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Introduction

Oncolytic viruses (OVs) are promising cancer therapeutics, and a variety of oncolytics are currently in clinical trials[1]. Results from early human trials indicate that this class of therapeutics is safe; however, there is still an opportunity to increase antitumour efficacy. One recent method of increasing antitumour activity is by incorporation of immunomodulatory genes into OVs in order to increase intratumoural replication[2,3]. While this strategy produces a more potent oncolytic it does not increase tumour specificity, potentially decreasing the therapeutic window. Clearly there exists a need for novel viral engineering strategies that enable targeting viral replication to tumours.

MicroRNAs are small (~21nt), endogenous, non-coding RNAs that direct the translational repression of target mRNAs having partial complementary sequence in their 3'UTR[4-6]. Differential microRNA expression is a hallmark of cancer cells and indeed the majority of microRNAs are downregulated in cancer cells[7,8]. The general downregulation of microRNA expression in cancer is often due to a decrease in posttranscriptional processing of these RNAs, and when this processing is abrogated it promotes tumourgenesis[8,9]. Therefore low microRNA represents a common defect in cancer cells that has yet to be exploited therapeutically.

One family of highly conserved microRNAs demonstrating low expression in cancer cells is let-7[10-17]. Decreased expression of let-7 is functionally linked to tumour cell biology, regulating the expression of proto-oncogenes[18,19], and reflecting the differentiation state of tumours[7,15,16]. Synthetic microRNA complementary sequences in the 3'UTR of a target gene has previously demonstrated let-7 specific repression[20]. Thus we exploited differential expression of this microRNA to enable expression of viral genes specifically in cancer cells by incorporation of synthetic let-7 complementary sequences. These sequences should inhibit viral gene expression and replication in normal, let-7 expressing cells while low let-7 activity in cancer cells would facilitate viral gene expression and subsequent oncolysis.

As a prototype virus for this strategy we chose vesicular stomatitis virus (VSV), a negative sense single-stranded RNA Rhabdovirus, that is naturally sensitive to microRNA mediated repression[21-23]. Specifically, we chose to subject the expression of the wildtype matrix protein of VSV (VSV M) to let-7 regulation, as this protein has an essential role in viral growth and replication, and serves to counteract antiviral responses[24-26]. This strategy facilitates the use of potent viral proteins while not sacrificing safety and is an important step in increasing therapeutic potency of VSV and other OVs.

Here we examine the potential of using differential microRNA expression, central to cancer biology, to achieve selective expression of VSV M in tumour cells. We show here that it is possible to engineer let-7 sensitive expression of individual VSV genes. We demonstrate that incorporation of let-7

microRNA target elements to regulate VSV M expression in a wildtype toxic strain of VSV results in a virus that is attenuated specifically in normal cells and avirulent *in vivo* while retaining antitumour activity.

RESULTS

Endogenous let-7 microRNA expression correlates with VSV$^{let\text{-}7wt}$ replication.

Recombinant vesicular stomatitis viruses carrying three repeats of a sequence of differing complementarity to the mature let-7a microRNA are depicted schematically in Fig 1. 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[27]. 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 translational repression and transcript degradation. The let-7mut sequence demonstrates no detectable inhibition in a luciferase reporter due to a high degree of noncomplimentarity to the mature let-7a microRNA and serves as a negative control[20]. These same sequences incorporated into VSV$^{let\text{-}7wt}$ and VSV$^{let\text{-}7mm}$ are translationally repressed when incorporated into the 3'UTR of a luciferase reporter gene in a let-7 dependent mechanism[20].

Functional activity as assessed by luciferase reporter assay and analysis of the expression of mature let-7a microRNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR) shows that GM38 cells express more functional let-7 than A549 cells (Figure 2a&b). This confirms observations by others that human A549 lung carcinoma cells express low let-7[13,14]. The primary human fibroblast cell line, GM38, has approximately 3 times more let-7 activity than A549 cells (Figure 2a). This was assessed using the let-7mm sequence as this was used previously as a sensitive detector of let-7 mediated translational inhibition[20]. This functional activity correlates well with expression levels of let-7a as measured by qPCR (Figure 2b). As such, A549 and GM38 cell lines serve as models of target cells and non-target primary cells respectively, for evaluating the specificity of VSV$^{let\text{-}7wt}$. Importantly let-7 activity and expression in GM38 cells correlates with an approximate 1000-fold replication deficit of VSV$^{let\text{-}7wt}$ as compared to VSV$^{let\text{-}7mut}$ after 48hours. This effect was not observed in A549 cells (Figure 2c). The growth curve also demonstrates that the greatest attenuation specific to VSV$^{let\text{-}7wt}$ occurred in GM38 cells, and after 72 hours of infection VSV$^{let\text{-}7wt}$ progeny were not detected. Therefore VSV$^{let\text{-}7wt}$ produced the fewest progeny in a let-7 target sequence specific manner in this primary cell line.

Replication of viruses containing let-7 complementary sequences is affected by exogenous let-7.

