Enhancing the Safety and Efficiency of Retroviral Replicating Vectors for Cancer Gene Therapy

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular and Medical Pharmacology

by

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Molecular and Medical Pharmacology

University of California, Los Angeles, 2015

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An improved Retroviral Replicating Vector (RRV), Toca 511, encoding an optimized yeast cytosine deaminase prodrug activator gene, is currently under investigation for recurrent high-grade glioma in Phase I/II clinical trials. RRVs exhibit inherent tumor selectivity, due to their intrinsic inability to enter post-mitotic cells, and unlike replication defective vectors, are capable of efficient gene transfer to tumors, as the virus continues to spread through the tumor mass after initial infection\(^1\). However, tumor transduction may still be limited by cellular anti-viral immune factors. Moreover, concerns remain about the use of RRVs for gene therapy given the possibility that viral integration might lead to off-target effects, or genotoxicity, in non-cancerous cells. Therefore, we investigated mechanisms to improve both the safety and efficiency of RRV-mediated gene therapy.

Enhancing the tumor specificity of RRVs

We examined whether the incorporation of miRNA target sequences (miRTs) into the RRV genome could restrict viral replication in a tissue-specific manner. Accordingly, we developed
novel RRVs with one to four copies of the target sequence for human miR142-3p, a lymphohematopoietic-specific miRNA, inserted into the ψ packaging signal, the 3’ LTR and/or the transgene cassette of the RRV. We showed that incorporation of the miR142-3p target sequence (miRT142) significantly reduced viral spread to hematopoietic tissues, without inhibiting intratumoral replication or cytotoxic effect. However, the genomic location of the miRT affected viral replication kinetics, transgene stability, and miRT sequence integrity, in some cases. Therefore careful placement of miRTs is necessary to preserve efficient viral replication and genomic stability in non-restricted tissues, while maximizing suppression of RRV replication in lymphohematopoietic cells. MicroRNA-based restriction can effectively suppress RRV replication in a tissue-specific manner, and this approach may add an additional safeguard to gene therapy applications.

**Improving the efficiency of RRV-mediated gene transfer**

To explore the effect of cellular factors on RRV replication, we monitored RRV spread in a panel of primary human glioblastoma (glioma) cell lines, and quantified the expression, in these same cells, of various cellular factors that may play a role in RRV replication. We confirmed the finding, by another group, that tetherin (BST2, CD317) is strongly overexpressed in glioma cells, and identified an additional antiviral factor, protein kinase R, whose expression may also be dysregulated. We demonstrated that tetherin expression is correlated with slower RRV spread and reduced viral titers in the supernatants of infected cells, and suggest a possible antitumor effect of tetherin knockdown in glioma. Our results can be extended to future studies of the effect of tetherin on RRV replication or glioma tumor progression, or may be used to inform either the development of novel RRVs with enhanced infectivity, or the stratification of patients in clinical trials.
The dissertation of Nina Darrah Timberlake is approved.

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2015
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Chapter One


Chapter Two

Chapter two is adapted from Timberlake N, et al. Reduced systemic spread, without loss of tumor toxicity, of a retroviral replicating vector incorporating miR142-3p target sequences in the ψ and 3'LTR (manuscript in preparation).

Chapter Three

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**Publications and presentations**


Timberlake N, *et al.* Reduced systemic spread, without loss of tumor toxicity, of a retroviral replicating vector incorporating miR142-3p target sequences in the ψ and 3'LTR. *Manuscript in preparation.*


Introduction

The use of viruses to treat cancer ("oncolytic virotherapy") dates back to the middle of the 20th century. Inspired by case reports from the late 1800s and early 1900s, which detailed spontaneous cancer regressions following naturally acquired viral infections, physicians in the 1950s began experimenting with the use of viruses as cancer therapeutics. In one of the earliest trials, 33 patients suffering from Hodgkin’s disease were inoculated with sera from hepatitis patients. Of these, at least 13 developed hepatitis, and while some short-lived clinical improvements were observed, several patients also died during treatment. Subsequent trials in the 1950s-1970s employed pathogens such as West Nile virus, adenovirus, and mumps virus, with few positive outcomes, and many side effects, thus this approach was largely abandoned following the success of chemotherapy and radiation for the treatment of cancer. With the advent of recombinant DNA technology came renewed interest in oncolytic virotherapy; the viral genome could now be manipulated to reduce pathogenicity, improve tumor specific targeting, and even increase tumor cell killing by the addition of exogenous transgenes. Since then, over 2000 gene therapy clinical trials have been initiated, and the vast majority of these trials have targeted cancer.

To maximize safety, early gene therapy vectors were rendered replication-defective by removal of genes required for viral replication. While this did reduce the possibility of uncontrolled spread and off-target toxicity, the efficiency of gene transfer, and therefore the clinical efficacy, of non-replicating vectors was very low. In one of the largest cancer gene therapy clinical trials to date, a non-replicating retroviral vector, encoding the Herpes simplex virus thymidine kinase (TK) suicide gene, achieved <0.002% transduction of patient gliomas, and no survival benefit was observed compared to standard therapy. Even a non-replicating adenoviral vector, which was produced at 1000-fold higher titers, remained confined to the area immediately surrounding the
needle track\textsuperscript{6}. Therefore, in an effort to improve the efficiency of gene transfer, replicating viruses are now under investigation as cancer therapeutics.

The ideal oncolytic virus replicates selectively in, and efficiently destroys, tumor cells, while causing minimal damage to normal, non-cancerous tissue. Specificity for tumor cells can arise from the natural preference of the virus for dividing cells and an immune suppressed environment\textsuperscript{7}, or by vector engineering, which can include manipulation of viral receptor specificity\textsuperscript{8}, use of tissue specific promoters\textsuperscript{9}, selective removal, or mutation, of genes required for replication in non-tumor cells\textsuperscript{10}, and/or incorporation of tissue-specific microRNA target sequences\textsuperscript{11,12}. Often, alterations made to limit extratumoral spread of oncolytic viruses also reduce the overall fitness of the virus, and can allow for swifter viral clearance by the innate immune system, which though generally impaired in tumor cells, may still be sufficient to exert antiviral activity, thereby potentially limiting clinical efficacy\textsuperscript{13}. Balancing the demands of safety and efficacy is critical to the development of viral vectors for cancer gene therapy, and achieving highly efficient, and tumor-specific gene transfer remains one of the foremost obstacles to the success of this approach.

Here we discuss work aimed at improving the tumor-specificity and gene-transfer efficiency of a retroviral replicating vector (RRV) derived from murine leukemia virus (MLV). This vector, Toca511, is currently under investigation in Phase I/II clinical trials for the treatment of advanced stage glioma (https://clinicaltrials.gov/; NCT01156584, NCT01470794, NCT01985256). Toca511 is cytocidal for infected cells only after administration of the anti-fungal prodrug 5-fluorocytosine (5-FC), which is converted to the anticancer drug 5-fluorouracil (5-FU) in situ, by the sequence-optimized yeast cytosine deaminase (yCD2) prodrug activator transgene\textsuperscript{14,15}. High levels of tumor-specific gene transfer, and the anti-cancer efficacy of Toca511, and related RRVs, have been demonstrated in a number of rodent models\textsuperscript{16–18}. However, RRVs may still be subject to
restriction by anti-viral immune factors in human patients, the effects of which cannot be perfectly recapitulated under cell culture conditions or in animal models. Furthermore, concerns remain with the use of retroviral vectors for gene therapy, as retroviral integration has been shown to cause oncogenesis in rodents, non-human primates, and humans\textsuperscript{19–21}.

RRVs harbor multiple inherent safety mechanisms that reduce the likelihood of viral integration in non-cancerous cells: (1) RRVs do not contain a nuclear localization signal and therefore can only productively infect actively dividing cells, (2) retroviral replication is generally restricted by innate and adaptive immune mechanisms that are operative in normal tissues, but frequently mutated or suppressed in tumors, enabling tumor-selective viral replication, and (3) the suicide gene carried by RRVs allows for simultaneous killing of all infected cells by administration of a prodrug\textsuperscript{17,22}. Preliminary results from clinical trials of Toca511 indicate transient levels of viral replication in peripheral blood that is well controlled by the immune system\textsuperscript{23,24}. However, this does not preclude the possibility of undesirable, off-target spread of the virus in the future as clinical protocols change and novel vector developments emerge. For instance, we are currently investigating the use of pseudotyped RRVs expressing different envelope proteins that exhibit broader cellular tropism, as well as the use of RRVs in combination with radiation or temozolomide (TMZ)\textsuperscript{25,26}, which are strong immunosuppressants and may therefore prevent successful immune control of RRV replication in normal cells. Furthermore, a third clinical trial was recently initiated, employing intravenous (IV) injection of high doses of Toca511. Changes to the RRV configuration and/or route of administration may result in increased replication of the virus in non-target tissues, therefore the investigation of additional safeguards is desirable in order to further increase the safety of RRV-mediated gene therapy in all situations.

