytopathic effect exclusively; other viral genes remained unaffected<sup>189</sup>. A similar gene specific inhibition was observed for RSV and it was suggested that the genome and antigenome of nonsegmented negative-strand RNA viruses are not subject to RNAi. It was argued that antiviral gene specific siRNA did not affect expression of all viral genes because the genome and antigenome are part of the nuclease resistant RNP. Therefore the observed gene specific inhibition was the result of siRNA activity on viral mRNA species containing complementary sequence<sup>191</sup>. The use of exogenous siRNA has potential as a nucleic acid-based therapeutic against a wide variety of viruses but it is not directly applicable to oncolytic viruses<sup>192</sup>. It does suggest, however, that a VSV containing miRTs in a viral mRNA would experience the same gene specific expression inhibition since, like siRNA, perfectly complementary microRNA triggers RNAi.

Examined in this work is the potential for attenuation of a wildtype, pathogenic VSV by incorporation of let-7 or mir-34a miRTs. It is hypothesized that let-7 and mir-34a miRT mediated attenuation of VSV will permit replication and oncolysis of tumour cells but eliminate or reduce the pathogenicity of VSV.

### **1.5 Hypothesis**

Incorporation of tumour suppressor miRTs into the 3'UTR of VSV M mRNA will result in a virus that has desirable oncolytic properties. Specifically, the differential expression of let-7 and mir-34a between cancer and normal cells can be exploited by incorporation of microRNA target sequences (miRTs) into a normally pathogenic VSV, producing an oncolytic virus with enhanced safety. Perfectly complementary miRTs, designed to initiate RNAi, will be more potent attenuators of VSV than partially complementary miRTs designed to initiate translational repression. The incorporation of let-7 miRTs in the 3'UTR of VSV M mRNA causes attenuation of VSV cytotoxicity and replication in normal cells. Attenuation is the result of decreased expression of wild-type M protein in infected cells due to the induction of RNAi (Figure 1). The reduction of VSV M protein in infected cells allows the protection of neighbouring normal cells by expression of type I interferons. Such a virus replicates well in a mouse tumour model but is less pathogenic than wild-type VSV. The incorporation of mir-34a miRTs in the 3'UTR of VSV M mRNA creates a virus that is sensitive to the p53 dependent expression induction of mir-34a. The activation of p53 with conventional chemotherapeutics inhibits VSV

containing mir-34a miRTs. This virus possesses reduced replication capacity in the CNS of mice that have previously been exposed to p53 activating agents as would occur during combination therapy. The objective of this thesis is to examine these hypotheses.

Specific rationale behind the choice of let-7 and mir-34a, VSV and VSV M mRNA as a target for miRTs are explained and reflected upon in section 4.

## **2 Materials and Methods.**

### **2.1 Cell lines.**

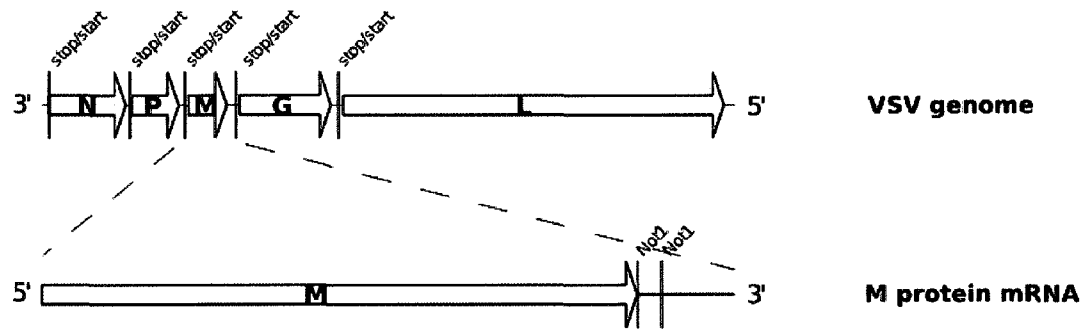
Human A549 lung carcinoma (American Type Culture Collection (ATCC) (Rockville, MD)), CT26 mouse colon carcinoma cell line (ATCC), Human Hela cervical carcinoma (ATCC), and Human GM38 primary fibroblasts (National Institute of General Medical Sciences Mutant Cell Repository (Camden, NJ)) were propagated in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal calf serum (Cansera, Etobicoke, Ontario, Canada). HCT116 p53+/+ and isogenic HC116 p53-/- were a gift from Dr. Bruce McKay and were originally from Bert Vogelstein's lab (John Hopkins University, Baltimore, MD)<sup>193</sup>. These cells were maintained in McCoy's 5A media with 10% fetal calf serum.

### **2.2 Viruses.**

Novel recombinant viruses were cloned as described in Figure 2 and rescued as described previously<sup>80</sup>, with the following modifications. This procedure uses a vaccinia virus that expresses bacteriophage T7 RNA polymerase. A high multiplicity of infection (MOI) infection of A549 cells precedes transfection with four plasmid DNAs. Three plasmid DNAs each driving the expression of one of Maraba N, P or L by a CMV promoter and a previously described plasmid encoding a positive-sense VSV (Indiana strain) genome transcribed by T7 were transfected into A549 cells<sup>194</sup>. Viruses containing luciferase were

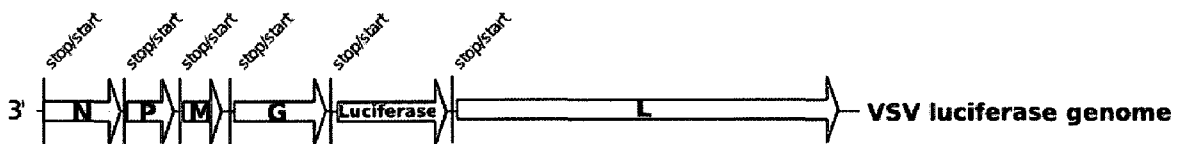
**Figure 2: Schematic of miRTs incorporated into VSV let-7mm, VSV let-7wt, VSV let-7mut and VSV mir34a-wt. (a)** The VSV genome and viral transcripts with the location of an introduced Not1 restriction site in the 3' untranslated region (3'UTR) of VSV M are represented. The mRNA of VSV M contains the original 3'UTR following the incorporated Not1 site. The sequence elements inserted into the Not1 site in VSVlet-7mm, VSVlet-7 and VSVlet-7wt show partial complementarity, complete complementarity or limited complimentary, respectively, to mature let-7a microRNA, while the miRT inserted into VSVmir34a-wt shows complete complementarity to mature mir34a microRNA. All miRTs are present in triplicate as this has been shown to increase inhibitory potency<sup>122</sup>. The viruses derived from insertion of let-7mut, let-7mm, let-7wt and mir34a-wt are designated VSVlet-7mut, VSVlet-7mm, VSVlet-7wt and VSVmir34a-wt, respectively. **(b)** Schematic of VSV genome containing luciferase transgene in between genes encoding VSV G and VSV L. In addition to the miRT containing VSVs depicted in (a), the let-7 miRTs in (a) have also been equivalently placed in the 3'UTR of luciferase in the VSV luciferase genome to create VSVLuciferaselet-7mut and VSVLuciferaselet-7wt. Additionally a VSV luciferase genome was created in which a mir34a miRT has been incorporated into the 3'UTR of VSV M.

**a**



STOP  
TGAGCGGCCGC(GCACAGCCTATTGAACTACCCCTCACTCGA)(x3)GCGGCCGC(3'UTR) **let-7mm**  
TGAGCGGCCGC(AACTATAACAACCTACTACCTCACTCGGA)(x3)GCGGCCGC(3'UTR) **let-7wt**  
TGAGCGGCCGC(GCACAGCCTATTGAACTACCTCACTCGGA)(x3)GCGGCCGC(3'UTR) **let-7mut**  
TGAGCGGCCGC(AACAACCAGCTAAGACACTGCCA)(x3)GCGGCCGC(3'UTR) **mir34a-wt**

**b**



cloned by insertion of the luciferase ORF (pGL3Basic, Promega, Madison, WI) followed by let-7 microRNA target sites were cloned into an Nhe1 site in a VSV genome engineered to carry transgenes previously described<sup>195</sup>. Propagation of all viruses was done in A549 cells. VSV infections *in vitro* were done in a minimal volume of serum free media for 35min before the replacement of serum containing media. For the growth curve (Figure 3c) the cells were washed once with phosphate-buffered saline (PBS) to remove free input viral particles. Virions were purified from cell culture supernatants by passage through a 0.2 µm Steritop filter (Millipore, Billerica, MA). Virions were quantified by plaque assay on Vero cells. For animal studies virions were concentrated by centrifugation at 30 000g and resuspension in PBS (Hyclone, Logan, UT). Resuspended virus was then sedimented through a linear sucrose gradient (5-50% sucrose) by ultracentrifugation in a Beckman SW28 rotor for 30 min at 24 000rpm. The thickest band was collected and concentrated by centrifugation at 30 000g and resuspension in PBS. Aliquots were kept at -80C and subsequently thawed only once. Quantification of a thawed aliquot was done by plaque assay on Vero cells. Assistance with rescue of recombinant VSV was given by Dr. Christopher Brown (University of Ottawa) and Dr. Brian Lichty (McMaster University). Removal of virions from cell supernatant, as required in experiments described in Figure 9, was performed using Amicon Ultra-15 Centrifugal Filters with a 100 kDa MW cutoff (Millipore, Billerica, MA) as per the manufacturers instructions. Original supernatant volumes were centrifuged in the Amicon Ultra-15 filter units until 2mL supernatant

had produced 1.5mL of virus free supernatant.

### **2.3 Mice and tumour models.**

Female mice were obtained from Charles River Laboratories (Wilmington, MA). CD-1 nude mice were injected subcutaneously with  $1 \times 10^6$  A549 cells to establish right hind flank tumors. Tumour bearing animals were treated intravenously with  $10^9$  pfu in 100  $\mu$ L. Tumour volume was measured using electronic calipers and volume calculated using the formula  $(\text{length}/2 * \text{width}^2)$ . Toxicity studies for the let-7 miRT containing viruses were conducted with intranasal administration of  $10^5$  pfu in 5  $\mu$ L of VSV to 6-week-old BALB/c mice or  $7.5 \times 10^7$  pfu in 5  $\mu$ L to CD-1 nude mice. Toxicity studies for the mir34a miRT containing viruses were conducted with intranasal infection of  $2 \times 10^7$  pfu or intravenous infection of  $1 \times 10^9$  pfu virus. All experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Service. Intravenous and subcutaneous injections were performed by Theresa Falls. Chemotherapeutic drugs were obtained from the Ottawa Hospital Regional Cancer pharmacy and mice were treated IV using undiluted drug as indicated.

### **2.4 Cell survival assay.**

Survival was determined by assaying mitogenic activity using a colourimetric MTS assay kit, CellTiter AQ 96 (Promega) as per the manufacturer's instructions.

## **2.5 Luciferase assays.**

For determination of let-7 functional activity in cell lines lysates from transformations of pGL3Basic with a let-7 target sequences in the 3'UTR along with pRL (Promega, Madison, WI) were prepared as described for the Dual Luciferase Reporter Assay System (Promega, Madison, WI). The activity was measured using a luminometer (Lumat LB 9509, EG&G Berthold, Bad Wildbad, Germany).

## **2.6 *In vivo* imaging.**

Mice were injected with *d*-luciferin (Molecular Imaging Products Company, Ann Arbor, MI) (200 $\mu$ L intraperitoneally at 10mg/ml in PBS) for firefly luciferase imaging. Mice were anesthetized under 3% isoflurane (Baxter Corp., Deerfield, IL) and imaged with the *in vivo* imaging system 200 Series Imaging System (Xenogen Corporation, Hopkinton, MA). Data acquisition and analysis was performed using Living Image v2.5 software. The images were captured under identical exposure, aperture and pixel binning settings, and bioluminescence is plotted on identical color scales as indicated.

## **2.7 qRT-PCR.**

VSV M mRNA was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions using oligo dT primers. qPCR was done using Platinum Taq (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions in the presence of SYBR green. Expression of microRNA was quantified using

mirVana qRT-PCR kit (Ambion, Austin, TX) using total RNA isolated using mirVana microRNA isolation kit (Ambion, Austin, TX) both used as per the manufacturer's instructions. All PCR was quantified in real time using a Rotor-Gene GR-3000A thermocycler (Corbett Research, Sydney, Australia).