To determine if the correlation of microRNA activity and inhibition of replication of these viruses was indeed due to let-7 levels we assessed the response of the viruses to exogenous let-7. Since both endogenous microRNA and exogenous siRNA enter the RISC complex and affect transcripts in a similar mechanism endogenous let-7 microRNA can be supplemented with exogenous siRNA of the same sequence[28,29]. Transfection of Hela cells with siRNA containing the sequence of mature let-7a microRNA specifically reduced the titres produced by both viruses containing let-7a complementary sequences (Figure 3a). The results presented in Figure 3a&b using exogenous siRNA to augment endogenous let-7 and the results from Figure 2 demonstrating a correlation of let-7 expression with $VSV^{let\text{-}7wt}$ replication together suggest that the observed correlation of let-7 functional activity and $VSV^{let\text{-}7wt}$ replication were due to the endogenous, sequence specific expression of let-7 microRNA.

Additional let-7 increases the survival of cells infected with viruses containing let-7 complementary sequences.

Ideally these oncolytic viruses will not only produce more progeny in low let-7 expressing cells, as demonstrated, but also preferentially kill these cells. To test the specificity of the engineered viruses for low let-7 expressing cells we assessed the survival of cells transfected with siRNA and then infected with the let-7 complementary sequence containing viruses. Transfection of A549 cells with let-7a siRNA resulted in 57% viability of cells infected with $VSV^{let\text{-}7wt}$. This protection

was not seen in A549 cells infected with VSV$^{let\text{-}7mut}$, with 15% viability, suggesting that cell survival was enhanced in a let-7 sequence specific mechanism (Figure 3b). Data from an identical experiment using Hela cells also showed specific inhibition of VSV$^{let\text{-}7wt}$ 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 cellular microRNA levels.

VSV$^{let\text{-}7wt}$ 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 3c). For these experiments we used Hela cells as these cells have measurable let-7 activity[18,20,30]. We also detected let-7 activity in these cells by luciferase assay (as in Figure 2a) as well as a reduced capacity to support VSV$^{let\text{-}7wt}$ replication (data not shown). Quantification of transcripts early in infection demonstrated the abundance of VSV transcripts is related to their position in the VSV genome (Figure 1). This supports previous findings that the nonprocessive nature of the viral RNA polymerase (VSV L) leads to this characteristic transcript abundance[31,32]. Also evident from this data was that in Hela cells the M protein transcript is less abundant early during infection with VSV$^{let\text{-}7wt}$ as compared to VSV$^{let\text{-}7mut}$ when expressed as a ratio to other genes in the VSV genome. Together this data suggests that the presence of let-7

microRNA decreases M mRNA in VSV$^{let\text{-}7mut}$ infected cells, resulting in decreased viral production and oncolysis.

The let-7 sensitive replication of VSV$^{let\text{-}7wt}$ is due to its reduced expression of VSV M.

Our intent when designing VSV$^{let\text{-}7wt}$ was to have VSV M mRNA be specifically regulated by let-7, however it is formally possible that let-7 could bind the engineered target sequence in the positive sense VSV genome that exists as an essential element in the viral lifecycle. To determine if the attenuation of VSV$^{let\text{-}7wt}$ is due to the placement of the let-7 target sequences and the decreased abundance of M mRNA specifically, new viruses were created. These viruses contained a firefly luciferase reporter gene incorporated with the let-7 target sequences, let-7mut and let-7wt in their 3'UTR. Viral luciferase expression in infected let-7 expressing Hela cells showed a let-7wt specific reduction while A549 cells, having low let-7 activity, displayed no such reduction (Figure 4a). In GM38 cells a similar let-7wt specific reduction of luciferase was observed (data not shown). Titres produced after 24 hours infection of Hela cells at an MOI of 0.1 with the Luciferase let-7mut and Luciferase let-7wt were very similar (data not shown). Infection of GM38 cells demonstrated that only VSV$^{let\text{-}7wt}$ had reduced titres and the associated cytopathic effect, observed upon VSV$^{let\text{-}7mut}$ and VSV$^{let\text{-}7mm}$ infection, was absent from the confluent monolayer of GM38 cells infected with VSV$^{let\text{-}7wt}$ (Figure 4b, c).

Prior infection of GM38 cells with VSV$^{let\text{-}7wt}$ protected these cells from subsequent infection with a VSV expressing an eGFP transgene suggesting that an antiviral state was induced following infection with VSV$^{let\text{-}7wt}$ (Figure 4d). The VSV M protein, as mentioned previously, has a role in counteracting antiviral responses[24-26]. This suggested that decreased wildtype M protein specifically is responsible for this observed phenotype of VSV$^{let\text{-}7wt}$.

This data suggests that incorporation into the 3'UTR of the let-7 target sequences affects only the expression of that particular viral gene and has no impact on the viral genome.

VSV$^{let\text{-}7wt}$ is less pathogenic in balb/c and CD-1 nude mice.