While minimizing extratumoral viral spread is an important consideration for the safety of RRV-mediated gene therapy, ensuring maximum intratumoral gene transfer is essential for achieving
clinical efficacy. Compared to other oncolytic vector systems, RRVs are relatively non-immunogenic\(^{27}\), and are therefore able to spread covertly through tumors without activating immune responses that can result in viral clearance. Furthermore, the viral genome integrates permanently into cellular DNA, allowing for stable and prolonged expression of viral proteins and encoded transgenes, as well as the formation of a viral reservoir, which allows for continued intratumoral production of virus after a single RRV injection\(^{15,18}\). Mutations within tumor cells tend to knock down immune function, and the natural environment of the brain is immune privileged, creating a seemingly ideal environment for viral replication. However, abnormal regulation of gene expression is a hallmark of cancer cells, and changes in the expression levels of viral receptors or anti-viral response genes may limit RRV replication in tumors\(^{15}\). For instance, the retroviral restriction factor, tetherin (aka Bst2, CD317), was found to be highly upregulated in tumors from patients with glioblastoma multiforme (glioma)\(^{28}\). The expression, in glioma cells, of other key factors involved in the progression or restriction of viral infection, and the effect of these factors specifically on RRV replication, has not been investigated.

As tumor specificity and efficiency of gene transfer are both critical components of a successful oncolytic virus, we investigated mechanisms to improve both aspects of RRVs. Here we explored the use of a microRNA (miRNA) “detargeting” strategy to specifically restrict the replication of RRVs in hematopoietic tissues. While this approach has shown promise in other vector systems, miRNA target sequences incorporated into RNA vectors are prone to mutation and deletion\(^{12,29–31}\). We therefore investigated whether insertion of the miRNA targets in various sites in the RRV genome, including in the highly conserved \(\Psi\) packing signal, could effect stable, more prolonged restriction of RRV replication. The results of these studies can be used to not only further the development of novel RRVs with reduced capacity for extratumoral replication, but also to inform the design of other vector systems incorporating miRNA target sequences. Finally, we examined the expression of various antiviral restriction factors, and viral
receptor proteins, in a panel of human glioma cell lines derived from patient tumors, and studied the replication kinetics of RRVs in these same cells. Identifying factors that promote or restrict RRV replication in glioma cells may inform the stratification of patients for clinical trials, or the development of novel RRVs with enhanced tumor transduction capabilities. With ongoing trials to test RRV-mediated gene therapy in human patients, improvements in RRV safety and efficiency, developed as a result of this research, are rapidly translatable to the clinic.
Chapter 1: MicroRNA 142-3p Attenuates Viral Spread of Murine Leukemia Virus-Based Retroviral Replicating Vectors in Hematopoietic-Lineage Derived Cells

Despite the demonstrated tumor selectivity of RRVs, off-target effects could potentially result from productive infection of healthy tissues. Here, we investigated whether incorporation of a hematopoietic lineage-specific microRNA target sequence in RRVs could further restrict replication in hematopoietic lineage-derived human cells in vitro, and in murine lymphoid tissues in vivo. Accordingly, one or four copies of the microRNA 142-3p target sequence (miRT142) were inserted into the 3'UTR of the RRV genome. In primary human peripheral blood mononuclear cells (PBMCs), vectors containing miRT142 showed a remarkable decrease in GFP expression relative to the parental vector, and viral spread was not observed over time. In hematopoietic lineage-derived human cell lines, spread of vectors containing miRT142 was strongly repressed in the early stages of infection, and four copies of miRT142 were more efficient than a single copy in achieving sustained repression of viral spread. In a syngeneic subcutaneous mouse tumor model, RRVs with and without miRT142 spread equally well in tumor cells and generated similar antiviral immune responses. In an immune-deficient mouse model, RRVs with miRT142 were strongly repressed in the blood, bone marrow and spleen 30 days post-intravenous vector administration, however by 60 days post administration, the viruses had escaped restriction. In summary, microRNA-based detargeting can selectively attenuate retroviral replication in a tissue-specific manner, and potentially provides an additional safeguard to this delivery platform for gene therapy applications.
Introduction

We are developing a retroviral replicating vector (RRV) encoding cytosine deaminase (CD) as an anticancer agent for the treatment of recurrent glioma\textsuperscript{15}. Despite its demonstrated natural selectivity for tumors, and other safety features, such a virus could potentially cause off-target effects by productively infecting healthy tissues. Here, we investigated whether incorporation of a hematopoietic lineage-specific microRNA target sequence in the RRV genome could further restrict replication in hematopoietic lineage-derived human cells \textit{in vitro} and in murine lymphoid tissues \textit{in vivo}.

MicroRNAs (miRNAs) are small, non-coding RNAs involved in biological processes including developmental timing, cell proliferation, apoptosis, migration, cell differentiation, and angiogenesis\textsuperscript{32}. MiRNAs are transcribed by RNA polymerase II to generate precursor polynucleotides called primary precursor miRNAs (pri-miRNAs). In the nucleus, pri-miRNAs are processed by the ribonuclease Drosha to produce the miRNA precursor (pre-miRNA). Subsequently, pre-miRNAs are transported to the cytoplasm via Exportin 5 and further processed by the ribonuclease Dicer to generate an active, mature miRNA that is around 20-24 nucleotides in length. The guide strand of the mature miRNA is then incorporated into an RNA-induced silencing complex (RISC) that binds to complementary target sequences primarily found in the 3’ UTR of messenger RNAs (mRNAs). The formation of the RNA duplex results in decreased expression of the target gene, either by repression of protein translation, or by degradation or destabilization of the mRNA\textsuperscript{33,34}.

The incorporation of a miRNA target sequence (miRT), in transcription-based vectors, has been utilized as a way to control undesirable expression of transduced genes in non-target cells or tissues. Brown \textit{et al}. first demonstrated that incorporation of the miRNA142-3p target sequence into a conventional, replication-defective lentiviral vector, abrogated transgene expression in
hematopoietic lineage-derived cells, thereby suppressing immune responses against the transgene product and allowing specific and stable transgene expression in the liver\textsuperscript{11,29}. Subsequently, a number of other groups demonstrated that incorporation of target sequences of tissue- or cell-enriched miRNAs into the viral genome can at least partially restrict off-target spread of replicating oncolytic viruses in a specific tissue or cell type\textsuperscript{30,31,35,36}. The application of this approach to RRVs presents a unique challenge, as the reverse transcription step provides an opportunity for mutational escape of the target sequence from the matching miRNA. However, given the potential for clinical use of RRVs, it is of interest to investigate the feasibility of this approach for restricting the replication of RRVs in a tissue-specific manner.

We designed RRVs containing one or four copies of the hematopoietic-specific miRNA142-3p target sequence (miRT142) in the 3'UTR of the viral genome, and examined the viral spread, gene expression and genomic stability of these RRVs in lymphoid and non-lymphoid cells and tissues. Our data showed that this miRNA-based approach could selectively repress RRV replication in human peripheral blood mononuclear cells (PBMCs), in human hematopoietic-lineage derived cell lines, and in murine hematopoietic tissues, \textit{in vivo}. Our results demonstrated that this miRNA-based “detargeting” strategy potentially offers an additional safeguard for the RRV delivery platform for gene therapy applications. However, optimization may be required for long-term restriction of RRV replication in these tissues, as mutant viruses that were resistant to miRNA-mediated restriction did eventually emerge.
Materials and methods

Plasmid construction. RRV-GFP, aka pAC3-emd or T5.0006, is an MLV-based RRV in which the yCD2 gene downstream of the IRES in pAC3-yCD2 vector\textsuperscript{15} has been replaced with an emerald GFP gene. Single or four tandem repeats of miRNA142-3p target sequence, completely complementary to the sequence of miR142-3p, were synthesized with an endonuclease restriction site NotI at both ends, and cloned into the corresponding NotI site downstream of the IRES-GFP region in 3'UTR of the vector. The sequence of the single 142-3pT: gcggccgcGTCGACTCCATAAAGTAGGAACACTACAgcggccgc and the sequence of the four tandem repeats of 142-3pT henceforth 142-3pT4X: gcggccgcGTCGACTCCATAAAGTAGGAACACTACAacccgtTCCATAAATGGAAAAACATACAGcggccgc were synthesized by BioBasic Inc. The underlined sequences represent sequences complementary to miR142-3p. The sequence of the synthesized DNA fragments was confirmed before and after cloning into the RRV-GFP vector using the primers: 5'-CTGATCTTACTCTTTGGACCTTG-3', and 5'-CCCCTTTTTCTGGAGACTAAATAA-3'.