Primers used are listed below

VSV M sense: 5'-CATCTGGAGCGTGGGTCCTGG; VSV M antisense: 5'-CTAGGCTCGAGAAGCTTGTCTG; VSV N sense: 5'-GCTGCATTGGCAACATTTGG; VSV N antisense: 5'-GGCATGTATGAATCGGCCTTG; VSV L sense: 5'-CCACCCCTTACCCAAAGATGC; VSV L antisense: 5'-CGGAGCCGTCTCCACAACCTC

## **2.8 Viral infections and exogenous siRNA.**

Dilutions of VSV in 100µL of DMEM were added to confluent cells in 6-well plates placed in a 5%CO<sub>2</sub> 37C incubator for 40min before 2mL of DMEM containing 10%FBS was added. 50pmol of exogenous siRNA (Dharmacon) or pre-miR (Ambion) when present were transfected using Oligofectamine (Invitrogen) for Hela cells and Lipofectamine 2000 (Invitrogen) for A549 cells as per the manufacturer's instructions 24hrs before infection.

Sequences of siRNA and pre-miR RNA are listed below:

let-7a: 5'-TGAGGTAGTAGGTTGTATAGTT

eGFP: 5'-AAGCTGACCCTGAAGTTCATC

mir34a: 5'-

GGCCAGCUGUGAGUGUUUCUUUGGCAGUGUCUUAGCUGGUUGUUGUGAGC  
AAUAGUAAG  
GAAGCAAUCAGCAAGUAUACUGCCCUAGAAGUGCUGCACGUUGUGGGGCC

## **2.9 Western Blot of viral proteins.**

Cell lysates were collected in RIPA buffer (50mM Tris pH 8.0, 150mM NaCl, 1% NP-40, 0.5% deoxycholic acid and 0.1% SDS), cleared by centrifugation and quantified by Bradford assay. Equal amounts of total protein were electrophoresed on a NuPAGE Bis-Tris 4–12% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with polyclonal anti-VSV serum from hyperimmune rabbits using standard protocols.

## **2.10 Transfection of plasmid DNA.**

Plasmids encoding VSV M were transfected using Lipofectamine 2000 (Invitrogen) 16hours after plating cells such that their density was approximately 90% at the time of transfection as per the manufacturer's instructions.

## **2.11 ELISA.**

The quantification of INF- $\beta$  used the INF Beta ELISA Kit from PBL InterferonSource (Piscataway, NJ) as per the manufacturer's instructions. The ELISA was performed by Dr. Fabrice Le Boeuf on collected cell supernatants precleared by 1min centrifugation at 1500g.

## **2.12 Microscopy.**

Images of *in vitro* cell culture were of a representative field of view using a Leica MZFLIII microscope using a standard eGFP filter set for fluorescent images. Images were captured with a Nikon Coolpix 100 camera.

### **3 Results**

#### **3.1 Rescue of novel vesicular stomatitis viruses containing miRT sequences.**

Recombinant vesicular stomatitis viruses carrying three repeats of differing sequence complementarity to mature let-7a and mir-34a microRNA are depicted schematically in Figure 2. Let-7a is one member of the let-7 family consisting of highly similar microRNAs. The let-7wt sequence is a perfect complement to the mature let-7a microRNA and, in a cell expressing let-7a, will be cleaved by the RISC complex resulting in transcript instability and potent inhibition of expression<sup>122,196</sup>. The let-7mm sequence is an imperfect complement to the mature let-7a microRNA, similar to sequences contained in the 3'UTR of endogenous mRNAs, and results in less potent inhibition of expression<sup>122</sup>. The let-7mut sequence demonstrates no detectable inhibition in a luciferase reporter assay due to a high degree of non-complimentarity to the mature let-7a microRNA and serves as a negative control<sup>122</sup>. These same sequences incorporated into VSV<sup>let-7wt</sup> and VSV<sup>let-7mm</sup> are repressed when incorporated into the 3'UTR of a luciferase reporter gene through a let-7 dependent mechanism<sup>122</sup>. The let-7wt, let-7mm and let-7mut sequences were also incorporated into a VSV in the 3'UTR of a luciferase reporter gene, contained in the VSV genome which is depicted schematically in Figure 1b. These viruses enable the evaluation of harnessing endogenous microRNA activity as a instigator of translational repression, as in let-7mm and as an instigator of RNAi, as in let-7wt, to attenuate VSV through a let-7 dependent mechanism. The mir34a-wt sequence consists of three repeats of a perfectly complementary sequence to the endogenous

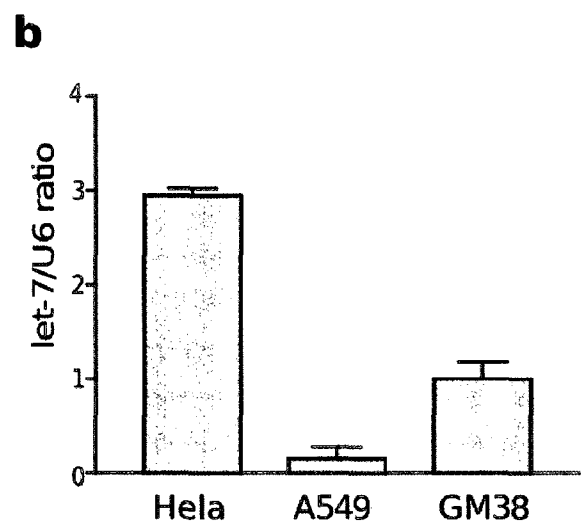
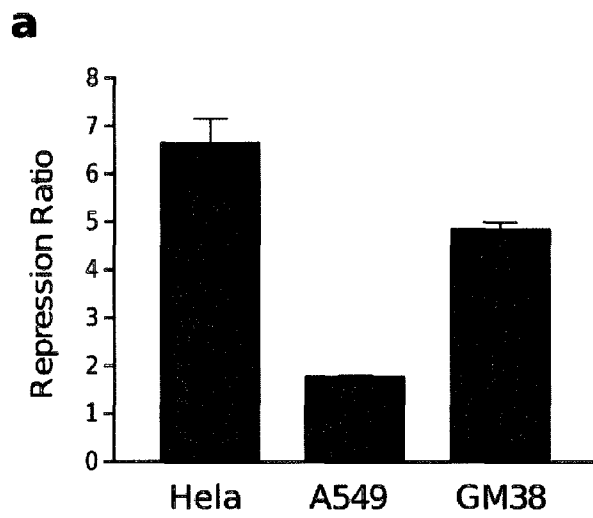
mature mir34a microRNA. This sequence, not previously evaluated for its sensitivity to mir34a microRNA expression, is incorporated into the 3'UTR of VSV M mRNA in VSV<sup>mir34wt</sup>. It has also been cloned into the 3'UTR of VSV M mRNA in VSVluciferase<sup>mir34a-wt</sup>, another VSV that carries a luciferase reporter gene. This sequence enables the evaluation of endogenous mir-34a to instigate RNAi and destabilize VSV M mRNA. All viruses contain the wild-type VSV genes of the Indiana serotype and were rescued from previously described plasmid DNA containing only the modifications described<sup>194</sup>. After plaque purification and subsequent amplification, RNA was isolated from infected cells and reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify viral M mRNA. Sequencing of these products confirmed the inclusion of the modified 3'UTR sequences in the M mRNA and that no mutations had occurred in this genomic region. The rescue of infectious VSV was accomplished using a slightly modified version of an established protocol (see Section 2.2). The successful rescue of all of the viruses from Figure 2 suggests that the rescue protocol is able to overcome any inhibition caused by VSV genomic miRTs. It was fortunate that the rescue protocol called for A549 cells as these cells contained low let-7 activity (Figure 3a). The activity of mir34a in A549 cells was not tested, however this virus was successfully rescued in A549 cells.

### **3.2 HeLa, A549 and GM38 cell lines provide differing let-7 activities to evaluate novel miRT containing viruses.**

As assessed by luciferase reporter assay and analysis of the expression of

mature let-7a microRNA by quantitative RT-PCR (qRT-PCR), GM38 cells express greater functional let-7 than A549 cells (Figure 3a&b). This confirms observations by others that human A549 lung carcinoma cells express low levels of let-7 microRNA<sup>148,197</sup>. The primary human fibroblast cell line, GM38, has approximately 3 times more let-7 activity than A549 cells (Figure 3a). This was assessed using the let-7mm sequence, as it has been used previously as a sensitive detector of let-7 mediated translational inhibition<sup>122</sup>. This functional activity correlates well with expression levels let-7a as a ratio to U6 snRNA as measured by qRT-PCR (Figure 3b). Previous studies examining low let-7a expression also normalize expression levels to U6 snRNA<sup>34,197</sup>. These observations confirm that A549 and GM38 cell lines can serve as models of target cancer cells and non-target normal cells, respectively. These cell lines are appropriate for evaluating the ability of oncolytic VSVs harbouring let-7 miRTs to distinguish between normal cells with let-7 activity and cancer cells with low levels of let-7 activity. Although Hela cells are a cervical cancer cell line they have high levels of let-7 activity and have been used extensively to examine many aspects of endogenous microRNAs, including let-7 specifically<sup>122,124,198,199</sup>. Additionally, their ease of culture, transfection and VSV infection make them a useful tool for examining the effect of let-7 activity on VSVs containing let-7 miRTs.

**Figure 3: Let-7 activity and expression levels in Hela, A549 and GM38 cells support a relationship between functional activity and expression level. (a)** Vectors containing the same let-7 sequences as VSV<sup>let-7mm</sup> in the 3'UTR of luciferase demonstrate greater repression in GM38 and Hela cells as compared to A549 cells. ( $p < 0.001$ ) The repression ratio is the ratio of the let-7mut luciferase activity to the let-7mm luciferase activity using a co-transfected renilla luciferase reporter to normalize for transfection efficiency. **(b)** Quantification of mature let-7 microRNA in the indicated cell lines expressed as a ratio to U6 small RNA. The lower abundance of mature let-7 microRNA relative to the U6 small RNA correlates with the low repression ratio in (a), and is significantly lower than GM38 cells ( $p < 0.002$ ). The data is presented as mean  $\pm$  SD.



### **3.3 Expression of the VSV M protein is subject to let-7 miRT repression.**

Expression of VSV M containing let-7 miRTs in the 3'UTR is repressed in transiently transfected Hela cells. Detection of VSV M by western blot clearly indicates let-7mm sequence specific repression (Figure 4a). While in this experiment transcription is driven from the CMV promoter of the mammalian expression vector pcDNA3.1, and not a viral polymerase in the context of VSV infection, it does demonstrate that the M protein itself, like luciferase, is subject to let-7 mediated translational repression. This is significant as there are examples, although controversial, of mammalian viral proteins that do inhibit microRNA mediated transcript instability<sup>187,200</sup>. In contrast, these results indicate that the VSV M protein is unable to completely block microRNA mediated translation repression of its own mRNA and is sensitive to the presence of imperfectly complementary let-7 miRTs in its 3'UTR and is therefore not an example of a viral inhibitor of microRNA mediated transcript instability. Thus exogenous siRNA, in the context of a VSV infection, and endogenous microRNAs in the context of a transiently transfected plasmid are both sufficient to inhibit the expression of VSV M<sup>189</sup>.

Cotransfection of a plasmid encoding DsRed, a fluorescent reporter protein, indicates a similar transfection efficiency at 24 hours (Figure 4b). Qualitative observation suggests that any differences in observed fluorescence are due to decreased cell survival. In cells transfected with M containing mock miRTs the observed manifestation of cell death is consistent with the cytopathic effect of

cellular VSV M expression<sup>201,202</sup>. Thus the cotransfection confirms similar transfection efficiency and the observed differential expression of functional VSV M (Figure 4a) as manifested in its cytopathic effect.

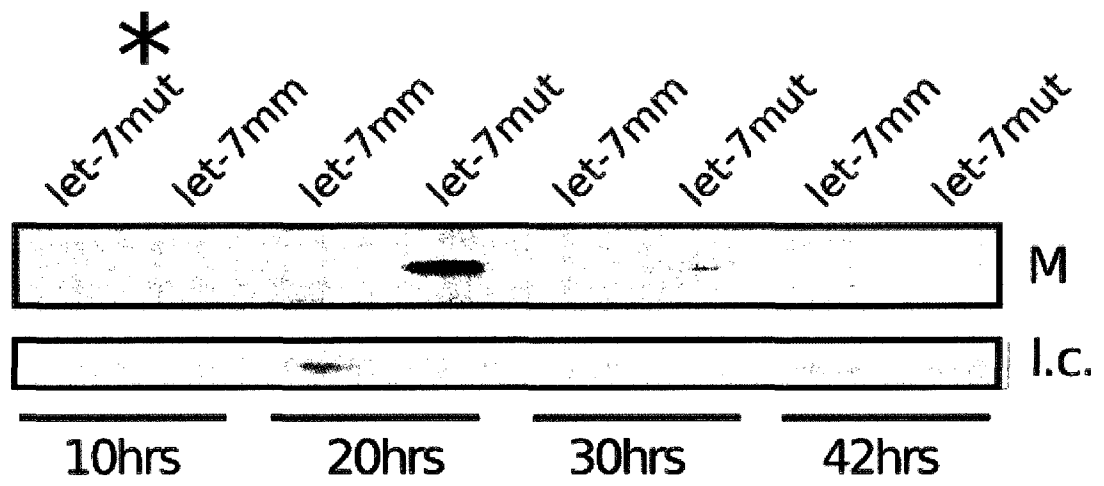
While this data supports the idea of creating VSVs containing miRTs in the 3'UTR of M, for miRT targeted viruses to be successful viral gene expression inhibition by microRNAs has to remain functional in the cell during infection.