The lack of VSV$^{let\text{-}7wt}$ cytotoxicity *in vitro* led us to test the toxicity of this virus *in vivo* (Figure 5). Intranasal infections of Balb/c mice resulted in the most significant transient weight loss in VSV$^{let\text{-}7mut}$ infected mice and an intermediate phenotype associated with VSV$^{let\text{-}7mm}$. No detectable transient weight loss was observed with VSV$^{let\text{-}7wt}$ (Figure 5a). In CD-1 nude mice no pathology was observed after intranasal infection with VSV$^{let\text{-}7wt}$. In contrast, one third of the mice infected with VSV$^{let\text{-}7mut}$ were found moribund on day 8 (Figure 5b) and had substantial virus found in their brains (>1E4pfu/mg tissue) whereas there was no detectable virus in the brains of two VSV$^{let\text{-}7wt}$ 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 target sequences renders VSV less pathogenic.

VSV$^{let\text{-}7wt}$ has antitumour activity.

Previous work has demonstrated antitumour effects of VSV on subcutenous CT26 tumours in balb/c mice using a M protein mutant of VSV[24,26,33]. Our observations of tumour specific replication *in vitro* led us to test the antitumour activity of VSV$^{let\text{-}7wt}$ *in vivo*. Indeed multiple intravenous injections of VSV$^{let\text{-}7wt}$ retarded CT26 tumour growth as compared to an identical schedule of PBS injections (Figure 5c). Mice receiving 10E9 pfu and 10E8 pfu were grouped as there was no dose dependence observed. No complete responses were observed in this model. Additionally multiple intravenous injections of VSV$^{let\text{-}7wt}$ administered to CD-1 nude mice harbouring established subcutaneous A549 tumours, retarded tumour growth as compared to an identical schedule of PBS injections (Figure 5d). One complete response was observed after treatment with VSV$^{let\text{-}7wt}$, however it was the smallest tumour at 4mm^3 on the first day of treatment. As a comparison for oncolytic activity this virus was compared to a previously engineered oncolytic VSV, Δ51 VSV[24]. In figure 5c 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 wildtype viral proteins that show toxicity.

Discussion

The strategy presented here demonstrates microRNA mediated suppression of viral gene expression can eliminate undesirable replication of VSV in non-target cells. Novel strategies to genetically suppress undesirable characteristics of viruses are needed to improve current oncolytic viral specificity and may enable use of previously unsuitable viruses for oncolytic viral cancer therapies.

Increasing evidence for an association between microRNA expression and cancer[34,35] along with the number of examples of endogenous cellular microRNAs with effective antiviral activity[36,37] led us to hypothesize that exploiting microRNA expression would be a novel strategy to direct replication of therapeutic viruses to target tissues. The observation that VSV is sensitive to siRNA and microRNA mediated repression combined with the virus's preliminary success as an oncolytic agent suggested that VSV would be an excellent prototype to test the concept of exploiting differential microRNA expression of cancer cells[21-23].

The let-7 family of microRNAs is highly conserved and found abundantly and ubiquitously expressed in mammalian cells[38-40]. The expression of let-7 is associated with differentiation and is expressed at low levels in cancer cells[10-16]. Most recently it has been shown that let-7 is downregulated specifically in less differentiated breast tumour initiator cells[15]. In addition to tumour specific low expression, let-7 was thought to be suitable for this strategy because of its high expression and activity in the majority of differentiated normal cells. 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*. 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[12,41], 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[22]. These or other naturally occurring microRNA target sites may also contribute 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.

While other potential oncolytic viruses could be designed using the same principles presented in this paper, VSV possesses some distinct advantages. As an RNA virus the VSV lifecycle is always subject to interaction with endogenous microRNAs. The negative sense microRNA targets in the genome of VSV are not complementary to the endogenous microRNA. 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 the data presented in Figure 3c as viral titres are not reduced by microRNA target incorporation into the 3'UTR of the irrelevant gene, luciferase. 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 4d, cells infected by VSV$^{let\text{-}7wt}$ 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 wildtype VSV expressing eGFP. 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[1]. 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[1,42,43]. This strategy has been employed with oncolytic VSV[33], influenza[42], and HSV-1[44]. One function of wildtype VSV M protein in the host cell is the suppression of the host cell innate immune response and this function is eliminated by mutation in an oncolytic strain of VSV[24]. While wildtype VSV M demonstrates toxicity in normal cells, mutant M proteins eliminate this toxicity, but may also display a reduced cytopathic effect in cancer cell lines[24,45,46]. Oncolytic VSV containing this mutant M protein induces an antiviral response in infected cells and this response may limit intratumoural replication and antitumour activity of OV viruses including VSV[2,3,47]. Therefore retaining wildtype viral protein functions that serve to counteract the antiviral response in cancer cells while not sacrificing safety is an important step in increasing therapeutic potency of VSV and other OVs. The strategy presented

here retains wildtype viral protein function while eliminating the associated undesirable toxicity by selective expression.