Cell Culture. 293T cells were obtained through a materials transfer agreement with the Indiana University Vector Production Facility and Stanford University deposited with ATCC (SD-3515; Lot# 2634366). Human astrocytoma cells U87-MG (ATCC, HTB-14), human prostate adenocarcinoma cells PC-3 (ATCC, CRL-1435), human lymphoblastic leukemia cells CEM (ATCC, CCL-119), and human histiocytic myeloid lymphoma cells U937 (ATCC, CRL-1593.2) were obtained from ATCC. CEM-T4, which is a sub-clone of CEM was obtained from the NIH AIDS Research & Reference Reagent Program (cat# 117). 293T, U87-MG, PC-3 cells were cultured in complete DMEM medium containing 10% FBS (HyClone), sodium pyruvate, glutaMAX (Invitrogen), and antibiotics (penicillin 100 IU/mL, streptomycin 100 IU/mL). CEM, CEM-T4, and U937 cells were cultured in complete RPMI medium containing 10% FBS, 1X glutaMAX and 100 IU/mL penicillin-streptomycin.
**MicroRNA142-3p expression assay.** miRNA-enriched RNA was extracted from PBMCs, U87-MG, 293T, CEM, CEM-T4, U937 and PC-3 cells with the mirVana miRNA isolation kit followed by DNase I treatment (Ambion P/N1560 and P/N1906) according to the manufacturers’ protocols. TaqMan microRNA Reverse Transcription Kit (ABI, P/N 4366596) was used with RT primers for miR142-3p (assay ID# 000464), and for RNU6 (assay ID# 001093) as an endogenous control to produce cDNA. Reverse transcription and quantitative PCR reactions (qRT-PCR) were carried out according to manufacturers’ protocols. $2^{-\Delta Ct}$ was calculated to obtain miR142-3p expression relative to RNU6B in each sample. In U87 cells, the Ct value for miR142-3p was at the lower limit of detection (Ct values between 38-40). When performing calculations of relative expression by $2^{-\Delta\Delta Ct}$, the value of $2^{-\Delta\Delta Ct}$ in U87 was assumed to be one.

**Virus production.** Virus stock was produced by transient transfection of 293T cells using the calcium phosphate precipitation method. Briefly, cells were seeded at $2 \times 10^6$ cells per 10-cm petri dish the day before transfection. Cells were transfected with 20 µg of RRV-GFP, RRV-GFP-142-3pT or RRV-GFP-142-3pT4X plasmid the next day. Eighteen hours post-transfection, cells were washed twice with PBS and incubated with fresh complete culture medium. Viral supernatant was collected approximately 42 hours post transfection and filtered through a 0.45 µm syringe filter unit. Viral supernatants were stored in aliquots at -80°C. Viral infection to determine titers was performed by adding viral stock at 1:50 dilution in 1mL total volume to each well. AZT (Sigma) at 40 µm was added 24 hours post infection to prevent further viral replication and cells were harvested 48 hours post infection for genomic DNA isolation. Viral titer was then determined by quantitative real time PCR (qPCR) using the following primer set and probe which will bind to proviral DNA derived from the vectors as well as proviral DNA containing deleted IRES-GFP region. 5-MLV-U3-B: 5’-AGCCCACACCCCC TCACTC-3’, 3-MLV3Psi: 5’-TCTCCCGATCCCCGACGA-3’, and probe: 5’-FAM-CCCCAAATG AAAGACCCCCGCTGACG-BHQ-3’. Reactions were performed in a total volume of 20 µL containing 2X iQ SuperMix
(BioRad); 0.3 μM of each primer and 0.1 μM of the probe. PCR reactions were performed in triplicates, using a CFX-96 (BioRad) thermalcycler with the following parameters: 95°C 5 min; and 40 cycles of 95°C 15s; 60°C 1 min. Viral titers reported in transducing units per milliliter (TU/mL) were determined by calculation of Ct values derived from a standard curve ranging from 1×10^1 copies to 1×10^5 copies of plasmid DNA and from known amount of genomic DNA input, number of cells, and dilution factor of viral stock per reaction used in each reaction.

**Viral replication monitored by GFP expression.** To monitor viral replication in U87-MG cells, 1×10^5 cells were infected with RRV-GFP, RRV-GFP-142-3pT, or RRV-GFP-142-3p4X at an MOI of 0.01. For U937, CEM, and CEM-T4 cells, 1×10^5 cells were plated in 24-well plates followed by infection with the vectors at an MOI of 2. Every 3-4 days, a portion of the cells was passaged with fresh culture medium for continued monitoring of viral replication, and a portion of the cells was harvested for flow cytometric analysis of GFP expression at the time. Cells harvested for flow cytometric analysis were washed with PBS and centrifuged at 1000 rpm for 5 minutes. Cell pellets were resuspended in PBS containing 1% PFA. The percentage of GFP positive cells was measured on a Canton II using the FL1 channel (BD Biosciences). Viral replication kinetic analyses were obtained by plotting % GFP positive cells over time.

**Vector stability assay and amplification of IRES-GFP region.** U87-MG cells at 5x10^4 cells per well in 6-well plate were infected with stock virus at an MOI of 0.1. At day 4 post-infection viral supernatant from ~70% infected cells was collected and filtered through a 0.45 μm filter unit. A 1:10 dilution of the viral supernatant was then used to infect fresh U87-MG cells seeded the night before. Four-day infected U87-MG cells were harvested for genomic DNA isolation for IRES-GFP PCR. This virus infection cycle was repeated at least 12 times. Genomic DNA extraction was carried out using the Maxwell 16 DNA purification kit (Promega). DNA concentration and quality were determined by spectrophotometer using a Nanodrop 1000 (Thermo Scientific). To assess the integrity of the IRES-GFP region in proviral DNA, standard PCR was performed using the following primer set. UCLA5-127: 5’-CTGATCTTACTCTTTT
GGACCTTG-3’, and UCLA3-37: 5’-CCCCTTTTCTGGAGACTAAATAA-3’. The reaction was performed in a total volume of 25 µL containing 0.4 µM of each primer, 0.4 mM dNTP and 2.5 unit of SuperTaq (Ambion), and the PCR reaction was performed with the following parameters: 95°C 2 min; and 40 cycles of 95°C 15s; 60°C, 30s; 72°C, 1min, followed by 72°C, 5 min. One fifth of the PCR reaction was loaded on a 1% agarose gel to resolve PCR products. The expected PCR product of an intact IRES-GFP region is ~1.4 kb. PCR products less than 1.4 kb indicate partial or complete deletion in the IRES-GFP region. For experiments in which the PCR products were excised from the gel for TA cloning, a gel extraction kit (Qiagen) was used to obtain the PCR product.

**Vector copy number of proviral DNA.** Genomic DNA extraction from cultured cells, whole blood, or tissues was carried out using the Maxwell 16 DNA purification kit (Promega). DNA concentration and quality were determined by spectrophotometer using Nanodrop 1000/8000 (Thermo Scientific). Quantitative real time PCR, using the same primer set and probe and PCR parameters as described above, was performed to determine the average vector copy number per cell or average vector copy number per µg of genomic DNA derived from tissues. The average vector copy number per cell was calculated by dividing the number of transduction events per reaction (derived from the Ct values according to a standard curve ranging from 1x10^5 copies to 1x10^1 copies of plasmid DNA) by the number of equivalent diploid genomes (derived from the amount of genomic DNA input per reaction and assuming 150,000 diploid genomes/µg).

**Relative expression of cellular viral RNA by qRT-PCR.** RNA was extracted from cells using the RNeasy Kit (Qiagen). Reverse transcription was carried out with 100 ng total RNA using the High Capacity cDNA Reverse Transcription Kit (ABI). Quantitative PCR analysis was performed to measure the mRNA expression level of unspliced and spliced cellular viral RNA with the following parameters: 95°C 10 min; 95°C 10s; 70°C 10s; 95°C 10s; 69°C 10s; 95°C 10s; 68°C 10s; 95°C 10s; 67°C 10s; 95°C 10s; 66°C 10s; and 34 cycles of 95°C 15s; 65°C 30s. The Pol2
primer set and probe: 5’Pol2: 5’- CAAGGGGCTACTGGAGGAAAG -3’, 3’Pol2: 5’-
CAGTCTGGTACATGGAGGAAAG -3, probe: 5’HEX-TATCGCTGGACCACGGATCGCA-
3’BHQ. The Env2 primer set and probe: 5’Env2: 5’-ACCCTCAACCTCCCCTACAAGT-3’, 3’Env2:
5’-GT TAAGCGCCTGATAGGCTC-3’, probe: 5’FAM-AGCCACCCCAAGA CTGGAGA TAGA-
3’BHQ. The average integrated vector copy number was used to determine a normalization
factor (ratio of parental vector copy number to miRT-RRV vector copy #) at each time point. The
normalized relative expression of cellular viral RNA was determined by multiplying the
normalization factor by the 2^−DD(Ct) value.