### **3.4 Replication of viruses containing let-7 miRTs are affected by exogenous let-7.**

To determine if let-7 microRNA could inhibit replication of VSV containing let-7 miRTs in the 3'UTR of VSV M mRNA we assessed the response of the engineered viruses to exogenous let-7. Since both endogenous microRNA and exogenous siRNA enter the RISC complex and affect transcripts by a similar mechanism, endogenous let-7 microRNA can be supplemented with exogenous siRNA of the same sequence<sup>198,199</sup>. Transfection of HeLa cells with siRNA containing the sequence of mature let-7a microRNA specifically reduced the titres produced by viruses containing let-7a miRTs (Figure 5a). The titres in Figure 5a are normalized to eGFP siRNA in order to highlight the effect of the exogenous let-7. Importantly VSV<sup>let-7wt</sup> titres were reduced by a factor of two in HeLa cells transfected with eGFP siRNA as compared to VSV<sup>let-7mut</sup>, presumably due to the endogenous let-7 activity observed in Figure 3a.

**Figure 4: Regulation of VSV matrix protein expression by incorporation of let-7 miRTs. (a)** Expression of M protein, as observed by western blot at the indicated time-points, in transiently transfected Hela cells. The miRT sequences were cloned into the 3'UTR as indicated in Figure 1a. The blot was also probed for beta-actin (l.c.). (\* The loading pattern in these lanes deviated and the labels are correct.) **(b)** Fluorescence microscopy pictures of the cells in (a). These pictures reveal expression from cotransfected vector encoding DSRed.

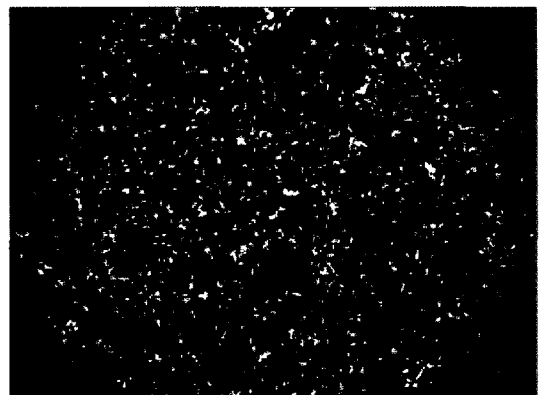
**a**



**b**

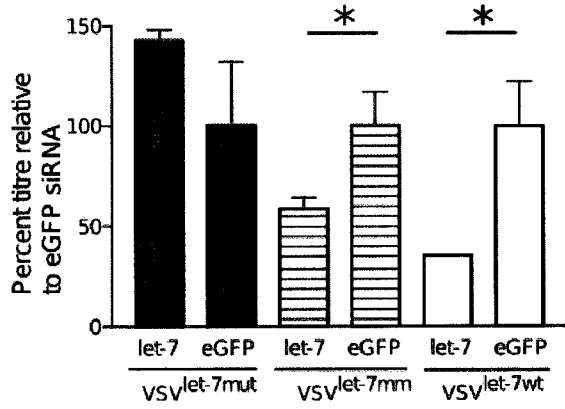
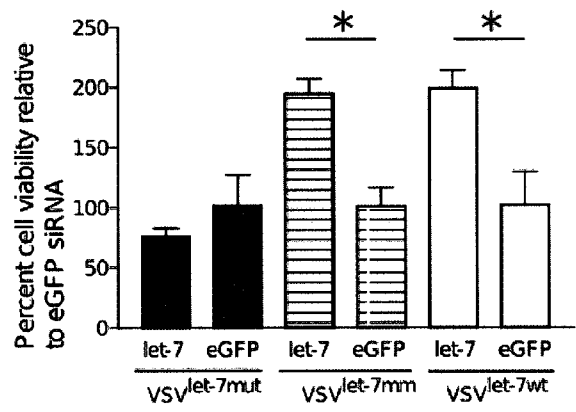


let-7mut



let-7mm

**Figure 5: Exogenous let-7 small interfering RNA reduces let-7 sensitive VSV replication and cytotoxicity. (a)** HeLa cells were infected at a multiplicity of infection (MOI) of 0.1 with the indicated viruses 24 hours after transfection with let-7 or eGFP siRNA. Titres were determined 20 hours after infection and are expressed as a percentage of titres from cells transfected with eGFP siRNA (mean + SD) (\*  $p < 0.05$ ). After transfection of eGFP siRNA, VSVlet-7mut infected HeLa cells produced titres of  $9.5 \times 10^7 + 3.5 \times 10^7$  pfu/mL. **(b)** Cell survival of A549 cells after infection with the indicated let-7 sensitive VSV at an MOI of 0.1. The indicated siRNAs were transfected 24 hours prior to infection. An MTS assay was used to quantify cell survival 24 hours after infection and is expressed as a percentage of survival compared to cells transfected with eGFP siRNA (mean + SD) (\*  $p < 0.05$ ).

**a****b**

### **3.5 Additional let-7 increases the survival of cells infected with viruses containing let-7 miRTs.**

Ideally the sensitivity of these oncolytic viruses to let-7 activity oncolytic viruses will, in addition to the observed decreased replication in let-7 microRNA expressing cells, also demonstrate decreased toxicity to let-7 expressing cells. To test the engineered viruses ability to kill low let-7 expressing cells, again let-7 activity was augmented with exogenous let-7 siRNA and survival of cells was assessed after infection with the let-7 miRT containing viruses. Transfection of A549 cells with let-7a siRNA resulted in 57% viability of cells infected with VSV<sup>let-7wt</sup>. This protection was not seen in A549 cells infected with VSV<sup>let-7mut</sup>, with 15% viability, suggesting that cell survival was enhanced in a let-7 sequence specific mechanism (Figure 5b). Data from an identical experiment using Hela cells also showed specific inhibition of VSV<sup>let-7wt</sup> induced cell death in the presence of exogenous let-7 siRNA (data not shown). These results demonstrate it is possible to design a virus where viral replication and cell killing are sensitive to the quantity of a specific microRNA.

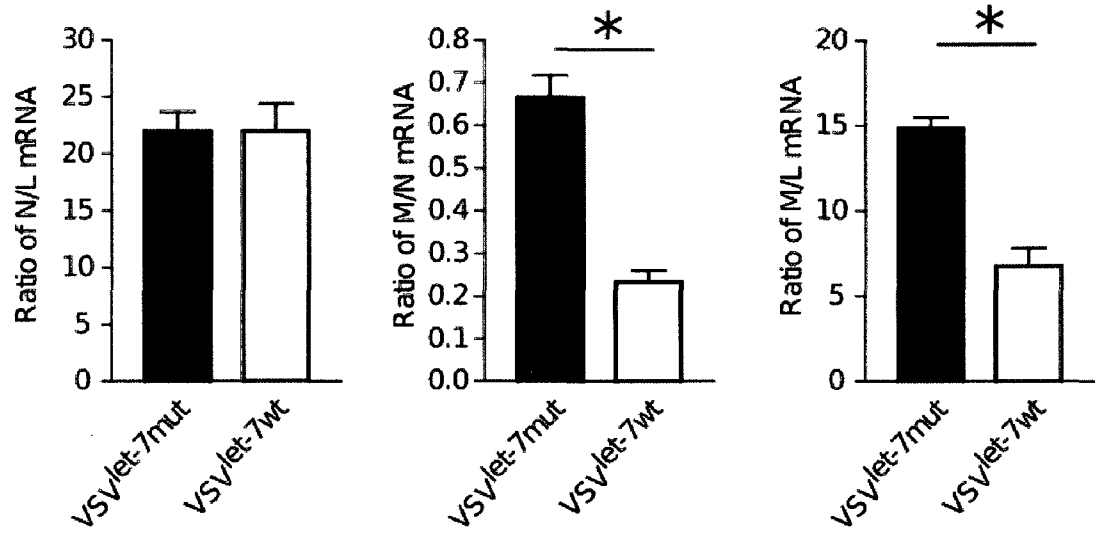
### **3.6 VSV<sup>let-7wt</sup> infected let-7 expressing cells contain reduced M mRNA.**

To determine whether the perfectly complementary let-7 target sites were initiating degradation of the M mRNA we chose to look at the ratio of VSV gene products by qRT-PCR (Figure 6). For these experiments we used Hela cells as these cells have measurable let-7 activity<sup>34,122,124</sup>. We also detected let-7 activity in

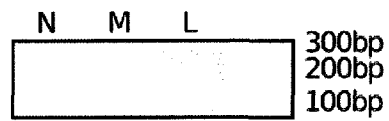
**Figure 6: Let-7 miRTs reduce the abundance of target viral mRNA specifically.**

**(a)** Hela cells were infected for 6hrs at an MOI of 1 and after 6hours total RNA was extracted and viral mRNAs were quantified by qPCR of oligo dT primed cDNA (mean + SD) (\* $p < 0.0001$ ). **(b)** Amplified DNA fragments from qRT-PCR of the VSV genes N, M and L, are a single species of the expected size as visualized by agarose gel electrophoresis.

**a**



**b**



these cells by luciferase assay (Figure 3a) as well as a reduced capacity to support VSV<sup>let-7wt</sup> replication (as mentioned previously). Quantification of transcripts early in infection demonstrated the abundance of VSV transcripts is related to their position in the VSV genome (see Figure 2). This agrees quantitatively with previous findings, using an independent method, that the nonprocessive nature of the viral RNA polymerase (VSV L) leads to this characteristic transcript abundance<sup>84,85</sup>. In 1976, Villarreal et al. measured a molar ratio of N/L as 18, but were unable to resolve M mRNA from P mRNA. Evident from this data is that in HeLa cells the M protein transcript is less abundant at six hours post-infection in VSV<sup>let-7wt</sup>, that contains let-7 miRNAs in the 3'UTR of the M mRNA, as compared to VSV<sup>let-7mut</sup>, when expressed as a ratio to other genes in the VSV genome. This data suggests that the presence of let-7 microRNA decreases M mRNA in VSV<sup>let-7wt</sup> infected cells, resulting in decreased viral production and oncolysis. The causal role of reduced M protein mRNA in decreasing viral titres *in vitro* has been observed previously<sup>87</sup>.

### **3.7 The let-7 sensitive replication of VSV<sup>let-7wt</sup> is due to its reduced expression of VSV M.**

Our intent in designing VSV<sup>let-7wt</sup> is to subject VSV M mRNA to let-7 regulation, however it is formally possible that let-7 binds miRNAs in the positive sense VSV genome that exists as an essential element in the viral lifecycle<sup>87</sup>. This could be an additional mechanism of let-7 influence on viral replication and

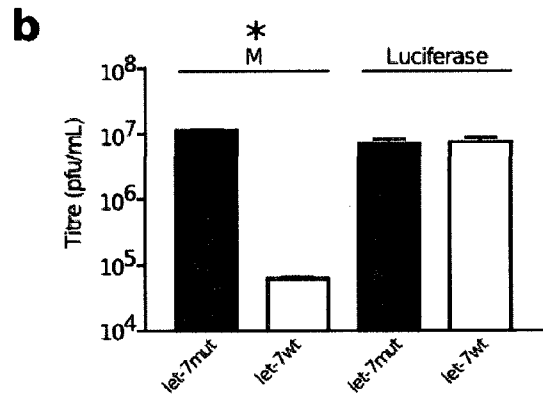
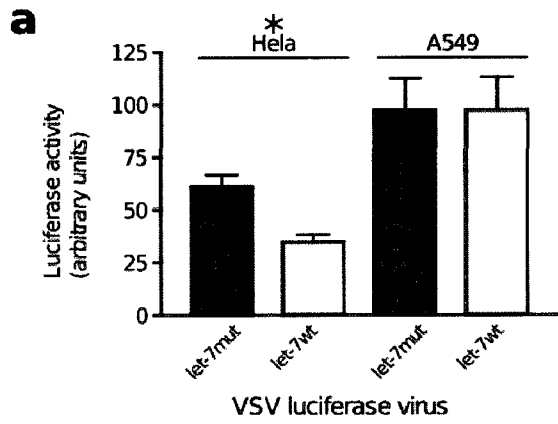
cytotoxicity as seen in Figure 5a and 5b. To determine if the observed attenuation of VSV<sup>let-7wt</sup> is due a let-7 mediated decrease in abundance of VSV M protein specifically, luciferase expressing VSVs were employed (Figure 2). These viruses contained a firefly luciferase reporter gene with the let-7 miRTs, let-7mut, let-7mm and let-7wt in its 3'UTR (see Figure 2). The luciferase activity in infected Hela cell lysates has a let-7wt specific reduction while A549 cells has no such reduction (Figure 7a). This let-7wt specific luciferase activity reduction correlates with the luciferase activity reduction observed in Figure 3a for these cell lines. The reduction in luciferase activity is presumably due to reduced luciferase protein, therefore when these same sequences are placed in the 3'UTR of VSV M presumably this results in less VSV M protein and produces the observed let-7 miRT specific phenotypes. This is consistent with the observed decrease in VSV M mRNA (Figure 6), the exogenous let-7 dependent decreased viral progeny (Figure 5a), and the exogenous let-7 dependent decreased viral cytopathic effect (Figure 5b). Titres produced after a 48 hour infection of the human primary fibroblast cell line, GM38, at an MOI of 0.1 with the VSVluciferase<sup>let-7mut</sup> and VSVluciferase<sup>let-7wt</sup> were very similar, however when VSV M contained let-7 miRTs titres were reduced drastically (Figure 7b). This indicates that only by including miRTs on an essential viral gene can titres be affected. This also suggests that any effects on the positive sense genome do not hinder the ability of the virus to replicate. This is not completely unexpected since the positive sense genome is found in a ribonucleoprotein complex within an infected cell and these complexes are nuclease resistant<sup>203</sup>.