While VSV$^{let\text{-}7wt}$ 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 2a, 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$^{let\text{-}7wt}$ directed oncolysis. Despite this, VSV$^{let\text{-}7wt}$ maintains antitumour activity in this model. This suggests that, while AV1 and AV2 had greater efficacy in this model, VSV$^{let\text{-}7wt}$ had antitumour activity against tumours with let-7 activity.

The data clearly show VSV$^{let\text{-}7wt}$ 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 wildtype M protein is not more effective than one containing a mutant M protein. Importantly, this observation may be dependant on the experimental model and also demonstrates that the oncolytic capacity of VSV$^{let\text{-}7wt}$ 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, VSV$^{let\text{-}7wt}$ is a promising prototype.

There are other viruses that show early promise as oncolytics that may be suitable for this type of engineering. Engineered OVs 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. Examples of OVs 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[1]. Picornaviruses may also be suitable for microRNA mediated regulation of replication, however they have demonstrated an ability to produce escape mutants when engineered to carry a let-7 microRNA target site[7,48]. It may be necessary for these viruses to carry multiple repeats or repeats of different microRNA target sites in order to prevent the production of escape mutants.

While there exists no specific microRNA expression profile common to cancerous cells such expression profiles reflect the developmental linage and differentiation state of tumours[7]. This is relevant since, not only could microRNA expression profiles be useful diagnostically, but perhaps direct an oncolytic therapeutic strategy such as presented in this work.

The incorporation of microRNA target sites in to 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 this strategy.

Materials and Methods

Viruses.

Novel recombinant viruses were cloned as described in Figure 1 and rescued as described previously[24]. Viruses containing luciferase were cloned by insertion of the luciferase ORF (pGL3Basic, Promega, Madison, WI) followed by let-7 microRNA target sites were cloned in an Nhe1 site in a VSV genome engineered to carry transgenes previously described[49]. Propagation of all viruses was done in A549 cells. Viral 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 2c) the cells were washed once with 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). For animal studies virons were concentrated by centrifugation at 30 000g and resuspension in phosphate-buffered saline (PBS) (Hyclone, Logan, UT). Quantification of virions was done by plaque assay on Vero cells.

Cell lines.

Human A549 lung carcinoma, Human Hela cervical carcinoma, murine CT26 colon carcinoma (American Type Tissue Collection) 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).

Mice and tumour models.

Female mice were obtained from Charles River Laboratories (Wilmington, MA), and injected subcutaneously with $5x10^5$ CT26 cells to establish hind flank tumors. Tumour bearing animals were treated intravenously with $1x10^8$-$1x10^9$ pfu in 100μL. CD-1 nude mice were injected subcutaneously with $1x10^6$ A549 cells to establish right hind flank tumors. Tumour bearing animals were treated intravenously with 10^9 pfu in 100μL. Tumour volume was measured using calipers and volume calculated using the formula (length/2 * width^2). Toxicity studies were conducted with intranasal administration of 10^5 pfu in 5μL of VSV to 6-week-old balb/c mice or $7.5x10^7$pfu in 5μL to CD-1 nude mice. All experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Service.

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).

qRT-PCR.

VSV M mRNA was reverse transcribed with Superscript III (Invitrogen, Carlsbad, CA) as per the manufacturers instructions using oligo dT primers. qPCR was done using Platinum Taq (Invitrogen, Carlsbad, CA) as per the manufacturers 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 manufacturers instructions. All PCR was quantified in real time using a Rotor-Gene GR-3000A thermocycler (Corbett Research, Sydney, Australia).

Viral infections and exogenous siRNA.

Dilutions of VSV in 100μL of DMEM were added to confluent cells in 6well plates placed in a 5%CO_2 37C incubator for 40min before 2mL of DMEM containing 10%FBS was added. 50pmol of exogenous siRNA (Dharmacon) when present were transfected using oligofectamine (Invitrogen) for Hela cells and lipofectamine 2000 (Invitrogen) for A549 cells as per the manufacturers instructions 24hrs before infection.