**Preparation of PBMCs and RRV infection.** Human T-lymphocytes from healthy human donors
were purified by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). Cells
were maintained in RPMI 1640 medium supplemented with 10% FBS and 100 IU/mL penicillin-
streptomycin, and T-lymphocytes were activated by 48-hour incubation with 1µg/mL anti-human
CD3 (eBioscience, clone OKT3, #16-0037-81). Activated T-lymphocytes were resuspended in
RPMI 1640 medium supplemented with 10% FBS, 100 IU/mL penicillin-streptomycin, and 100
U/mL recombinant human IL-2 (R&D systems) and plated at 5 x10^6 cells/mL on tissue culture
plates coated with 25 µg/mL Retronectin (Takara). Retrovirus supernatant from frozen stocks
was added to each well at an MOI of 4. Plates were spun at 860xg for 2 hours at room
temperature, and then incubated overnight at 37ºC. After 24 hours, cells were harvested,
resuspended, and transduced again, as above. Cells were analyzed by flow cytometry 3, 6, and
10 days after initial transduction. Cells were harvested, washed and resuspended at 5 x10^6
cells/mL in 200 µL Stain Buffer with BSA (BD Pharmingen). Aliquots of 100 µL were incubated
with 5 µL of either PE-conjugated mouse anti-human CD3 (BD Pharmigen, #555340) or an
isotype control antibody (BD Pharmingen, 555574) for 20 minutes on ice. Cells were washed
twice and resuspended in 500 µL Stain Buffer and then analyzed by flow cytometry for GFP
expression with gating on the CD3^+ T-lymphocyte population.
Titration of infectious viral particle from infected hematopoietic-lineage derived cell lines. Infected hematopoietic-lineage derived cells at various indicated time points were seeded at 1x10^6 in 5 mL of culture medium 48h prior to collection of viral supernatant. Viral titers were determined by qPCR using the same primer set and probe and PCR parameters, as described above.

RRV infection in vivo and biodistribution analysis. All animal studies were conducted under protocols approved by the UCLA Animal Research Committee. Female, athymic nude mice (HSD: Athymic Nude-Foxn1nu) were purchased from Harlan Laboratories (Indianapolis, IN) and housed in microisolator cages under specific pathogen-free conditions. At six-weeks of age, mice were injected, via the tail vein, with 4 x 10^5 TU of RRV in 200 µL total volume. Control mice were injected with 200 µL of PBS. Eleven experimental mice per group, and five control mice, were used for each time point. Mice were sacrificed on day 15 and day 30 after RRV injection. Upon sacrifice, peripheral blood was collected by cardiac puncture, the spleen was harvested, and bone marrow cells were flushed, with PBS, from the femurs and tibias of both hind legs. Serum was isolated from half of the collected peripheral blood. All samples were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

For biodistribution analysis, qPCR was performed to determine the range of infectivity in vector copy number per microgram of tissue derived genomic DNA. Ct values >38 were scored as non-detectable, and copy numbers less than 250 copies/µg were scored as below the lower limit of quantification (LLOQ). T-test (Mann-Whitney) was performed for statistical analysis using Graph Pad Prism. In the analysis, values from samples scored as LLOQ were included in the calculation. Value for statistically significant difference was set at < 0.05.

Anti-MLV ELISA. 96-well assay microtiter plates were coated with 100 µL of diluted (1:160) Toca 511 stock of ≥ 2E8 TU/mL at 4°C overnight. The next day, the coated plates were blocked with blocking buffer (PBS, 1% BSA, 0.5% Tween 20) for 1 hour at room temperature followed by a 2 hour incubation, at room temperature, with 100 µL diluted serum from mice bearing RRV-
infected tumors. Serum samples from experimental groups were serially diluted to 1:100, 1:200 and 1:400, whereas the positive control (serum from a mouse immunized with Toca 511) was serially diluted in a range from 1:100 to 1:12,800. A negative control serum was diluted to 1:100 and eight replicates were added to the plate. After serum incubation, unbound antibodies were removed by washing (PBS, 0.5% Tween 20) followed by a 2-hour incubation with HRP-conjugated goat anti-mouse IgG antibody (1:2000) at room temperature. Chromogenic TMB and subsequently stop solution were added to obtain a colorimetric reading at the absorbance of 450 nm. In this assay, a positive serum control was serially diluted to generate a reference curve to assess assay performance. Test samples were determined as being anti-MLV positive or negative by comparing the mean OD values to a given threshold value (Mean value of OD of the negative control + 2 × standard deviation value).

Detection of viral protein. At the end of infection, cells were seeded at $1 \times 10^6$ in 5 mL of culture. At 48 h post-seeding, cells were harvested and lysed for immunoblotting. Goat anti-human GAPDH (Abcam cat# ab9483) was used to detect the expression of GAPDH. Rat anti-gp70 (83A25) antibody was used to detect expression of viral envelope glycoprotein. Rat anti-gag (R187) was used to detect the expression of viral capsid protein followed by secondary antibodies conjugated with HRP. Protein expression was visualized using Immun-Star WesternC kit (Bio-Rad, cat# 170-5-70).
Results

**MiRNA142-3p expression in hematopoietic-lineage cells.** Expression levels of miR142-3p (miR142) are enriched in hematopoietic-lineage cells compared to some monocytic and lymphoblastic cell lines\(^{37,38}\). We confirmed this result in primary resting and activated PBMCs from five healthy individuals, and three established human cell lines (CEM, CEM-T4, and U937) of hematopoietic origin, and compared these to non-hematopoietic cell lines (U87-MG glioma, and PC-3 prostate cancer, Figure 1-1a). The expression of miRNA142 in both U87-MG (U87) and PC-3 cells was at the lower limit of detection (C\(\text{t}\) values between 38-40) by qRT-PCR. The miR142 expression in primary PBMCs did not vary appreciably among individuals, or between cells in the resting or activated state. In lymphoid derived cell lines (CEM, CEM-T4 and U937) miR142 expression levels were comparable to those in PBMCs. Thus, miR142 expression is enriched in hematopoietic-lineage cells, and activation state appears to have little effect on miR142 expression level in primary mononuclear cells.

**RRV-GFP vectors containing miRT142 replicate efficiently in U87 cells.** RRV-GFP, in which the CD gene of RRV-yCD2 was replaced by an emerald GFP gene\(^{14}\), generates infectious vector, is less stable than the clinical product candidate, Toca 511, in serial passage experiments, but is as stable as previous CD encoding RRVs with therapeutic activity in rodent tumor models\(^{39}\). We constructed RRV-GFP vectors containing either a single copy (RRV-GFP-142-3pT) or four tandem repeats (RRV-GFP-142-3pT4X) of miRT142. The target sequences were inserted into the 3’-UTR, downstream of the IRES-GFP region (Figure 1-1b-d). All three vectors generated similar transient transfection titers, in the range of 1\(\times\)10\(^6\) – 2\(\times\)10\(^6\) TU/mL on PC-3 prostate cancer cells (Figure 1-2a) and the replication kinetics and transgene expression levels in U87 cells were also comparable for all three vectors (Figure 1-2b,c). As expected, neither repression of GFP expression, nor restriction of viral replication was observed in U87
cells infected with vectors containing miRT142, as miR142 expression was at the lower limit of detection in these cells.

**RRV-GFP vectors containing miRT142 are stable through multiple rounds of infection in U87 cells.** We examined the stability of these vectors over serial infection cycles, by transferring diluted viral supernatant from fully infected U87 cells to naive U87 cells for 12 cycles, harvesting the genomic DNA after each infection cycle, and amplifying a 1.4 kb PCR product to assess the integrity of the proviral genome (Figure 1-2d), as previously described. PCR products <1.4 kb represent viruses with deletions in the IRES-GFP region. The RRV-GFP-142-3pT and RRV-GFP-142-3pT4X vectors showed complete stability up to infection cycle six, similar to the parental vector. After infection cycle six, emergence of deletion mutants, indicated by PCR products <1.4 kb, was observed in all three vectors, however, the 1.4 kb band carrying the intact IRES-GFP region could still be detected up to infection cycle twelve, consistent with previous data. Thus, our results demonstrated that the RRV-GFP vector could tolerate insertion of miR142 target sequences in the 3’ UTR without significant impairment of viral replication or vector stability.

**Transgene expression from vectors containing miRT142 is repressed in PBMCs.** Activated PBMCs from one of the five donors were infected with RRV-GFP, RRV-GFP-142-3pT and RRV-142-3pT4X vectors, respectively, at a multiplicity of infection (MOI) of 4. Due to the short-term viability of PBMCs in culture and their low infectivity by amphotropic MLV, in vitro viral replication kinetics, monitored by GFP expression, could only be assessed up to day 10 post-infection. At day 3 post-infection, there was little difference in the percentage of GFP-positive cells among the 3 vectors. Subsequently, however, the parental vector continued to spread, whereas the number of GFP positive cells, among those exposed to RRV-GFP-142p3pT or RRV-GFP-142-3pT4X, remained static, or decreased slightly over time. By day 10 post-
infection, a significant difference in GFP-positive cells was observed between the three vectors (Figure 1-3a). Furthermore, despite relatively small differences in viral spread at day 3, there was a remarkable difference in the GFP expression level, as indicated by mean fluorescence intensity (MFI), between the parental vector and the miRT142-restricted vectors (Figure 1-3b). Notably, the vector containing four copies of miRT142 appeared to be more effective in repressing GFP expression than the vector containing only a single copy. Confirming the validity of our flow cytometry results, the IRES-GFP transgene region of the virus was stable in the genomic DNA of the PBMCs for the duration of the experiment (Figure 1-3c).