**Figure 7: Let-7 miRTs on M reduce viral replication in Hela cells while miRTs on a virally encoded reporter gene have no observable effect on replication.**

**(a)** Hela and A549 cells were infected at an MOI of 10 with VSV containing luciferase with identical let-7 target sites as depicted in Figure 2 and after 2 hours luciferase expression was assayed. Data are represented as mean + SD (\*p<0.005)

**(b)** VSV titres resulting from infection of GM38 cells for 48hours with VSV having either let-7 target sequences in the 3'UTR of M or luciferase. (mean + SD)

(\*p<0.001).



Importantly, the distinctive result of this experiment is that the observed reduction in titres of VSV<sup>let-7wt</sup> was most pronounced in the primary human fibroblast cell line GM38. We then sought to examine this further.

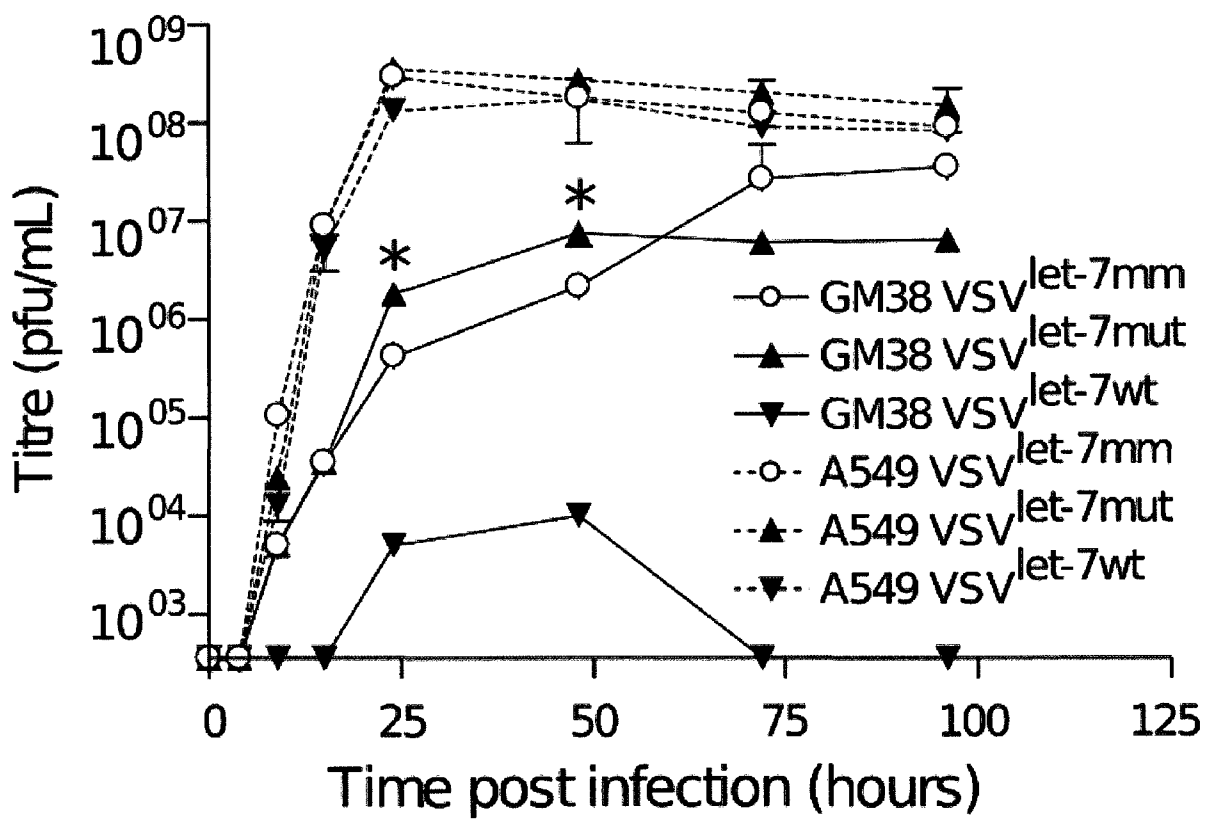
### **3.8 VSV<sup>let-7wt</sup> is strongly attenuated in the primary human fibroblast cell line GM38.**

As mentioned previously, GM38 cells are a human primary fibroblast cell line that has significantly more let-7 activity than A549 cells and we are employing these cells as representative non-target cells for viral oncolysis (Figure 3a). Importantly, let-7 activity and expression in GM38 cells correlates with approximately 1000 times less replication of VSV<sup>let-7wt</sup> as compared to VSV<sup>let-7mut</sup> after 48 hours. After 72 hours of infection VSV<sup>let-7wt</sup> progeny were not detected. This effect was not observed in A549 cells (Figure 8). Therefore VSV<sup>let-7wt</sup> produced the fewest progeny in a let-7 target sequence specific manner in this non target cell line.

More strikingly perhaps is an absence of cytopathic effect, specific to VSV<sup>let-7wt</sup>, observed in an infected confluent monolayer of GM38 cells at 72 hours post infection (Figure 9a). The cytopathic effect is observed upon infection with all of the luciferase reporter containing VSVs and confirms that miRT placement within the VSV genome and decreased VSV M are relevant to the let-7 specific attenuation of VSV<sup>let-7wt</sup> in GM38 cells.

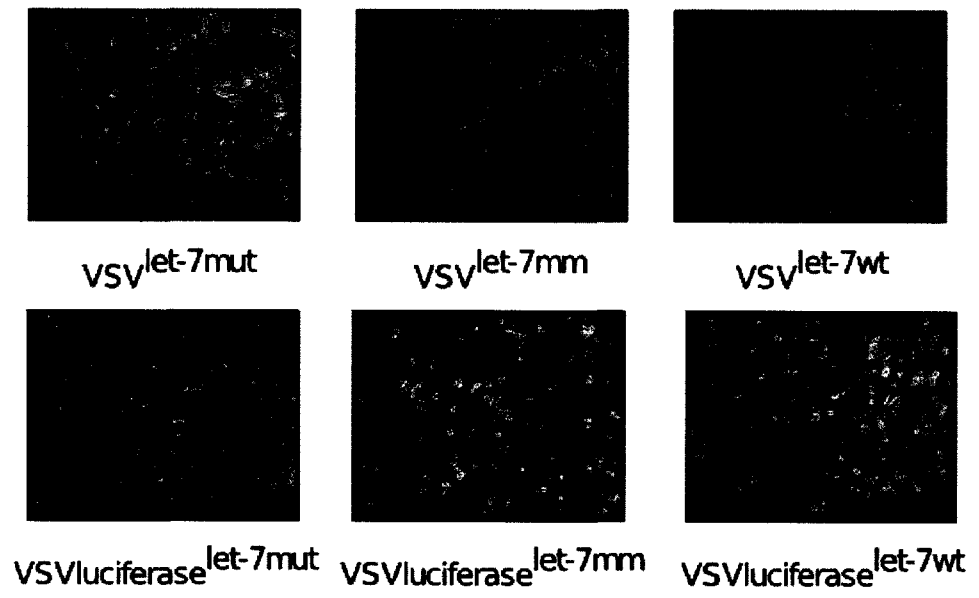
Initiation of the innate immune response by extracellular cytokine signals can protect cells from VSV infection<sup>13,204</sup>. The experiments in Figure 9b&c demonstrate

**Figure 8: Growth curve of miRT containing VSVs in A549 and GM38.** An MOI of 0.1 was used to infect monolayers of A549 and GM38 cells. The x-axis denotes the limit of detection for the titring assay. Data are represented as mean  $\pm$  SD. For GM38 infected with VSVlet-7wt and VSVlet-7mut, \* indicates a  $p < 0.0005$ .

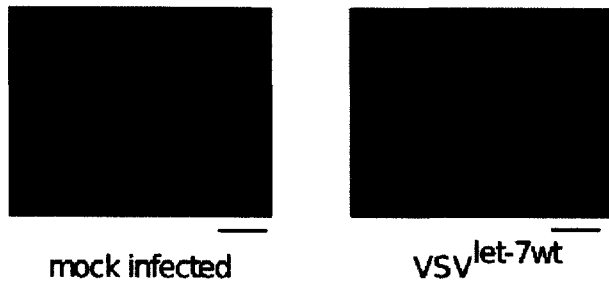


**Figure 9: A monolayer of the primary human fibroblast cell line, GM38, survives low MOI infection with VSV<sup>let-7wt</sup> and is protected from subsequent infection from a wt-VSV by a soluble factor. (a)** Phase contrast pictures of infected GM38 cells 72hrs after infection at an MOI of 0.1, scale bar = 100µm. **(b)** GM38 cells mock infected or infected with VSV<sup>let-7wt</sup> 24hrs before infection with wt VSV expressing eGFP. After another 24hrs, fluorescence microscopy was used to visualize eGFP expression, scale bar = 100µm. **(c)** A monolayer of GM38 cells was infected with the indicated virus. Twenty-four hours later the supernatants from these infections were placed individually on another monolayer of GM38 cells, after filtration to remove virus particles. After 24hours exposure to the supernatants these GM38 cells were infected by a wt VSV expressing eGFP for 24hours at which time the bright field and fluorescence microscopy pictures were taken.

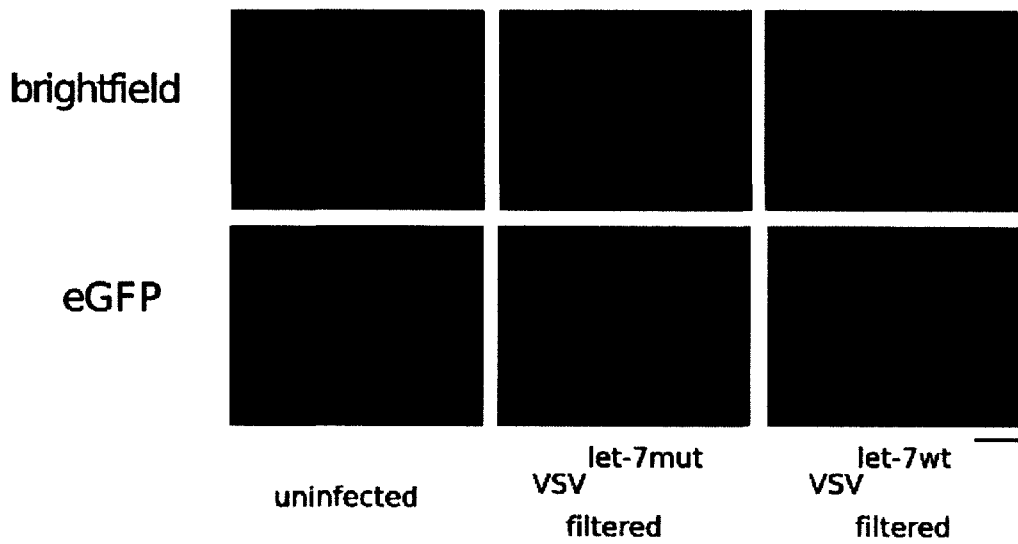
**a**



**b**



**c**



that an extracellular soluble factor was involved in the resistance of GM38 cells to VSV<sup>let-7wt</sup> infection. Prior infection of GM38 cells with VSV<sup>let-7wt</sup> protected the monolayer from subsequent infection with a VSV expressing an eGFP transgene (VSV-eGFP) demonstrating induction of an antiviral state following infection with VSV<sup>let-7wt</sup> (Figure 9b). Furthermore, protection from subsequent VSV-eGFP infection can be transferred to cells in a virus free supernatant, implicating a soluble mediator of VSV protection (Figure 9c). As mentioned previously the VSV M protein has a role in counteracting antiviral responses including the production of soluble antiviral cytokines<sup>80,205,206</sup>. Together the presented *in vitro* data suggest that decreased accumulation of VSV M mRNA during infection, mediated by let-7 sequence specific miRTs, permits normal cells with let-7 microRNA activity to release antiviral cytokines. It is unlikely that the miRT reduction of VSV M on its own is responsible for the degree of attenuation seen in the GM38 cells since at an MOI of 1, VSV<sup>let-7wt</sup> destroys a monolayer of GM38 cells (data not shown). Additionally, enzyme-linked immunosorbent assay (ELISA) results show that 24 hours after infection, the supernatants of GM38 cells infected with VSV<sup>let-7wt</sup> have an average of 114.3pg/mL of IFN- $\beta$  while those infected with VSV<sup>let-7mut</sup> have an average of 20.8pg/mL of IFN- $\beta$ . IFN- $\beta$  is an antiviral cytokine produced by fibroblasts in response to viral infection. These results are highly suggestive, but not conclusive, that IFN- $\beta$  plays a role in the resistance of GM38 cells to VSV<sup>let-7wt</sup>. While intracellular dsRNA can signal the production of IFN- $\beta$ , the dsRNA that forms from endogenous microRNA binding miRTs is not stimulating this response since VSVluciferase<sup>let-7wt</sup> still kills the

fibroblast monolayer<sup>207</sup>.