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Figure Legends

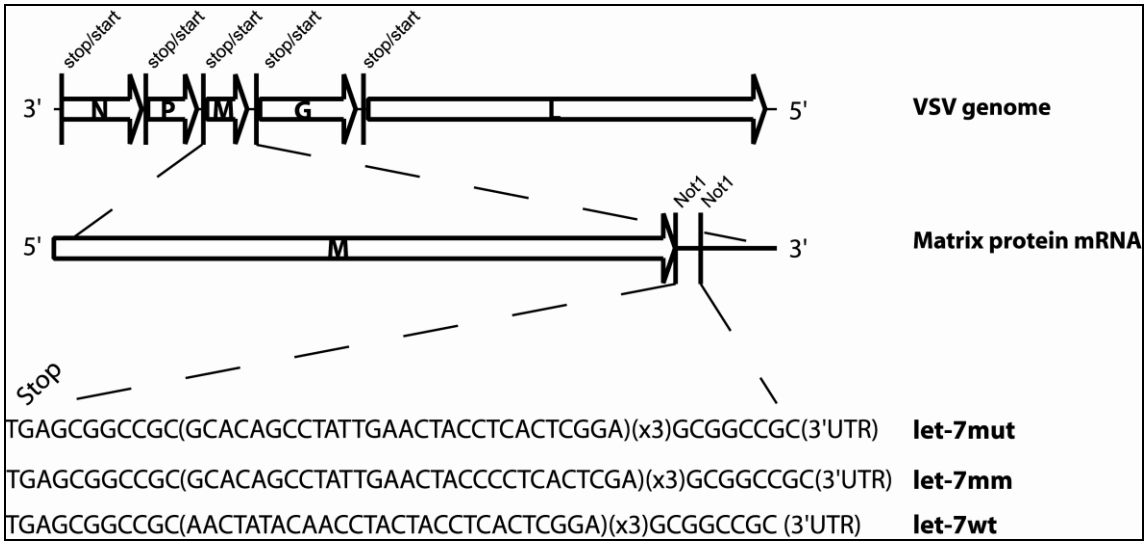

Figure 1: Schematic of let-7 microRNA target elements incorporated into VSV$^{let\text{-}7mut}$, VSV$^{let\text{-}7mm}$ and VSV$^{let\text{-}7wt}$. The VSV genome and viral transcripts with the location of an introduced Not1 restriction site in the 3'UTR of VSV M are represented. The mRNA of VSV M contains the original 3'UTR following the incorporated Not1 site. The sequence elements inserted into the Not1 site in VSV$^{let\text{-}7mut}$, VSV$^{let\text{-}7mm}$, VSV$^{let\text{-}7wt}$ and show limited complimentary, partial complimentarity or complete complimentarity respectively, to mature let-7 microRNA and are present in triplicate as this has been shown to increase inhibitory potency. The viruses derived from insertion of let-7mut, let-7mm and let-7wt into the 3'UTR of VSV M are designated VSV$^{let\text{-}7mut}$, VSV$^{let\text{-}7mm}$ and VSV$^{let\text{-}7wt}$ respectively.