**Repression of viral spread in PBMCs is mediated by selective reduction of viral mRNA.**

We compared the levels of cellular viral transcript from PBMCs infected with parental or miRT142-restricted vectors at all time points by qRT-PCR, using both the env2 and GFP primer sets (Figure 1-3d). Cellular viral RNA levels were first normalized to GAPDH and subsequently further normalized to the average proviral copy number per cell. The normalization to the proviral copy number is based on the assumption that, in the absence of RNA interference, each copy of integrated proviral DNA should generate the same amount of viral RNA in the same host cell population. When using the pol2 and env2 primer sets for measurement of cellular viral RNA levels, reductions in normalized cellular viral RNA were observed at all time points for both miRT-restricted vectors, as compared to the parental vector (Figure 1-3e), with day 10 levels appearing, qualitatively, to be the most markedly suppressed (around 25% of control or less). Therefore, our data showed selective repression of transcripts from RRV-GFP-142-3pT and RRV-GFP-142-3pT4X, consistent with the proposed RNAi mechanism of action.

To further examine the stability of the vectors containing miRT142, we analyzed the target sequences to determine whether mutations had occurred during the course of infection. We therefore isolated and cloned IRES-GFP PCR products from genomic DNA of PBMCs, 10 days post-infection with RRV-GFP-142-3pT or RRV-GFP-142-3pT4X. Sequencing data revealed A-
to-G mutations in 2 of 10 clones from cells infected with RRV-GFP-142-3pT. Similarly, C-to-T and G-to-A mutations were found in 1 of 10 clones from cells infected with RRV-GFP-142-3pT4X (Figure 1-4a, b). In summary, our results indicated that the spread of RRV incorporating miRT142 could be restricted in cultured PBMCs by selective depletion of viral RNA, and without significant mutation to the miRNA target sequences.

**Transgene expression from vectors containing miRT142 is repressed in hematopoietic-lineage derived cell lines.** To investigate whether incorporation of miRT142 results in effective restriction of RRV replication in hematopoietic-lineage cells, over a longer period of time than could be examined in primary human PBMCs, we examined established cell lines of myeloid (U937) and lymphoid origin (CEM). Cells were infected with RRV-GFP, RRV-GFP-142-3pT, or RRV-GFP-142-3pT4X, at an MOI of 2, and viral spread was monitored by flow cytometry. Both cell lines supported replication of the parental vector, RRV-GFP, (Figure 1-5a, 1-6a). Low levels of GFP expression were observed in U937 cells infected with RRV-GFP-142pT during the entire course of infection, whereas GFP expression was fully repressed in cells infected with RRV-GFP-142-3pT4X (Figure 1-5a).

There was evidence of deletion of the IRES-GFP region, which presented at about the same time in both RRV-GFP and RRV-GFP-142-3pT, but became almost complete for RRV-GFP-142-3pT, while the majority of the parental vector population retained the full-length transgene. In contrast, the RRV-GFP-142-3pT4X vector remained stable throughout the entire course of infection (Figure 1-5b) and the presence of the intact, full-length 1.4 kb product, a low abundance product arising from the small percentage of cells transduced by the initial viral inoculum, is consistent with suppression of replication both at the proviral DNA level (vector copy number, Figure 1-5c), and at the RNA level (Figure 1-5d), after initial infection.

Quantification of cellular viral RNA levels, by qRT-PCR, revealed effective repression of RRV-GFP-142-3pT replication up to 21 days post-infection, after which emergence of deletion
mutants was observed, and proviral and viral RNA levels increased. In contrast, no escape mutants were observed in cells infected with RRV-GFP-142-3pT4X, and proviral and viral RNA levels showed sustained suppression through day 28. This reduction in cellular viral RNA expression correlated with the titers of infectious virus produced in infected cells, with no detectable titer from U937 cells infected with RRV-GFP-142-3pT4X (Figure 1-5e). Furthermore, sustained repression of viral capsid and envelope gene expression was observed in U937 cells infected with RRV-GFP-142-3pT4X vector (Figure 1-5f).

In CEM cells, deletion of the IRES-GFP region in cells infected with the parental vector was observed in the early stages of infection. In this case, the observed lack of complete infection, as monitored by GFP expression, is partly due to the emergence of deletion mutations (Figure 1-6b). However, CEM cells infected with RRV-GFP-142-3pT4X showed prolonged repression of viral replication without emergence of deletion mutants, a reduction in cellular viral RNA levels, and undetectable viral titers in the supernatant, consistent with our results in U937 cells (Figure 1-6a-e).

Collectively, data from hematopoietic-lineage cell lines suggested that the lack of GFP expression in cells infected with vectors containing miRT142 was mediated by miR142 regulation, at least during the early time points of infection, and that incorporation of four copies of miRT142 resulted in more efficient and durable reduction of viral spread and transgene expression.

**Vectors containing miRT142 spread efficiently in tumors of immune-competent mice.** We demonstrated that RRVs incorporating miRT142 can efficiently spread in tumor cells *in vitro*, and can effectively suppress viral spread in primary human PBMCs and hematopoietic-lineage cell lines. Since the mature miR142-3p sequence is identical in humans and mice, the replication of vectors containing miRT142 was further evaluated *in vivo* by monitoring the biodistribution of the vectors in immune-competent, tumor-bearing mice. Tu-2449 mouse glioma
cells containing 0.01% cells fully transduced with RRV-GFP, RRV-GFP-142-3pT or RRV-GFP-142-3pT4X were implanted subcutaneously to the right flank of B6C3F1, immune-competent mice. Genomic DNA from tumor, whole blood, bone marrow and spleen was harvested 20 days post-tumor engraftment, and qPCR was performed to determine the vector copy number per microgram of tissue-derived genomic DNA. In addition, sera were also collected to measure the anti-MLV immune response by ELISA, as suppression of expression of xenoantigens in PBMCs has been associated with a reduced immune response to these antigens\textsuperscript{29}. Our data showed that the tumor growth rate among the control and experimental groups was comparable (Figure 1-7a) and that viral spread was almost completely restricted to tumors for all three vectors (Table 1-1). In this experiment, RRV-GFP-142-3pT4X appeared to spread slightly more efficiently than the parental vector (Figure 1-7b). There was no significant difference in ELISA detected immune responses among the experimental groups bearing RRV-infected tumors (Figure 1-7c). Together, these data suggested that incorporation of the miRT142 into the RRV did not appreciably affect viral replication in tumor cells, or the host anti-MLV immune response.

**Vectors containing miRT142 are repressed in lymphoid tissues of immune-deficient nude mice.** In the experiments described above, the spread of vectors containing miRT142 to lymphoid tissues was not robust enough to demonstrate suppression of viral spread. Therefore, we evaluated the efficacy of miRNA-mediated viral suppression in lymphoid tissues *in vivo* using immune-deficient nude mice, to allow more robust systemic spread of the virus. Mice were injected, via the tail vein, with 4E5 transducing units (TU) of RRV-GFP, RRV-GFP-142-3pT or RRV-GFP-142-3pT4X. Genomic DNA from whole blood, bone marrow and spleen was harvested 15 or 30 days post-IV administration of RRV, and qPCR was performed to determine the vector copy number per microgram of tissue. At day 15 post-infection, virus was detectable in the blood and bone marrow, but not in the spleen, in mice infected with the parental vector. In contrast, viral spread was mostly below the lower limit of quantification or undetectable in
tissues from mice infected with either RRV-GFP-142-3pT or RRV-GFP-142-3pT4X (Table 1-2). By day 30 post-infection, the RRV was detectable in all tissues in each of the groups. However, mice infected with RRV-GFP-142-3pT or RRV-GFP-142-3pT4X continued to show marked repression of viral spread in all tissues compared to the parental vector (Figure 1-8a-c, Table 1-2). However, by day 60 post-infection, there was no longer an observable difference in vector copy number, between the parental and targeted vectors, for any of the tissues tested, suggesting that the RRVs containing miRT142 had mutated to escape restriction. Intriguingly, the vector copy number of the parental vector appeared to be higher in the bone marrow than in the blood and spleen at all time points tested. Therefore, we investigated whether bone marrow lineage negative stem and progenitor cells (lin-) could be infected by RRV, and if so, whether incorporation of miRT142 could restrict viral spread in this particular cell population. We waited 30 days following IV administration of RRV and harvested BM samples from 3 sets of 3 randomly pooled mice in each group in order to obtain sufficient numbers of lin- cells for measuring GFP expression levels and vector copy number. RRV-GFP does indeed infect lin- cells in immune-deficient nude mice, however, vectors incorporating miRT142 were effectively suppressed in this population, as analyzed either by GFP expression or vector copy number (Figure 1-8d, e) Similar results were obtained in the lin+ cell population (Table 1-3). All together, our results indicated that the spread of RRV incorporating miRT142 could be temporarily restricted in lymphoid tissues in vivo, and that the restriction is more effective when multiple miRT142 sequences are present in the vector. This in vivo effect is presumably by the same mechanisms observed in primary human PBMCs and hematopoietic-lineage-derived cell lines.
Discussion

The results presented above represent the first use of miRNA-mediated restriction to modulate the tissue-specific spread of RRVs. Restriction of off-target infection by RRV has been explored through the use of various other strategies for tissue-specific targeting\textsuperscript{40,42–44}. However, lymphohematopoietic cells were not specifically detargeted in these studies, and as these are natural host cells for MLV, restriction of RRV replication in lymphohematopoietic cells has the potential to greatly improve the safety of MLV-based replicating vectors, and RRV-mediated gene therapy. In this study, we examined whether tissue-specific repression of viral replication could be achieved by the interaction between endogenous miR142-3p and artificial miR142-3p target sequence in the 3'UTR of the vector.