The successful attenuation of VSV<sup>let-7wt</sup> in the non-target cell line GM38 led us to investigate its *in vivo* properties.

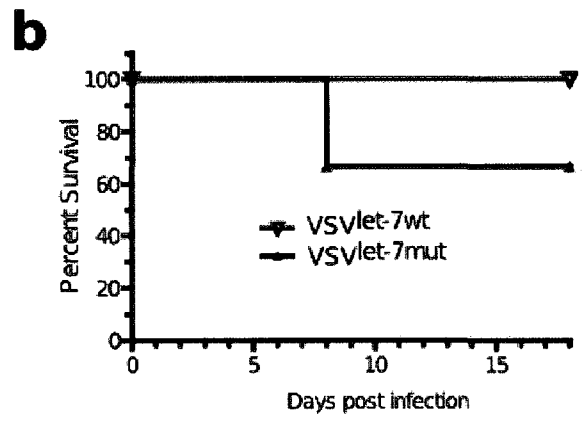
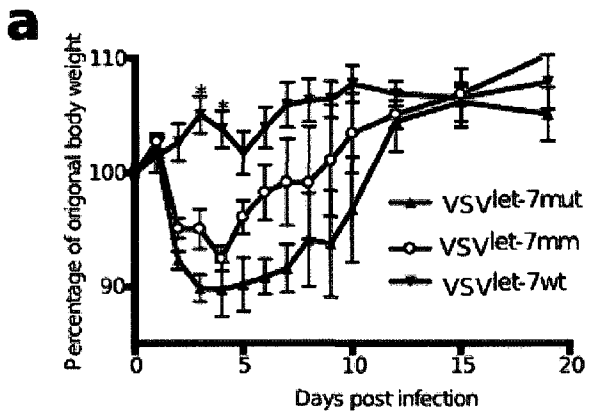
### **3.9 VSV<sup>let-7wt</sup> is less pathogenic in BALB/c and CD-1 nude mice.**

The lack of VSV<sup>let-7wt</sup> cytotoxicity *in vitro* led us to test the toxicity of this virus *in vivo* (Figure 10). Intranasal infections of BALB/c mice resulted in the most significant transient weight loss in VSV<sup>let-7mut</sup> infected mice and an intermediate phenotype associated with VSV<sup>let-7mm</sup>. No detectable transient weight loss was observed with VSV<sup>let-7wt</sup> (Figure 10a). In CD-1 nude mice no pathology was observed after intranasal infection with VSV<sup>let-7wt</sup>. In contrast, one third of the mice infected with VSV<sup>let-7mut</sup> were found moribund on day 8 (Figure 10b) and had substantial viral titres in their brains ( $>1 \times 10^4$  pfu/mg tissue) whereas there was no detectable virus in the brains of two VSV<sup>let-7wt</sup> infected CD-1 nude mice removed from the study at the same time. This result clearly demonstrates that down regulation of the VSV M protein by inclusion of let-7 miRTs renders VSV less pathogenic.

### **3.10 VSV<sup>let-7wt</sup> has antitumour activity.**

Previous work has demonstrated antitumour effects of VSV on subcutaneous CT26 tumours in BALB/c mice using a M protein mutant of VSV<sup>13,80,83</sup>. Our observations of tumour specific replication *in vitro* led us to test the antitumour

**Figure 10: VSV<sup>let-7wt</sup> has reduced pathogenic activity in immune competent and immunodeficient mice. (a)** BALB/c mice infected intranasally with  $1 \times 10^5$  pfu of the indicated virus. Body weights were expressed as a percentage of initial body weight before infection and averaged. (mean  $\pm$  SD) n=5. (\*p<0.005). **(b)** Survival of CD-1 nude mice infected with VSV<sup>let-7mut</sup> or VSV<sup>let-7wt</sup>. Mice were infected at a dose of  $7.5 \times 10^7$  pfu of the indicated virus intranasally (n=6).

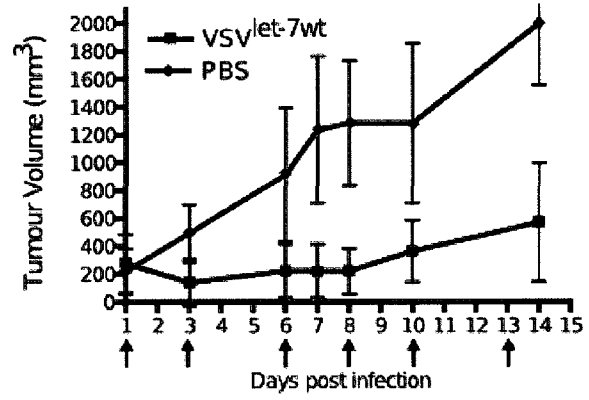
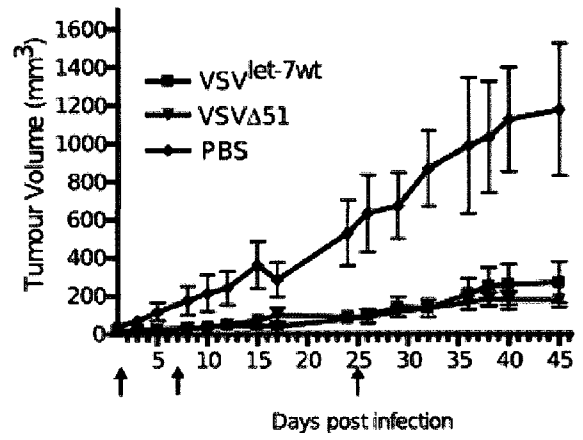


activity of VSV<sup>let-7wt</sup> *in vivo*. Indeed multiple intravenous injections of VSV<sup>let-7wt</sup> retarded CT26 tumour growth as compared to an identical schedule of PBS injections (Figure 11a). Mice receiving  $1 \times 10^9$  pfu and  $1 \times 10^8$  pfu were grouped as there was no dose dependence observed. No complete responses were observed in this model. Additionally multiple intravenous injections of VSV<sup>let-7wt</sup> administered to CD-1 nude mice harbouring established subcutaneous A549 tumours retarded tumour growth as compared to an identical schedule of PBS injections (Figure 11b). One complete response was observed after treatment with VSV<sup>let-7wt</sup>, however it was the smallest tumour, at  $4 \text{mm}^3$  on the first day of treatment. As a comparison for oncolytic activity this virus was compared to a previously engineered oncolytic VSV,  $\Delta 51$  VSV<sup>80</sup>. From Figure 11b it is apparent that these viruses demonstrate similar antitumour activities in this model. These results suggest that the tumour specificity of this virus was maintained, despite containing coding sequences for wild-type viral proteins that normally have pathogenic consequences.

### **3.11 Replication of VSV containing a mir34a miRT is affected by exogenous mir-34a.**

Figure 2 outlines schematically the viruses incorporating mir-34a miRTs. As discussed in section 1.432, mir-34a is p53 dependently transcribed. It was hypothesized therefore that VSV<sup>mir34a-wt</sup> would be sensitive to cellular mir-34a levels, much like VSV<sup>let-7wt</sup> is sensitive to cellular let-7 levels, and therefore be indirectly

**Figure 11: VSV<sup>let-7wt</sup> retains antitumour activity in vivo.** (a) Subcutaneous CT26 tumours were seeded with  $3 \times 10^5$  cells 10 days before first treatment (day 1) in BALB/c mice. Tumour volume was monitored during a treatment course of intravenously injected  $1 \times 10^8$ - $1 \times 10^9$  pfu VSV<sup>let-7wt</sup> (n=8) or PBS (n=4), injections are indicated with arrows on the x-axis (mean  $\pm$  SD). At day 14, the difference between PBS treated and VSV<sup>let-7wt</sup> mice has a p value of less than 0.05. (b) Subcutaneous A549 tumours were seeded with  $1 \times 10^6$  cells 12 days before first treatment (day 1) in CD-1 nude mice. Tumour volume was monitored during a treatment course of intravenously injected  $1 \times 10^9$  pfu VSV<sup>let-7wt</sup> (n=5), VSV<sup>let-7mut</sup> (n=5), VSV  $\Delta$ 51 (n=5) or PBS (n=3), injections are indicated with arrows on the x-axis (mean  $\pm$  SD). At day 45, the difference between PBS treated and VSV<sup>let-7wt</sup> treated mice has a p value of less than 0.05.

**a****b**

dependent on p53 deficiencies of a target cancer cell. This oncolytic VSV<sup>mir34a-wt</sup> would then be targeted to p53 deficiencies, common in cancer cells.

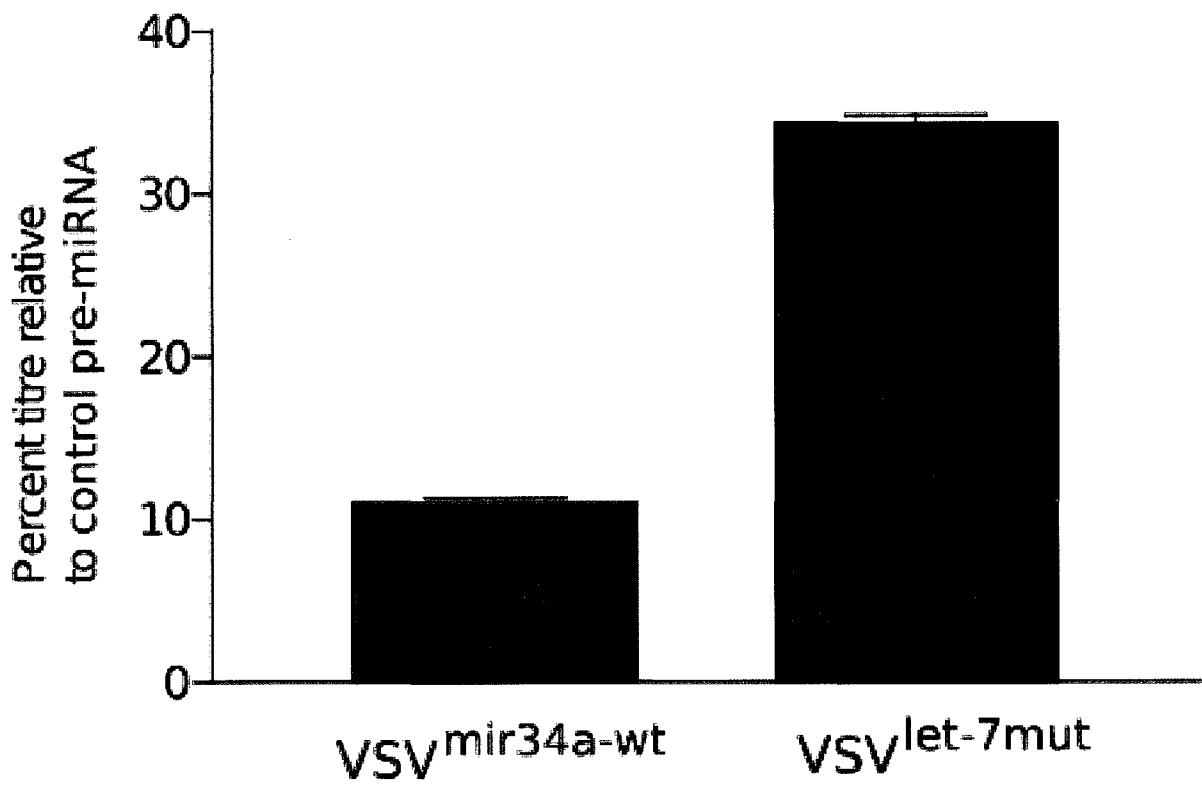
First it was necessary to determine if mir-34a miRTs rendered VSV sensitive to increased mir-34a microRNA. As seen in Figure 12, the titres resulting from infection of Hela cells with VSV<sup>mir34a-wt</sup> are reduced by approximately 90% by prior transfection of a mir-34a pre-miRNA as compared to an irrelevant control, while titres of VSV<sup>let-7mut</sup> are reduced by one-third. This experiment demonstrates that VSV<sup>mir34a-wt</sup> has greater sensitivity to increased levels of mir-34a than a virus containing irrelevant miRTs and supports the concept of an oncolytic virus that is responsive to p53 dependent transcription of mir-34a microRNA.