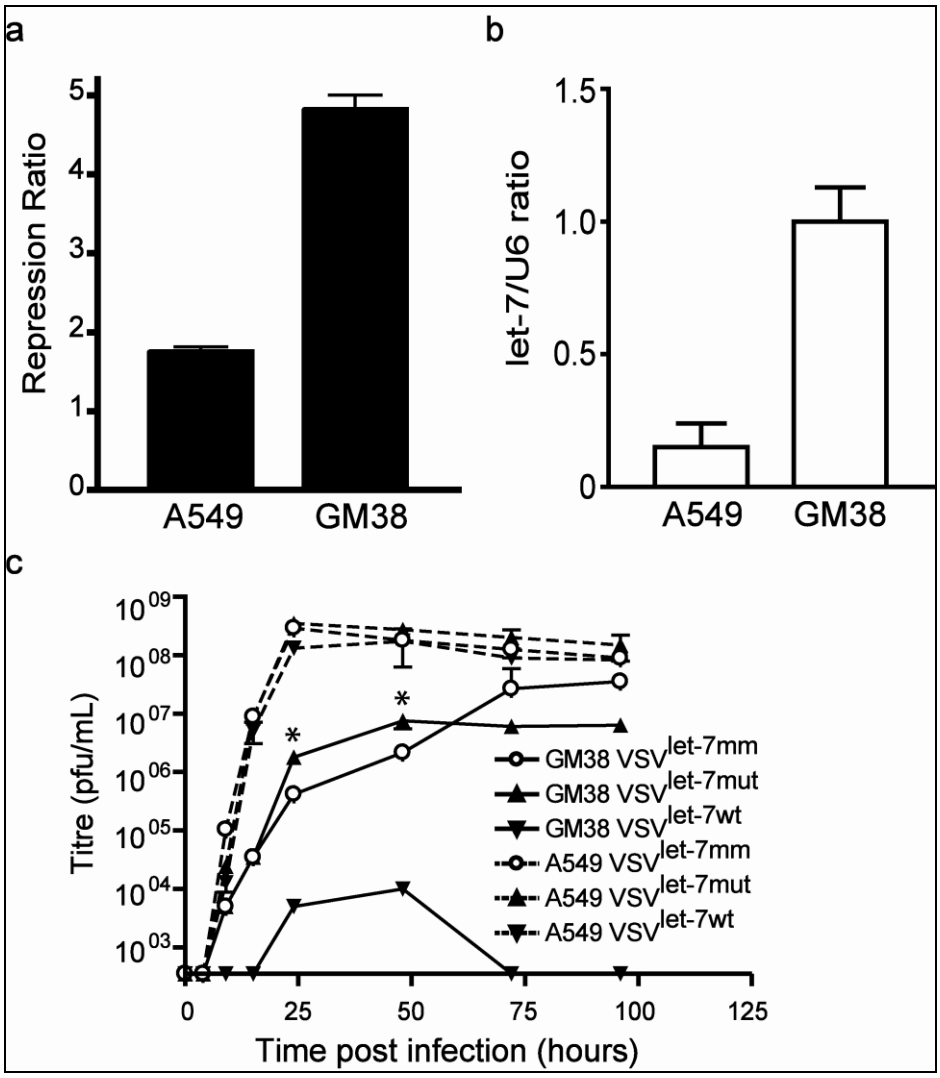

Figure 2: VSV$^{let\text{-}7wt}$ replication correlates with let-7 activity and expression in A549 and GM38 cells. (**a**) Vectors containing the let-7mm sequence in the 3'UTR of luciferase demonstrate greater repression in GM38 cells as compared to A549 cells. The repression ratio is expressed as a ratio of the let-7mm luciferase to the let-7mut luciferase using a co-transfected renilla luciferase reporter to normalize for transfection efficiency. ($p<0.001$) (**b**) Quantification of mature let-7 microRNA in the indicated cell lines expressed as a ratio to U6 small RNA. The data is presented as mean + SD. ($p<0.002$) (**c**) Growth curve of the engineered viruses in A549 and GM38 at an MOI of 0.1. The x-axis designates the limit of detection. Data are represented as mean ± SD. For GM38 infected with VSV$^{let\text{-}7wt}$ and VSV$^{let\text{-}7mut}$, * indicates a $p<0.0005$.