We demonstrated that RRVs can tolerate the insertion of miRT142, and that vectors incorporating miRT142 are able to replicate efficiently and remain stable over multiple rounds of viral infection in U87 glioma cells. Furthermore, the vectors incorporating miRT142 replicated as efficiently as the parental vector in tumors \textit{in vivo}. The tolerability of sequence insertion within the 3'UTR in our study is consistent with data previously reported\textsuperscript{39}. After the initial infection event, spread of RRVs incorporating miRT142 was effectively repressed in PBMCs, and in U937 and CEM cell lines over the course of longer experiments. Although the use of GFP expression as one of the readouts for repression of viral spread was complicated by the emergence of deletion mutants in the cell lines, the repression of viral replication was confirmed by additional methods including assessment of vector stability, measurement of the relative cellular viral RNA levels, and detection of viral proteins.

In the syngeneic tumor model, both targeted and untargeted vectors exhibited similar intratumoral viral spread, tumor growth rate, and host anti-MLV immune response, in addition to an absence of viral spread to lymphoid tissues. These results suggest that active viral
replication in hematopoietic lineage cells is not required for efficient infection of tumor cells \textit{in vivo}. In addition, the absence of viral spread in lymphoid tissue for all vectors, shows that lack of infection of lymphoid cells does delay the onset of the anti-MLV immune response. The opposite effect (inhibition of anti-xenoantigen immune responses) has been reported for non-replicating lentiviral vectors incorporating miR142-3p target sequences\textsuperscript{11,29,45}. The difference may be due to incomplete inhibition of viral gene expression in lymphoid tissue with the RRV, or simply to differences in routes of immune stimulation.

The relative cellular viral RNA expression, in cell lines and PBMCs infected with miRT-RRVs, was significantly reduced compared to cells infected with the parental vector. However, when the relative cellular viral RNA expression levels were normalized to average vector copy number per cell, we did not observe 100\% RNA degradation even in the early time period following initial infection, an effect that was most pronounced in PBMCs. Strong repression of GFP expression despite only moderate reductions to viral RNA levels observed in PBMCs suggests that there may be additional mechanisms involved besides RNA degradation in the miRNA-mediated restriction of RRVs. Possible additional mechanisms are translational inhibition of GFP protein synthesis or diversion of RNA to viral particle assembly and away from the RNAi cellular compartment. We speculate that both mechanisms can occur concomitantly, as the emergence of deletion mutants observed in the later stage of infection presumably could only arise from new infections via the pool of viral RNA directed to viral particle assembly.

The sustained repression of viral replication in PBMCs, U937 and CEM cells infected with RRV-GFP-142-3pT4X, and the marked repression of viral spread to hematopoietic tissues at early time point \textit{in vivo}, demonstrate that this miRNA-mediated “detargeting” approach can be effective in restricting retroviral replication. However, the emergence of mutated RRVs that are resistant to miRNA-mediated restriction is a barrier to the long-term success of this approach for
restricting extratumoral RRV replication. Kelly et al. examined the stability of inserted miRNA target sequences in a replicating oncolytic picornavirus (Coxsackie) up to day 45 post-virus administration and observed that nearly 50% of the virus from viremic mice had mutated sequences. We found that restriction of RRV incorporating four copies of miRT142 was, in general, more robust and durable than that of RRVs with only a single copy. Therefore, increasing the total number of target sequences in the vector may be one method to improve this targeting strategy. Alternatively, miRNA-mediated restriction could be combined with other targeting methods to more effectively reduce extratumoral replication and further reduce the likelihood of escape mutant formation.

Here, we demonstrated that the incorporation of miR142-3p target sequences into the 3’ UTR of the viral genome is one way of potentially further restricting RRV spread. We observed mutation of the inserted target sequences, especially in long-term experiments, which is a major barrier to the success of this approach in vectors based on RNA viruses. However, optimization of miRT copy number and/or insertion sites may allow for more robust restriction of RRV replication. The development of novel RRVs with greater infectious potential, as well as the continuing escalation of doses administered to clinical trial patients, could result in increased off-target effects associated with RRV-mediated gene therapy. Therefore, further investigation of this and other mechanism for restricting the spread of RRVs in non-cancerous cells are important for ensuring the safety and tolerability of RRV-mediated gene therapy.
Figure 1-1. (a) Relative miRNA142-3p expression in PBMCs of 5 healthy individuals, and in hematopoietic- and non-hematopoietic-lineage derived cell lines. R: resting PBMCs, A: activated PBMCs. (b) Schematic diagram of plasmid DNA of replicating retroviral vector RRV-GFP incorporating the miR142-3p target sequence. (c) Identical sequences of mature human and murine miRNA142-3p (accession # MIMAT0000434, accession # MIMAT000155). (d) Sequence of miR142-3p targets, and flanking nucleotides, inserted into RRV-GFP to generate RRV-GFP-142-3pT and RRV-GFP-142-3pT4X vectors.
Figure 1-2: (a) Viral titer of RRV-GFP, RRV-GFP-142-3pT and RRV-GFP-142-3pT4X on PC-3 cells. (b) Replication kinetics of RRV-GFP, RRV-GFP-142-3pT and RRV-GFP-142-3pT4X in U87-MG cells infected at an MOI of 0.01. (c) Mean fluorescence intensity (MFI) of GFP expression over the course of infection. (d) Schematic diagram of proviral DNA and locations of PCR primer sets. (e) Stability of the IRES-GFP transgene in RRV-GFP, RRV-GFP-142-3pT and RRV-GFP-142-3pT4X proviral DNA during serial passaging in U87-MG cells. DNA molecular marker (1kb plus marker) is included in the first lane of each gel. The numbers above each lane indicate the number of infection cycles for each vector. Arrows indicate size of the PCR product expected for undeleted IRES-GFP region (1445 bp for RRV-GFP; 1492 bp for RRV-GFP-142-3pT and 1575 bp for RRV-GFP-142-3pT4X vector). NTC, no template control; +, plasmid positive control.
Figure 1-3: (a) Replication kinetics of RRV-GFP, RRV-GFP-142-3pT and RRV-GFP-142-3pT4X in PBMCs infected at an MOI of 4. (b) Mean fluorescence intensity of GFP positive PBMCs infected with parental vector or miRT-RRVs. (c) Transgene stability of RRV-GFP (GFP), RRV-GFP-142-3pT (142-3pT) and RRV-GFP-142-3pT4X (142-3pT4X). NC: negative control; arrow indicates expected product for undeleted IRES-GFP transgene. (d) Schematic diagram of cellular viral RNA isoforms and qRT-PCR primers used. (e) Cellular viral RNA expression levels in PBMCs infected with miRT-RRVs relative to the parental vector.
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**Figure 1-4a:** Sequence alignment of clones from genomic DNA of PBMCs infected with RRV-GFP-142-3pT. IRES-GFP PCR product, amplified from genomic DNA of PBMCs infected with RRV-GFP-142-3pT, harvested 10 days post-infection, was subcloned into pSC-A-amp/kan PCR cloning vector. Ten positive clones containing the insert were randomly chosen for sequencing analysis using PCR primers spanning the 3'end of GFP gene. Line indicates the location of miRT142 insertion.
**Figure 1-4b**: Sequence alignment of clones from genomic DNA of PBMCs infected with RRV-GFP-142-3pT. IRES-GFP PCR product, amplified from genomic DNA of PBMCs infected with RRV-GFP-142-3pT, harvested 10 days post-infection, was subcloned into pSC-A-amp/kan PCR cloning vector. Ten positive clones containing the insert were randomly chosen for sequencing analysis using PCR primers spanning the 3'end of GFP gene. Lines indicate the location of miRT142 insertions.
Figure 1-5: (a) Replication kinetics of RRV-GFP, RRV-GFP-142-3pT and RRV-GFP-142-3pT4X in U937 cells infected at an MOI of 2. (b) Stability of miRT-RRVs and parental vector in U937 cells. DNA molecular marker (1kb plus marker) included in the first lane of the gel. The numbers above each lane indicate the time in days post infection. Arrow indicates size of the PCR product expected for undeleted IRES-GFP region, NC = naive cells, negative control. (c) Proviral copy number per cell in genomic DNA from infected U937 cells. (d) Cellular viral RNA expression levels in U937 cells infected with miRT-RRVs relative to the parental vector. (e) Viral titers in the supernatants of infected U937 cells. Asterisk (*) indicates deletion of IRES-GFP.
cassette in vectors containing the 142-3pT sequences. Viral proteins produced by infected U937 cells. Lanes 1-4: non-infected cells, cell infected with RRV-GFP, RRV-GFP-142-3pT and RRV-GFP-142-3pT4X vectors, respectively.