The reduction in titres resulting from exogenous mir-34a VSV<sup>mir34a-wt</sup> (Figure 12) are more pronounced than the reduction in titres resulting from exogenous let-7 on VSV<sup>let-7wt</sup> (Figure 5) and the reason for this is unknown. It is possible that mir34a alters endogenous gene expression such that infected Hela cells produce less progeny virus. This possibility is supported by the reduction in titres of VSV<sup>let-7mut</sup> in response to exogenous mir-34a. VSV is partially inhibited by p53<sup>208</sup>. These results suggest that perhaps some of this inhibition is mediated by p53-dependent transcription of mir-34a.

### **3.12 HCT116 cells exposed to doxorubicin prior to infection are more resistant to VSV<sup>mir34a-wt</sup>.**

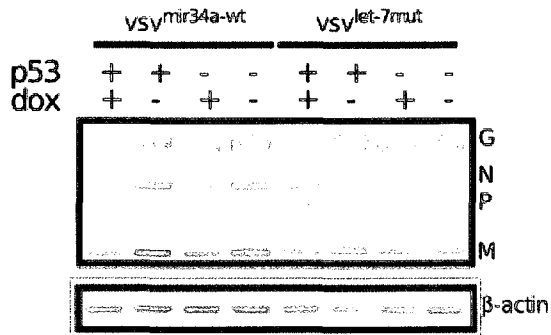
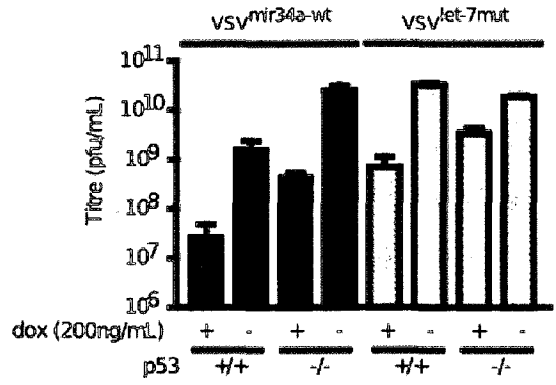
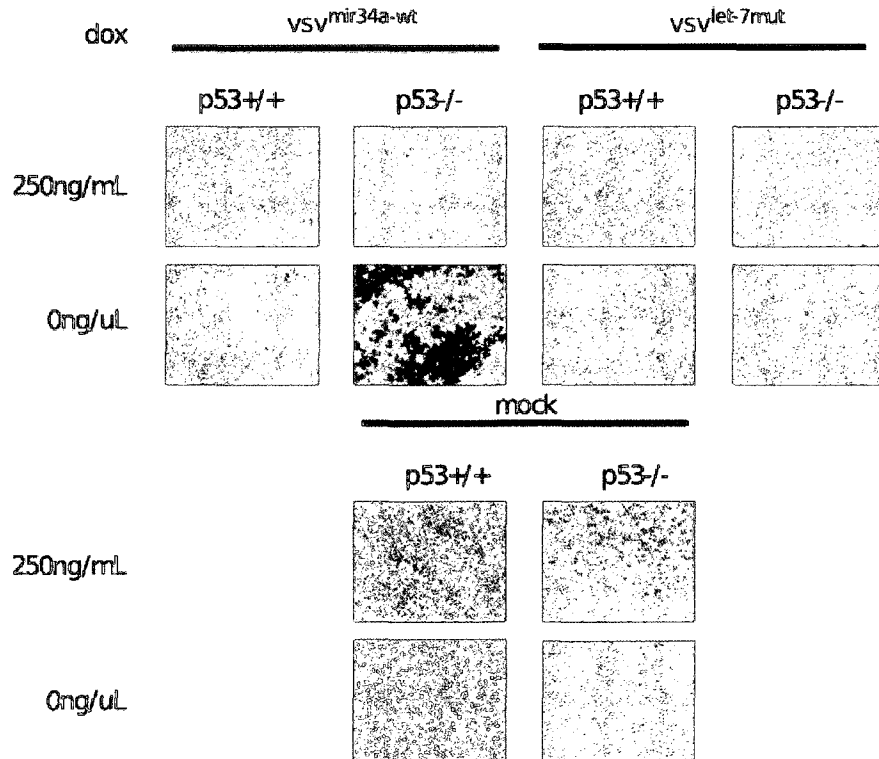
Doxorubicin is a topoisomerase II inhibitor that causes DNA damage and

**Figure 12: VSV<sup>mir34a-wt</sup> is sensitive to increased cellular mir-34a microRNA from an exogenous source.** HeLa cells were transfected with a mir-34a or control synthetic pre-miRNA forty-eight hours before infection with the indicated viruses. After 24 hours of infection the supernatants were titred on Vero cells. The titres are expressed as a percentage of the viral output from HeLa cells transfected with the control synthetic pre-miRNA.



activation of p53, which in turn initiates the transcription of mir-34a<sup>170</sup>. To examine the sensitivity of VSV<sup>mir34a-wt</sup> to p53 dependent transcription of mir-34a HCT116 cells were used as these cells have previously been shown to upregulate mir-34a after exposure to doxorubicin<sup>170</sup>. HCT116 cells exposed to doxorubicin prior to infection produce less viral protein (Figure 13a), less progeny virus (Figure 13b) and have delayed cytotoxicity in a mir-34a miRT specific and p53 dependent mechanism (Figure 13c). The production of less viral protein occurs only when cells are exposed to doxorubicin and then infected with VSV<sup>mir34a-wt</sup> (Figure 13a). All viral proteins detected (VSV G,N,P and M as labeled) appear to be affected approximately equally, despite the presence of mir-34a miRTs only in the M 3'UTR. A similar effect was observed for VSV<sup>let-7wt</sup> (data not shown). Perhaps this is due to a negative impact on accumulation of VSV proteins by the repression of VSV M. Another possibility is at this time-point the impact of microRNA mediated repression on VSV M has been overcome and viral gene expression is no longer inhibited, making the difference in VSV M expression no longer evident. This is supported by the fact that at high MOI, a difference in luciferase activity is only observed at very early time-points after infection (Figure 7a), a time-point when VSV proteins were not detectable by western blots (data not shown). Doxorubicin negatively impacts production of viral progeny in cells with or without p53, however it most significantly affects the replication of VSV<sup>mir34a-wt</sup> in a p53 dependent mechanism (Figure 13b). It also appears as if the presence of p53 alone, without doxorubicin treatment, negatively impacts replication of VSV<sup>mir34a-wt</sup>. It is possible that this is partially a

**Figure 13: HCT116 cells exposed to doxorubicin exhibit resistance to viral gene expression, viral replication and cytopathic effect of VSV<sup>mir34a-wt</sup>.** HCT116 cells exposed to 100ng/mL doxorubicin (dox) for 48hours were subsequently infected with wt VSV or VSV carrying mir-34a miRTs (VSV<sup>mir34a-wt</sup>) in the 3'UTR of the matrix protein. Cells were infected at an MOI of 0.1 with the mir-34a sensitive VSV or an equivalent wt VSV as indicated. **(a)** At 16hours post-infection cell lysates were collected and probed with a polyclonal VSV antibody and  $\beta$ -actin as a loading control. The VSV immunoreactive bands are labeled G (VSV glycoprotein), N (VSV nucleoprotein), P (VSV phosphoprotein), and M (VSV matrix protein). **(b)** Supernatants from infected cells were collected at 24hours post infection and titred on Vero cells. **(c)** Prior to infection these HCT116 cells were exposed to 250ng/mL doxorubicin for 48hours and phase contrast microscopy pictures were taken at 16hours post-infection.

**a****b****c**

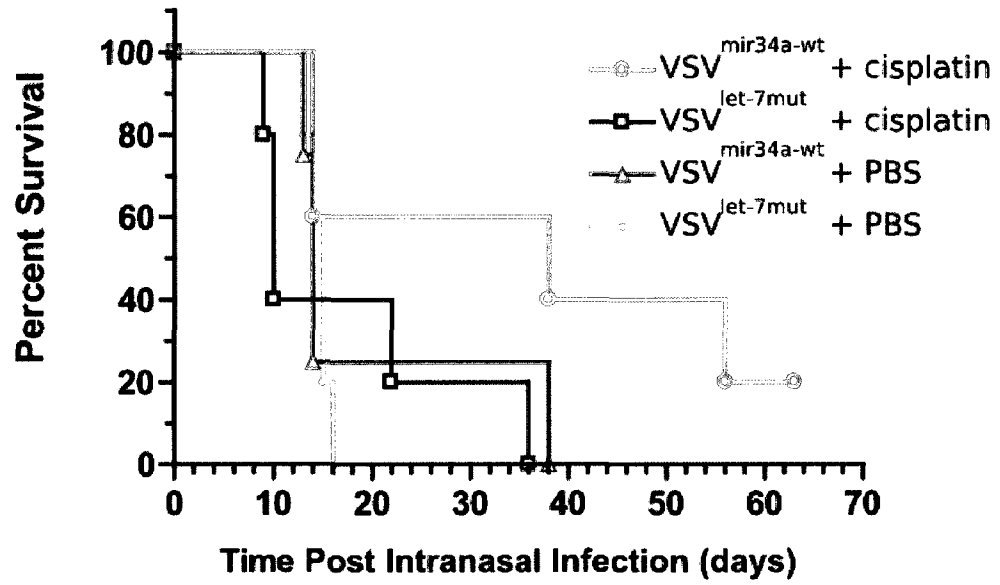
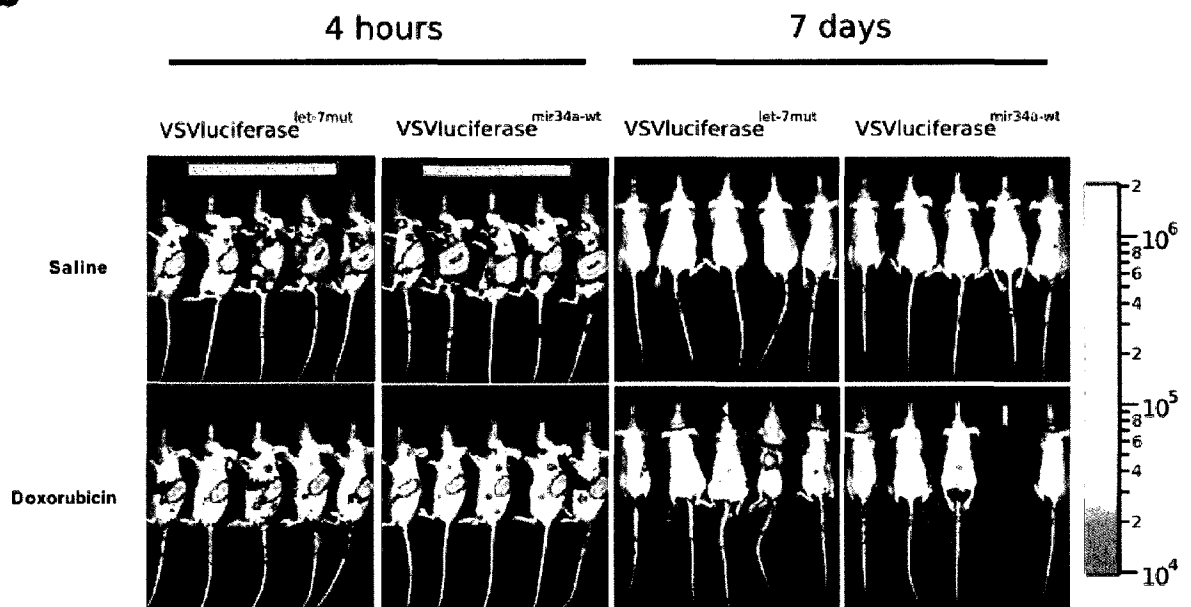
consequence of p53 +/+ HCT116 cells containing about twice as much mir-34a microRNA without chemically inducing additional p53 transcriptional activity<sup>178</sup>. Even without activation by chemically induced DNA damage, the observed p53 inhibition of viral replication is a desirable property in an oncolytic virus. Additionally desirable is the strong inhibition of VSV<sup>mir34a-wt</sup> induced cytopathic effect, also observed with prior treatment of HCT116 cells with doxorubicin (Figure 13c). Together the *in vitro* data indicate that VSV<sup>mir34a-wt</sup> is inhibited by activation of p53 in a mir-34a miRT dependent mechanism. These qualities suggest that VSV<sup>mir34a-wt</sup> can preferentially replicate and kill target cancer cells with defective p53 transcriptional activity of mir-34a microRNA. These results led us to test a combination treatment regimen *in vivo*.