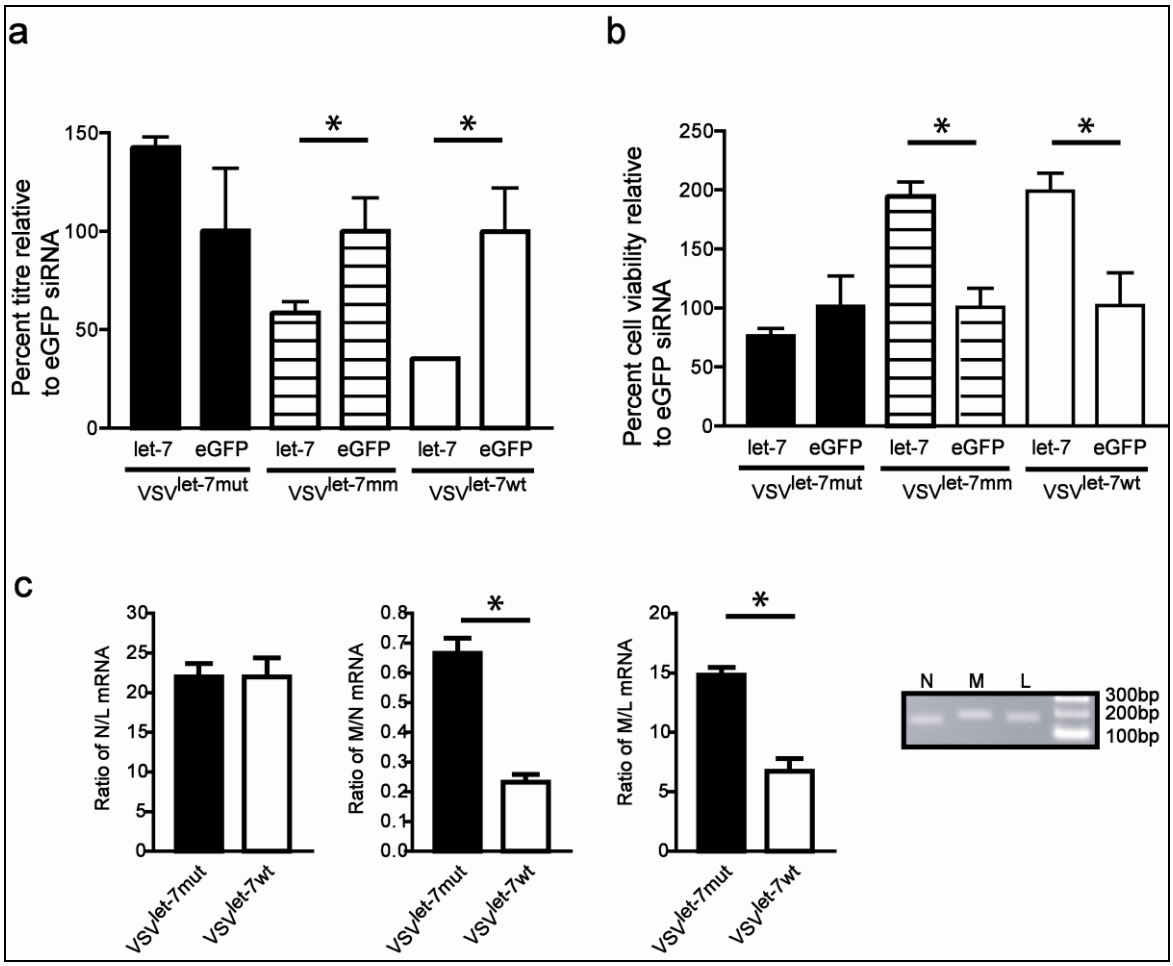

Figure 3. Let-7 microRNA effects replication, cytotoxicity and M mRNA levels in VSV$^{let\text{-}7wt}$ infected cells. (**a**) Hela cells were infected at an MOI of 0.1 with the engineered let-7 viruses 24hrs after transfection with the indicated siRNA, Let-7 or eGFP. Titres were determined 20hrs post 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, VSV$^{let\text{-}7mut}$ infected Hela cells produced titres of 9.5E7 ± 3.5E7pfu/mL while those infected with VSV$^{let\text{-}7wt}$ produced 5.6E7 ± 1.5E7 pfu/mL. (**b**) Cell survival of A549 cells after transfection with the indicated siRNAs 24hrs before infection with the indicated let-7 VSV at an MOI of 0.1. Cell survival was determined by MTS assay 24hrs after infection and is expressed as a percentage of survival compared to cells transfected with eGFP siRNA (mean + SD) (*p<0.05). (**c**) 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). 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.

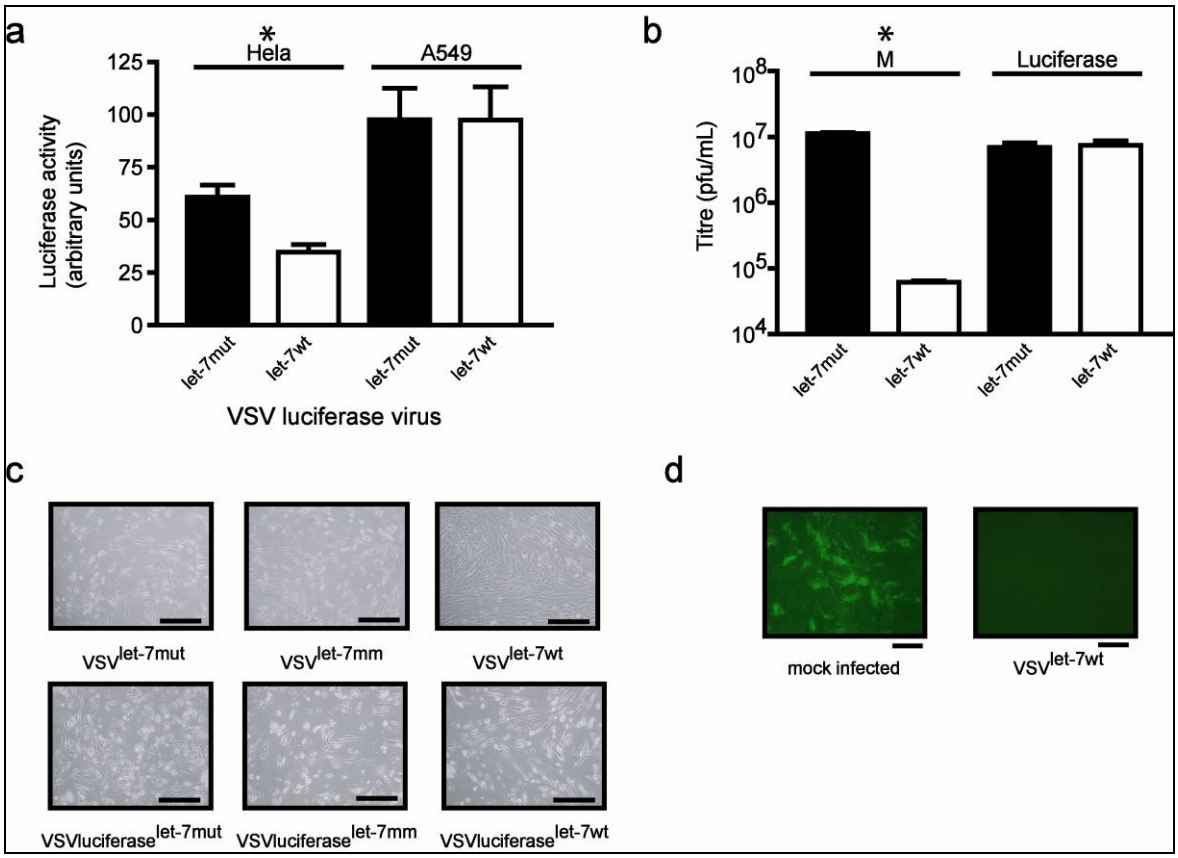

Figure 4: Let-7 target sites reduce target gene expression specifically and targetting VSV M strongly attenuates replication in normal human fibroblasts. (**a**) Luciferase expression of Hela and A549 infected at an MOI of 10 for 2 hours with VSV expressing an additional gene, luciferase, with identical let-7 target sites as depicted in Figure 1. 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) (**c**) Phase contrast pictures of infected GM38 cells 72hrs after infection at an MOI of 0.1, scale bar = 100µm. (**d**) GM38 cells mock infected or infected with VSV$^{let\text{-}7wt}$ 24hrs before infection with wt VSV expressing eGFP. After another 24hrs, fluorescence microscopy was used to visualize eGFP expression, scale bar = 100µm.