**Figure 1-6:** (a) Replication kinetics of RRV-GFP, RRV-GFP-142-3pT and RRV-GFP-142-3pT4X in CEM cells infected at an MOI of 2. (b) Transgene stability of proviral DNA in infected CEM cells. DNA molecular marker (1kb plus marker) included in the first lane of the gel. The numbers above each lane indicate the time in days post infection. Arrows indicate size of the PCR product expected for the undeleted IRES-GFP cassette (1445 bp for RRV-GFP; 1492 bp for RRV-GFP-142-3pT and 1575 bp for RRV-GFP-142-3pT4X. NC= naive cells, negative control. (c) Cellular viral RNA expression levels in U937 cells infected with miRT-RRVs relative to the parental vector. * indicates deletion of IRES-GFP cassette. (d) Viral titers in the supernatants of infected CEM cells.
Figure 1-7: (a) Growth of subcutaneous tumors infected with miRT-RRVs or parental vector. (b) Proviral copy number in the genomic DNA of harvested tumors 20 days post-tumor engraftment. Each dot represents one mouse. (c) anti-MLV immune response in the sera of mice 20 days post-tumor engraftment. Control group are mice engrafted with uninfected subcutaneous tumors. Mean values and standard deviations are as shown. One-way ANOVA was performed for statistical analysis, and values from samples scored as LLOQ (less than 250 copies/µg) were included in the analysis. *, significant difference (p<0.05); ns = not significant.
### Tumor (copies/microgram)

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<tr>
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<th>RRV-GFP-142-3pT4X</th>
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<tbody>
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### Blood (copies/microgram)

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<th>RRV-GFP-142-3pT4X</th>
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<tbody>
<tr>
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<td>&lt;LLOQ</td>
<td>&lt;LLOQ</td>
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</tr>
<tr>
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<th>RRV-GFP-142-3pT4X</th>
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<tbody>
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### Bone Marrow (copies/microgram)

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<th>RRV-GFP-142-3pT4X</th>
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<tbody>
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<td>ND</td>
<td>ND</td>
<td>&lt;LLOQ</td>
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<tr>
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<td>ND</td>
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<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>&lt;LLOQ</td>
<td>ND</td>
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**Table 1-1:** Biodistribution of miRT-RRVs in mice implanted with subcutaneous RRV-infected tumors. Proviral vector copy number per microgram of tissue derived genomic DNA, as measured by qPCR. ND=non-detectable, (Ct values>38); LLOQ=lower limit of quantification, (less than 250 copies/µg).
Figure 1-8: Proviral vector copy number per microgram of tissue derived genomic DNA at day 30 post-administration in the (a) blood, (b) bone marrow and (c) spleen of nude mice. (d) Viral spread, as measured by flow cytometry, to lineage negative (lin-) bone marrow cells 30 days post-administration. (e) Proviral vector copy number in the genomic DNA of lin- bone marrow 30 days post-administration of RRV. Control mice were injected with PBS. Mean values and standard deviations are as shown. One-way ANOVA was performed for statistical analysis, and values from samples scored as LLOQ (less than 250 copies/µg) were included in the analysis. *, significant difference (P < 0.05); ns = not significant.
<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th># Positive</th>
<th>Range of Positivity (Copy #/µg)</th>
<th>&lt;LLOQ</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
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<tr>
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<td>0 - 0</td>
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<td>0</td>
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<td>0</td>
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<td>11</td>
<td>628 - 10,462</td>
<td>1</td>
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<td>0</td>
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<tr>
<td>RRV-GFP-142-3pT, D15</td>
<td>11</td>
<td>9</td>
<td>0 - 0</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RRV-GFP-142-3pT4X, D15</td>
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<td>9</td>
<td>0 - 0</td>
<td>9</td>
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<tr>
<td>NC, D30</td>
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<td>0 - 0</td>
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<td>762 - 5,800</td>
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Table 1-2. Range of proviral copy number in hematopoietic tissues of nude mice infected with RRV-GFP, RRV-GFP-142-3pT or RRV-GFP-142-3pT4X vector. The range of infectivity was set as 0 = non-detectable (Ct value >38); low = less than 15,000 copies/µg (<0.1 copy/cell equivalent); medium = 15,000 to 600,000 copies/µg (0.1- 4 copies/cell equivalent); and high = more than 600,000 copies/µg (>4 copies/cell equivalent).
**Table 1-3:** Proviral vector copy number in genomic DNA harvested from lin+ and lin- bone marrow cells from nude mice infected with RRV-GFP, RRV-GFP-142-3pT or RRV-GFP-142-3pT4X vector. ND = non-detectable (Ct values >38); LLOQ = lower limit of quantification (less than 250 copies/µg).

<table>
<thead>
<tr>
<th>Group</th>
<th>NC</th>
<th>RRV-GFP</th>
<th>RRV-GFP-142-3pT</th>
<th>RRV-142-3pT4X</th>
</tr>
</thead>
<tbody>
<tr>
<td>lin+</td>
<td>lin-</td>
<td>Lin+</td>
<td>Lin-</td>
<td>Lin+</td>
</tr>
<tr>
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<td>ND</td>
<td>47,609</td>
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</table>
Figure 1-9: a-c) Proviral vector copy number per microgram of tissue-derived genomic DNA at day 60 post-administration in the (a) blood, (b) bone marrow and (c) spleen of athymic nude mice. Control mice were injected with PBS. Mean values and standard deviations are as shown. One-way ANOVA was performed for statistical analysis, and values from samples scored as LLOQ (less than 250 copies/µg) were included in the analysis.
Chapter 2: Reduced systemic spread, without loss of tumor toxicity, of a retroviral replicating vector incorporating miR142-3p target sequences in the ψ and 3'LTR

In the previous section, we demonstrated the use of microRNA targeting to restrict RRV replication in a tissue-specific manner. The application of this targeting approach to RRVs presents a unique challenge, as stochastic mutation of the miRNA target sequences, by error prone reverse transcriptase, can result in virus escape from restriction. Here we investigated whether incorporation of the miRNA target sequences in functional viral elements could prevent or delay the emergence of escape mutants and prolong the efficacy of miRNA-mediated RRV restriction. We evaluated the cell-type specificity and genomic stability of RRVs containing the miR142-3p target sequence (miRT142) in the 3'UTR, 3'LTR and/or the ψ packaging site of the virus. RRVs containing at least two copies of miRT142 were significantly repressed in lymphocytic cell lines, and primary human peripheral blood mononuclear cells, however, in some cases, the placement of the miRNA targets affected RRV replication in U87 glioma cells, the stability of the viral transgene, and the mutation rate of the miRNA target sequences themselves. Of the vectors tested, an RRV incorporating miRT142 in the 3’UTR and ψ packaging site was the most stable and was robustly restricted in hematopoietic tissues both in vitro and in vivo, while retaining the ability to spread effectively in tumors. In mice implanted with intracranial xenograft tumors, there was no difference in overall survival between those treated with parental RRV or the miRNA-targeted RRV (miRT-RRV), and there was a significant reduction in systemic spread from the miRT-RRV. MicroRNA targeting is a viable method for further increasing the tumor specificity of RRVs, and therefore the safety of RRV-mediated gene therapy. Careful selection of miRT insertion sites can prolong the effect of miRNA-mediated restriction of RRV, and may improve the efficacy of this targeting approach in other vectors systems as well.
Introduction

Over the last decade, incorporation of miRNA target sequences has become a popular method for the tissue-specific restriction of viral vectors\textsuperscript{12,31,45}. We previously demonstrated that insertion of miR142-3p target sequences into the transgene cassette of the RRV genome could restrict RRV replication in hematopoietic lineage-derived cell lines, primary human PBMCs and the blood, bone marrow, and spleen of both immune-competent, tumor-bearing mice, and immune-deficient mice\textsuperscript{46}. A major concern with this strategy is whether sufficient knockdown of viral replication can be achieved without the emergence of escape mutants\textsuperscript{45}. This is of particular importance in the application of this strategy to vectors based on RNA viruses, including RRVs, due to the high frequency of retroviral recombination and the low fidelity of RNA dependent polymerases\textsuperscript{12}. Mutations in the muscle-specific miRNA target sequences used to regulate an oncolytic coxsackievirus, resulted in viral escape and increased muscle damage in some treated mice\textsuperscript{30}. In our own studies using miRT142 to restrict RRV replication, we observed low levels of ongoing viral replication in hematopoietic cell lines and tissues, which in some cases was sufficient to allow mutation of the target sequences and the emergence of resistant viruses. This was particularly evident in RRVs carrying only one copy of the miRT142\textsuperscript{46}. Inclusion of multiple target sequences in a single vector not only results in more robust restriction of viral replication, thus minimizing the opportunity for stochastic mutation, but also increases the number of mutations required for escape, and can therefore prolong miRNA-mediated restriction.