### **3.13 DNA damaging agents do not protect mice from VSV<sup>mir34a-wt</sup> pathogenesis.**

Attempts to replicate p53 dependent *in vitro* resistance to VSV<sup>mir34a-wt</sup> in a mouse model have been largely unsuccessful. Intravenous injection of cisplatin, another DNA damaging agent that activates p53, in CD-1 nude mice results in no significant survival advantage to VSV<sup>mir34a-wt</sup> (Figure 14a). These mice succumbed to symptoms similar to those treated with VSV<sup>let-7mut</sup>, independent of cisplatin treatment, and were euthanized. A larger proportion of the animals died as compared to the similar experiment described in Figure 10b, and is attributed to the larger intranasal dose in this experiment.

In order to have a better idea of the replication of VSV<sup>mir34a-wt</sup> *in vivo* a luciferase expressing virus that contained the mir34a miRT in the 3'UTR of VSV M was used and replication was monitored using *in vivo* fluorescence imaging. Images taken four hours post intravenous infection indicate that doxorubicin treatment resulted in decreased viral gene expression in an area of the mouse consistent with the spleen. This observation is consistent with many other unpublished observations (Lemay CG, Power AT, personal communication) (Figure 14b). Seven days after infection the images show clear evidence of VSVluciferase<sup>let-7mut</sup> replication in the brain of three out of five mice and VSVluciferase<sup>mir34a-wt</sup> replication in the brain of two out of five mice. The mouse that is absent from the image was infected with VSVluciferase<sup>mir34a-wt</sup> and was euthanized prior to imaging due to severe morbidity consistent with VSV infection in the brain. Together these data demonstrate that the dominant effect of doxorubicin treatment prior to oncolytic IV VSV treatment is an increased risk of pathogenic infection. The role of the decreased viral gene expression early after infection in the spleen (Figure 14b), if any, is unknown. Perhaps with an optimized treatment regimen miRT specific, DNA damaging agent dependent attenuation of VSV could be observed, however, in light of the evidence presented here of increased virus pathogenesis after doxorubicin treatment the concept of combination treatment with wild-type M containing oncolytic VSV may be unwise.

**Figure 14: Prior treatment with p53 activating agents do not increase significantly survival of CD-1 nude mice infected with VSV<sup>mir34a-wt</sup> and enhances long term VSV gene expression and CNS infection independent of incorporated miRT. (a)** Survival of CD-1 nude mice infected IN with  $2 \times 10^7$  pfu of the indicated virus 24 hours after IV injection of 150ug cisplatin or PBS. Survival of the mice was followed for about two months. (n=5, except VSV<sup>mir34a-wt</sup> + PBS n=4) **(b)** Nude mice given 0.3mg doxorubicin or saline IV 24 hours before infection with  $1 \times 10^9$  pfu of the indicated VSV derivative virus IV. Four hours post infection mice were injected IP with 200  $\mu$ L luciferin (10mg/mL in PBS) and imaged. These same mice were imaged similarly one week later.

**a****b**

## **4 Discussion.**

The novel strategy of oncolytic viral targeting presented in this work could possibly be classified as a genetic complementation strategy (see section 1.24), wherein a microRNA activity deficit of cancer complements a miRT mediated attenuation of VSV. Novel strategies to genetically suppress undesirable characteristics of viruses are needed to improve current oncolytic viral specificity. The prototype viruses presented in this work demonstrate that microRNA mediated suppression of viral gene expression can eliminate undesirable replication of VSV in non-target cells, eliminating pathogenicity while maintaining oncolytic activity. The basic mechanism of action of miRT attenuation was also examined and determined to be viral gene specific in the case of VSV. Decreased VSV M mRNA containing perfectly complementary let-7 miRTs resulted in strong attenuation in normal human fibroblasts possibly due to the induction of IFN- $\beta$ . This let-7 sensitive VSV retained ability to replicate in target low let-7 expressing cell lines and reduce tumour burden in mouse models. Mir-34a miRTs in the 3'UTR of VSV M mRNA resulted in a virus sensitive to p53 activation with doxorubicin. Contrary to a major hypothesis of this work, however, p53 activating agents did not protect mice from pathogenesis resulting from infection with the mir-34a sensitive VSV. These viruses are the first pre-clinical therapeutic to establish that it is possible to exploit the differential expression of microRNAs in order to selectively kill cancer cells.

Crucial elements of this strategy include the choice of miRTs and the site of incorporation into an oncolytic virus. The difference in pathogenicity in mice

between VSV<sup>let-7wt</sup> and VSV<sup>mir-34a</sup> clearly demonstrate that miRT choice is vital, while the VSVluciferase viruses clearly demonstrate that miRT sequences must be on a relevant viral mRNA. This discussion will provide justification and related implications for the choice of miRT sequences, the choice of VSV as the prototype oncolytic virus, and the choice of the 3'UTR of VSV M as the target for microRNA mediated translation repression used in this thesis.

#### **4.1 miRT sites.**

This work using let-7 miRTs was the first published work on using miRTs to target an oncolytic virus to cancer<sup>209</sup>. Subsequent work in the field has utilized alternative miRTs to reduce viral gene expression and pathogenicity in normal tissues<sup>210-212</sup>.

Using tissue specific microRNA derived miRTs to eliminate pathogenic replication, instead of using tumour suppressor miRTs, is another successful approach to this strategy. Kelley *et al.*, (2008) report that coxsackie virus A21 (CVA21) is a potent oncolytic picornavirus that causes severe myositis and by including miRTs for muscle specific microRNAs, mir-133 and mir-206, the incidence of myositis is greatly reduced while oncolytic potency in a immunocompromised mouse model remains intact<sup>212</sup>. While all mice treated with mock miRT containing CAV21 succumbed to myositis, only approximately 20% of the mice treated with the recombinant oncolytic were euthanized during oncolytic virus treatments due to viral pathology and this was a result of sequence alterations of the incorporated miRTs.

Ylösmäki *et al.* (2008) also report using a similar strategy to reduce pathogenic liver replication of oncolytic adenovirus by incorporation of liver miRTs designed to complement the liver specific microRNA, mir-122<sup>213</sup>. No *in vivo* oncolytic activity was assessed in this study, however, using a very similar strategy Cawood *et al.* (2009) show that adenoviral gene expression in the liver and pathological liver histology can be greatly reduced using an almost identical virus<sup>214</sup>. Hepatotoxicity is a major issue for adenoviral oncolytic and gene therapy applications and this adenoviral engineering strategy may at least partially address this issue<sup>215</sup>. There are many potentially novel oncolytic viruses that could be created by eliminating pathogenic replication using tissue specific microRNA derived miRTs, however this approach may not be as broadly applicable as using tumour suppressor microRNA derived miRTs<sup>211</sup>.

Gürlevik *et al.* (2009) examined the potential of using miRTs in adenovirus using a unique strategy to restrict viral gene expression. Encoded in the engineered genome are microRNA precursors that are designed to complement naturally occurring viral sequences in essential viral genes. The promoter used to drive expression of the microRNA precursors is p53 dependent. Therefore in a normal cell that has p53 transcriptional activity this adenovirus expresses microRNAs that will destabilize its mRNAs. This virus produces almost 100-fold less virus in some p53 containing cell lines and performs significantly better than ONYX-015 in one out of two mouse models tested. It is possible that this strategy could help optimize oncolytic adenoviruses, however it is an approach only possible in viruses that can

utilize cellular promoters.

This more recent data suggests that there are many possibilities for targeting oncolytic virus to cancer cells by incorporation of miRTs. While there exists no specific microRNA expression profile common to cancerous cells such expression profiles reflect the developmental lineage and differentiation state of tumours<sup>130</sup>. This is relevant since not only could microRNA expression profiles be useful diagnostically, but perhaps direct a miRT containing oncolytic therapeutic strategy. While a customized oncolytic therapeutic may be possible, perhaps the most broadly successful strategy would take the approach presented in this work, rendering oncolytic viruses sensitive to tumour-suppressor microRNA expression.

#### 4.1.1 *Let-7*.

As discussed previously, the let-7 family of microRNAs is highly conserved and found abundantly and ubiquitously expressed in mammalian cells<sup>216-218</sup>. The expression of let-7 is associated with differentiation and is expressed at low levels in cancer cells<sup>135,147,148,152,153,197,219</sup>. Most recently it has been shown that let-7 is downregulated specifically in less differentiated breast tumour initiator cells<sup>152</sup>. In addition to tumour specific low expression, let-7 was deemed suitable for this strategy because of its high expression and activity in the majority of differentiated normal cells<sup>216-218</sup>. High expression may be required in order to compete with a rapidly replicating virus such as VSV while microRNA expression in the majority of non-target tissues may be required in order to eliminate pathogenic replication *in*

*vivo*. It is also fortunate that let-7 is perfectly conserved in both mice and humans allowing *in vitro* analysis using human cell lines and *in vivo* analysis in mouse models using the same virus<sup>145,216-218</sup>. Let-7 has these properties and may perhaps be one of only a few microRNAs useful for this particular method of targeting cancer cells with an engineered oncolytic. On the other hand, with many microRNAs serving a role as tumour suppressors<sup>147,168</sup>, other microRNA target sites or combinations of microRNA target sites may be useful in optimizing VSV oncolysis. Indeed the observation that VSV is sensitive to endogenous microRNAs, namely mir-24 and mir-93, suggest that the tropism of the virus may already be, in part, regulated by microRNAs<sup>185</sup>. These or other naturally occurring microRNA target sites may also contribute to VSVs inherent tumour tropism. Another possibility is that more general deficiencies acquired during tumourgenesis, in microRNA processing or activity for example, contribute to VSV replication in tumour cells.

The data presented here support the assertion that imperfectly complementary miRTs are less potent inhibitors of VSV gene expression. VSV<sup>let-7mm</sup> is less inhibited by exogenous let-7 (Figure 5), less attenuated in primary cells (Figure 8) and causes more significant transient weight loss in Balb/c mice (Figure 10a) than VSV<sup>let-7wt</sup>. The intermediate phenotype of VSV<sup>let-7mm</sup>, between VSV<sup>let-7wt</sup> and VSV<sup>let-7mut</sup>, suggests that exploiting the translational inhibition activity of microRNA could perhaps be a useful in an alternative oncolytic targeting strategy. In the case of targeting wild-type VSV M with let-7 miRTs, the elimination of observable pathogenesis and replication in primary cells requires induction of RNAi

and perfectly matched miRTs.

The incorporation of let-7wt miRTs into oncolytic VSV results in a virus that is attenuated in normal cells and has reduced *in vivo* toxicity while retaining antitumour activity. As a proof of concept, this work suggests a multitude of additional applications of incorporation of let-7 miRTs into OVs.

#### 4.1.2 *Mir-34a*.

Mir-34a also has some tumour suppressor-like properties, as discussed in section 1.432, and is therefore similar to let-7 in its suitability for this study. The differences between mir-34a and let-7 also make mir-34a an attractive candidate. Unlike let-7, mir-34a is upregulated in response to DNA damage caused by conventional cancer therapeutics in a p53-dependent mechanism<sup>168-170</sup>. Therefore, as demonstrated, a mir-34a miRT containing oncolytic can be made sensitive to p53, the same strategy used to develop the only approved oncolytic virus, ONYX-015. It also facilitates examining the effect of endogenous upregulation of a microRNA on a miRT regulated oncolytic using conventional cancer therapeutics. Thus mir-34a miRT incorporation can provide data on the effectiveness of combining conventional cancer therapeutics with an oncolytic virus. Examined in this work is the hypothesis that the upregulation of mir-34a by p53 in response to DNA damaging therapeutics would further protect cells from a mir-34a regulated VSV, while importantly, cancer cells lacking p53 activity will remain sensitive. The properties in common with let-7, namely tumour suppressor-like properties and

perfect conservation between mouse and human, and its unique properties, namely p53-dependent transcriptional upregulation, makes mir-34a an excellent candidate for this approach.