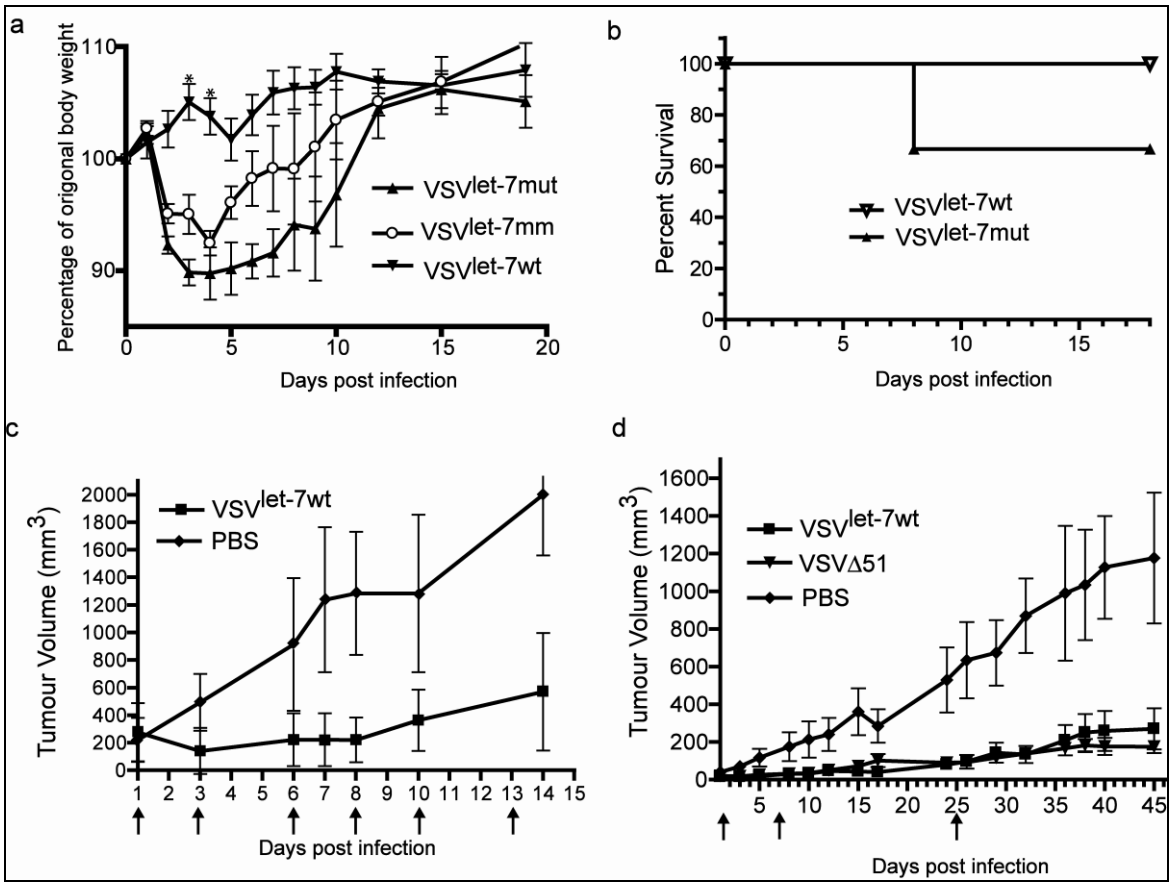

Figure 5: VSV$^{let\text{-}7wt}$ has reduced pathogenic activity while retaining antitumour activity in immune competent and immunodeficient mice. (**a**) Balb/c mice infected intranasally with 1x105pfu of the indicated virus. Body weights were expressed as a percentage of initial body weight before infection and averaged. (mean ± SD) n=5. (*p<0.005). (**b**) Survival of CD-1 nude mice infected with VSV$^{let\text{-}7mut}$ or VSV$^{let\text{-}7wt}$. Mice were infected at a dose of 7.5E7 pfu of the indicated virus intranasally (n=6). (**c**) Subcutaneous CT26 tumours were seeded with 3x10^5 cells 10 days before first treatment (day 1) in balb/c mice. Tumour volume was monitored during a treatment course of intravenously injected 1x10^8-1x10^9 pfu VSV$^{let\text{-}7wt}$ (n=8) or PBS (n=4) (mean + SD). At day 14, the difference between PBS treated and VSV$^{let\text{-}7wt}$ mice has a p value of less than 0.05. (**d**) Subcutaneous A549 tumours were seeded with 1E6 cells 12 days before first treatment (day 1) in CD-1 nude mice. Tumour volume was monitored during a treatment course of intravenously injected 1x10^9 pfu VSV$^{let\text{-}7wt}$ (n=5), VSV$^{let\text{-}7mut}$ (n=5), VSV Δ51 (n=5) or PBS (n=3) (mean ± SD). At day 45, the difference between PBS treated and VSV$^{let\text{-}7wt}$ treated mice has a p value of less than 0.05.