The data presented in Figure 13, support the idea that incorporation of mir-34a miRTs in VSV results in a virus sensitive to p53 and more sensitive to p53 activation. Unfortunately using combination therapy to activate p53 in *in vivo* mouse models results in increased pathogenesis of VSV. One hypothesis to explain this increased pathogenesis is that the transient immunosuppression, known to result from treatment with DNA damaging agents permits the observed VSV pathogenesis in the infected animals<sup>220</sup>. The observed decreased viral gene expression in the spleen a short time after infection is perhaps related to this (Figure 14). These results suggest that DNA damaging agents in combination with oncolytic VSV will require a different approach than presented here. Perhaps this data should be considered in oncolytic virus clinical trials where patients have previously been treated with conventional chemotherapeutics.

#### 4.1.3 *Let-7 vs mir-34a*.

The differences observed between VSV containing let-7 miRTs and mir-34a miRTs reveal that the choice of miRT is a crucial element of this oncolytic engineering strategy. Infection at a low MOI with VSV<sup>mir34a-wt</sup> kills a monolayer of GM38 cells (data not shown) whereas VSV<sup>let-7wt</sup> leaves a monolayer of GM38 cells intact (Figure 9a). Additionally, while VSV<sup>let-7wt</sup> has no observable adverse pathology

(Figure 10), VSV<sup>mir34a-wt</sup> is not significantly attenuated *in vivo*. Additionally, one hypothesis to explain these differences is related to the abundance of endogenous let-7 and mir-34a. While there are not many studies where a direct comparison of let-7 and mir-34a abundance can be compared, those that are available suggest that let-7 is expressed at a much higher level than mir-34a<sup>221</sup>. Perhaps a mir-34a miRT can not significantly attenuate VSV by repression of VSV M when the miRT complementing microRNA abundance is not sufficient.

## 4.2 VSV

While there is potential for designing other oncolytic viruses using the same principles presented in this thesis, VSV possesses some distinct advantages. Most notably, previous data indicates VSV is susceptible to RNAi<sup>189</sup> (see section 1.44). During its entirely cytoplasmic lifecycle, VSV, an RNA virus, is always available for interaction with endogenous microRNAs and therefore potentially microRNA induced RNAi. However, not all viral RNA in the cytoplasm is subject to RNAi. The negative sense miRTs in the genome of VSV are not complementary to the endogenous microRNA, and the presented results indicate that the positive sense genome that occurs during the viral lifecycle is not affected by the microRNA target sites, leaving only the viral mRNA transcript as the specific target using this method. This assertion is supported by data presented in Figure 7b as viral titres are not reduced by miRT incorporation into the 3'UTR of the irrelevant gene, luciferase and by the data in Figure 7 showing only VSV M mRNA is affected relative to other viral

genes. Thus incorporation of miRTs into the 3'UTR of a VSV gene allows for viral gene specific microRNA regulation. More specific manipulation of VSV containing a single miRT is therefore possible than would be if all viral RNAs were subject to microRNA induced RNAi. This is in contrast to a coxsackie virus that was engineered to contain muscle specific miRTs, CAV21, where all genes are affected by miRT incorporation<sup>16</sup>. Another distinct advantage of using VSV in this strategy is the virus's sensitivity to innate cellular antiviral responses which could protect against escape mutant viruses. Spontaneous generation of viruses containing functional mutations in incorporated microRNA target sites is of obvious concern with this strategy. As supported by the data in Figure 9b and c, cells infected by VSV<sup>let-7wt</sup> induce an antiviral state that protects all cells from a subsequent infection with an escape mutant. The escape mutant is represented in the experiment by a wild-type VSV expressing eGFP. In the case of an oncolytic CAV21 containing miRTs, escape mutants are an issue, not surprisingly, as picornaviruses have previously been observed to escape regulation by microRNAs<sup>16,222</sup>. It is possible that VSV is one of only a handful of viruses where this strategy would work so effectively.

Various strategies have been used to engineer oncolytic viruses in order to limit replication to cancer cells<sup>223</sup>. Viral genes required for countering host cell innate immunity are deleted or mutated in a variety of oncolytics. The approach of employing deletions or mutations in viral genes that have evolved to counter the host antiviral response is a common strategy in safe OV design<sup>52-54</sup>. This strategy

has been employed with oncolytic VSV<sup>83</sup>, influenza<sup>29</sup>, and HSV-1<sup>225</sup>. As stated earlier, the strategy presented here retains wild-type viral protein function while eliminating the associated undesirable toxicity by selective expression and may be applicable in designing these other oncolytic viruses.

There are other viruses that show early promise as oncolytics that may be suitable for this type of engineering. Engineered OV<sup>s</sup> that have genomic deletions in viral genes in order to increase safety could instead have these genes under the regulatory control of incorporated microRNA target sites. Thus expression is attenuated in normal cells to minimize toxicity, while expression of these genes is maintained in cancer cells to maximize efficacy. The previously mentioned examples of OV<sup>s</sup> and the corresponding viral gene deleted in current oncolytics that may be suitable for this approach include HSV-1 and the gamma 34.5 gene, influenza virus and the NS-1 gene, and vaccinia virus and viral thymidine kinase<sup>1</sup>. Recently, it has been demonstrated that miRT mediated regulation of the influenza nucleoprotein is effective, supporting the concept of applying this strategy to an oncolytic influenza virus<sup>226</sup>. Even more recently, this strategy has been successfully used to target an oncolytic HSV-1 by placing miR-143 or miR-145 into the 3'UTR of the HSV-1 essential gene, ICP-4<sup>227</sup>. Mir-143 and mir-145 are expressed at low levels in many cancer cells but are expressed in normal tissues which attenuated these viruses ability to replicate in non-target tissues <http://www.boingboing.net/> while remaining efficacious in a xenograft mouse model of cancer.

### 4.3 VSV M

There are many reasons to choose to use the matrix protein of VSV as a miRT regulated gene in this oncolytic prototype. The foremost reason is that VSV M protein expression is required for viral growth and replication, as are all five VSV genes. As discussed in section 1.32, one function of wild-type (wt) VSV M protein in the host cell is the suppression of the innate immune responses ability to induce interferon and this function is eliminated by mutation in an oncolytic strain of VSV<sup>80</sup>. Wt VSV M containing VSV demonstrates toxicity in normal cells and mice, and is therefore unsuitable as an OV. Oncolytic VSV containing mutant VSV M has much less associated toxicity but has reduced cytopathic effect in some cancer cell lines<sup>77,80,99,100,101</sup>. Therefore, a possible improvement to the virus would permit expression of the more potent oncolytic wt VSV M protein in infected tumour cells while enabling infected normal cells to repress wt VSV M expression. Let-7 miRT mediated repression of wt VSV M in normal cells permits induction of an antiviral state, possibly due to induction of type 1 interferons (Figure 9b and 9c). By selective expression of VSV M and thus induction of interferon in normal cells, these cells are spared whereas cancer cells, unable to induce an effective antiviral state in response to interferon, remain permissive to oncolysis. MiRT-dependent induction of interferon also ensures protection against escape mutants, as described above. Another possible justification for choosing VSV M as a miRT target, as opposed to alternative VSV genes, is that downregulation of VSV M in infected normal cells may also permit nucleocytoplasmic transport to continue during infection. This

transport is not only required for an effective interferon response but also for the biogenesis of microRNAs. Without continued production of microRNAs, a miRT regulated VSV might escape the proposed mechanism of regulation during infection. This idea was also considered when choosing VSV M as the viral gene to be regulated by miRT, however no experiments conducted during this work definitively demonstrated whether VSV M protein had any effect on microRNA activity in an infected cell (data not shown). Together, previous evidence that VSV M expression can be reduced with RNAi, that VSV M protein can play a role VSV tumour tropism and that VSV M protein expression may inhibit microRNA accumulation makes VSV M a reasonable choice for miRT mediated regulation in order to produce a safer oncolytic virus.

While VSV<sup>let-7<sup>wt</sup></sup> was not directly compared to other oncolytic strains of VSV in the subcutaneous CT26 mouse tumour model, previous work with the AV1 and AV2 strains have shown greater efficacy. Luciferase assays, as in Figure 3a, demonstrated that CT26 cells had high let-7 activity relative to A549 cells (data not shown). It was thought that this model would therefore be a challenging model for VSV<sup>let-7<sup>wt</sup></sup> directed oncolysis. Despite this, VSV<sup>let-7<sup>wt</sup></sup> maintains antitumour activity in this model. This suggests that, while AV1 and AV2 had greater efficacy in this model, VSV<sup>let-7<sup>wt</sup></sup> had antitumour activity against tumours with let-7 activity. The data clearly show VSV<sup>let-7<sup>wt</sup></sup> has similar oncolytic properties to Δ51 VSV in a subcutaneous A549 tumour model in CD-1 nude mice. This may suggest that an oncolytic VSV containing a wild-type M protein is not more effective than one

containing a mutant M protein. Importantly, this observation may be dependent on the experimental model and also demonstrates that the oncolytic capacity of VSV<sup>let-7<sup>wt</sup></sup> is not significantly reduced in this low let-7 expressing tumour model. Indeed this observation indicates that, as an oncolytic employing microRNA target sites to ensure tumour specific expression of viral genes, miRT targets on VSV M make VSV<sup>let-7<sup>wt</sup></sup> a promising prototype.

Interestingly, while targeting individual viral genes is possible with VSV it is likely not possible with picornaviruses. In a picornavirus infected cell a viral polyprotein is produced from a single RNA species. Therefore miRT regulation affects the translation of all viral proteins. While potent attenuation of CAV21 is achieved with miRT incorporation, the inability to target different viral genes limits the potential phenotypes that may be observed when different viral genes can be regulated using miRTs. If indeed the attenuation observed in a miRT targeted CAV21 is more potent than VSV, then perhaps using mir-34a miRT in CAV21 might be more sensitive to p53 transcriptional activity.

While let-7 miRTs on VSV M produced a better oncolytic virus perhaps placing mir-34a miRTs on another VSV gene would produce an oncolytic VSV with more desirable properties. Placing miRTs on alternative VSV genes is currently under investigation in order to optimize the safety and potency of VSV oncolysis.

#### **4.4 3'UTR miRTs**

To accomplish selective expression of wt VSV M in cancer cells miRTs were

cloned in the 3'UTR of VSV M mRNA. RNAi can be induced by incorporation of miRTs in the 3'UTR of transiently transfected reporter genes. The previously described let-7 miRTs, used in this study, were evaluated for inhibitory activity when placed into the 3'UTR of a reporter gene. Therefore miRTs were incorporated in the 3'UTR of VSV M mRNA to specifically inhibit VSV M protein expression. The data strongly support that the expression of individual viral genes can be regulated by incorporation into the 3'UTR of VSV mRNAs.

Interferon can be produced as a cellular response to intracellular dsRNA that occurs during viral lifecycles. Data presented here indicates that dsRNA resulting from endogenous microRNAs binding to complementary miRTs does not stimulate production of an antiviral state such as would be produced from IFN since VSVluciferase<sup>let-7wt</sup> destroys a monolayer of GM38 cells whereas VSV<sup>let-7wt</sup> does not. Both contain the same let-7 complementary sequences therefore it is the position of these sequences that is important when engineering oncolytic viruses using this strategy.

Triplicate repeats of microRNA complementary sequence were used in order to stay consistent with previously published results on these sequences<sup>122</sup>. However, it is clear that inhibitory potency increases with the number of repeats, so 'fine-tuning' of viruses that are regulated by miRTs may be as simple as changing the number of these repeats. It is possible, however, that the cellular microRNA machinery is saturated extremely quickly in many situations using this strategy and the number of repeats may not have any effect.

The proceeding reasoning prompted construction of the viruses described in this work. The reasoning, however, does not exclude the possibility that there are many other variations of this strategy that could produce excellent oncolytics. Other than confirming that targeting M was more effective than targeting an irrelevant reporter gene, none of the previously mentioned parameters were tested in this thesis. However, this work establishes that differential microRNA expression between cancer cells and normal cells is a viable target for therapeutic oncolytic viruses.

The more obvious short-term future work using this strategy is the elimination of VSV neurovirulence by the incorporation of brain specific miRTs. Also targeting alternative VSV genes in order to enhance differential replication between microRNA expressing cells and target low microRNA expressing cells would help in optimizing this approach for VSV. While an argument for targeting VSV M was constructed in this thesis there are good arguments for targeting other viral genes. For example, it may be that the most potent inhibition would occur by targeting the viral polymerase, VSV L, as it is essential for viral RNA transcription and replication. As mentioned previously, targeting alternative VSV genes may increase the safety of *mir-34a* miRT containing VSVs. Other future work includes applying this strategy to other potential oncolytic viruses. Currently this strategy is also being employed using vaccinia virus (unpublished), CAV21<sup>33</sup>, adenovirus<sup>214,228</sup> and HSV-1<sup>227</sup>. These studies demonstrate that this strategy is robust, there are a number of different applications of this strategy and it will likely continue to be investigated as a tool for

optimization of oncolytic agents.

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