Furthermore, inserting the identical sequences in different regions of the genome, rather than in tandem, may reduce the likelihood of recombination between the homologous sequences during reverse transcription. Insertion of miRNA target sequences in functional viral elements has also been proposed as a possible mechanism to delay or avoid the emergence of escape mutants\textsuperscript{31}. For instance, there were no detectable escape mutants after either in vitro or in vivo infections with an influenza A virus incorporating miRNA target sequences in the open reading frame of the nucleoprotein gene\textsuperscript{47}. 
We hypothesized that optimizing the design of miRNA-restricted RRVs could delay the emergence of escape mutants. Therefore, using the criteria discussed above, we generated novel RRVs incorporating miRT142 in the 3’UTR (Mlu site), 3’LTR (Nhe site) and the highly conserved ψ packaging site (Psi site). As insertion of exogenous sequences into functional regions of the viral genome can significantly impair viral replication, we carefully examined the replication kinetics of miRT-RRVs in permissive and non-permissive cells, as well as the stability of the viral transgene, and screened for mutations in the miRT sites and the emergence of escape mutants. Our data demonstrated that RRVs can tolerate insertion of miRT142 in some functional regions of the virus, without significant loss of replicative ability or tumor toxicity. However, the insertion of miRT142 in certain locations in the RRV genome resulted in significant loss of viral fitness, genomic instability, and selective pressure for RRVs with mutated miRT sequences. Therefore, our results also emphasize the importance of thoroughly screening viruses incorporating miRT sequences.
**Materials and Methods.**

**Plasmids.** RRV-GFP, also known as pAC3-emd, and RRV-yCD2, also known as pAC3-yCD2, were constructed as previously described\(^1\). RRV-GFP-142Mlu was created by PCR amplification from RRV-GFP using primers 5'-142.3-Mlu (5' - CACCAGCTAAACGCGTCCATAAAGTAGGAACACTACATTACTGGCCGAAGCCGCTTG-3') and 3-Mlu-miRT-PsiI (5' - TAAATCGTGTTTTTCAAAGG-3'), followed by cloning of the PCR product into the MluI and PsiI endonuclease sites of RRV-GFP. The underlined sequence is complementary to miR-142-3p. RRV-GFP-142Nhe was created by PCR amplification from RRV-GFP using primers 5'-142.3-NheI (GGGGGAATGAAAGCTAGCTCCATAAAGTAGGAACACTACATTACTGGCCGAAGCCGCTTG-3') and 3-NheI-miRT-Sacl (5' - GGGTTGTGGGCTCTTTTATTG-3'), followed by cloning of the PCR product into the NheI and Sacl endonuclease sites in RRV-GFP. To construct the RRV-GFP-PsiA-G plasmids, we synthesized seven different 365bp oligos (BioBasic Inc), spanning from the beginning of the 5' LTR into the \(\psi\) packaging region, in which the sequence complementary to miR142-3p was substituted for viral sequence at locations PsiA through Psi G (Figure 1a). The synthesized fragments were cloned into the Ascl and AatII sites in RRV-GFP. RRV-GFP-142PsiMlu and RRV-GFP-142PsiNhe were constructed by digesting RRV-GFP-142Mlu or RRV-GFP-142Nhe with MluI and NotI or NotI and Sacl, respectively, and cloning the fragment into the corresponding endonuclease sites in RRV-GFP-142Psi. RRV-GFP-142MluNhe was constructed by digesting RRV-GFP-142Nhe with NotI and Sacl, and cloning the fragment into the corresponding endonuclease sites in RRV-GFP-142Mlu. RRV-GFP-142PsiMluNhe was constructed by digesting RRV-GFP-142Nhe with NotI and Sacl and cloning the fragment into the corresponding endonuclease sites in RRV-GFP-142PsiMlu. All plasmids were confirmed by sequencing.

**Cell Lines.** Human embryonic kidney 293T cells (ATCC, CRL-3216), human glioblastoma U-87 MG cells (ATCC, HTB-14), human prostate adenocarcinoma PC-3 cells (ATCC, CRL-1435),
and human T-cell lymphoblastic lymphoma SUP-T1 cells (ATCC, CRL-1942) were obtained from ATCC. The TU-2449 mouse glioma cell line was obtained from Tocagen Inc. 293T, U87-MG and TU-2449 cells were cultured in complete DMEM medium containing 10% fetal bovine serum (FBS) (Hyclone) and 100 IU/mL penicillin-streptomycin (PS). PC3 and Sup-T1 cells were cultured in complete RPMI medium containing 10% FBS and 100 IU/mL penicillin-streptomycin.

**Virus Production.** Virus stock was produced by transient transfection of 293T cells using the calcium phosphate precipitation method. Briefly, 5x10⁶ 293T cells were seeded on 0.01% Poly-L-Lysine coated plates. The following day, cells were transfected with 22.5 µg of plasmid. Approximately fifteen hours post transfection, cells were washed with PBS and incubated with fresh DMEM medium containing 20mM HEPES and 10mM Sodium Butyrate. Approximately 24 hours post-transfection, cells were washed again with PBS and incubated with fresh DMEM with 20mM HEPES. Viral supernatant was collected approximately 40 hours post transfection and filtered through a 0.45 µm syringe filter unit. Aliquots were stored at -80°C. Virus stocks were used to infect U87-MG cells to create producer cell lines. Viral supernatant from 100% transduced, super-confluent producer cells was collected and filtered through a 0.45µm syringe filter. Aliquots were stored at -80°C. Viral titer was determined either by flow cytometry, for viruses expressing GFP, or by qPCR, for viruses expressing yCD2. Briefly, U87 cells were infected by addition of varying dilutions of viral supernatant from frozen stocks. 50µm AZT was added 24 hours post-infection to prevent further viral replication and cells were collected 48 hours post-infection for analysis by qPCR or flow cytometry to determine titer.

**Flow Cytometry.** Replication of RRV expressing the emerald GFP (GFP) transgene was monitored by flow cytometry. Cells were infected at a multiplicity of infection (MOI) of 0.01 for U87-MG and PC3 cells, at an MOI of 0.1 for Sup-T1, and at an MOI of 4 for PBMCs. Kinetics plots were obtained by plotting the percentage of GFP-positive cells over time. All flow cytometric data were acquired on a BD FACSCanto II cytometry running BD FACSDiva (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (TreeStar, Ashland, OR).
**PBMCs.** Isolation, infection and analysis of human PBMCs were performed as described in the previous chapter\(^{46}\).

**Vector Copy Number Analysis.** Proviral vector copy numbers in genomic DNA were determined by quantitative PCR (qPCR) as previously described\(^{14}\). All qPCR experiments were carried out on the iQ5 thermal cycler (Biorad).

**Relative Expression of Cellular Viral RNA by qRT-PCR.** RNA was isolated from cells using the Aurum Total RNA Mini Kit (BioRad). Reverse transcription was performed with the High-Capacity Reverse Transcription Kit (Applied Biosystems). cDNA was quantified by qPCR to determine the relative expression of viral RNA from each proviral copy as previously described\(^{46}\).

**Vector Stability.** Vector stability was assayed by amplifying the transgene region of the integrated provirus from gDNA of infected cells using primers 5'-4070A-env-1669 (5'-CACACGGGGCTAGTGAGAGAC-3') and 3-MLV-LTR-438 (5'-GGTTGTGGGCTCTTTTATTG-3'). Amplification of the full-length transgene yields a band 2060bp in size, while proviruses with deletions within the transgene region yield smaller products. To investigate the transgene stability during serial passaging, 1% supernatant from infected U87 cells was transferred to naïve U87 cells every 7 days for 8 weeks. gDNA was collected each week to measure vector stability as described above.

**Sequencing of miRT sites.** Proviral DNA surrounding the Psi, Mlu, and Nhe miRT insertion sites was amplified from gDNA of infected cells using the following primer sets respectively: 5-MLV-PBS (5'-TTTCAATTGGGGCTCGTC-3') and 3-MLV-Psi-mid (5'-CTCAGGGGCTGCAAAA-3'), 5-TGSFenv (5'-GTCAATTTGTTAAAGACAGGAT-3') and 3-endofIRES (5'-TTTAAAGGAAAACCACGTC-3'), and 5-endofGFP (5'-GTCCTGCTGGAGTTGTCGTGA-3') and 3-Nhel-miRT-Sacl (5'-GGGTGTGGGCTCTTTTATTG-3'). PCR products were ligated to the PCR-Blunt vector (Invitrogen) and individual clones were sequenced using the M13 Reverse primer (Laragen).
**In vivo experiments.** All animal experiments were conducted under protocols approved by the University of California, Los Angeles Animal Research Committee. Intravenous delivery of RRV to nude mice was performed as previously described\textsuperscript{46}. Genomic DNA from blood, bone marrow, and spleen was harvested 55 days post infection and vector copy number was analyzed by qPCR as previously described\textsuperscript{14}. For the survival study, 5-6 week old nude mice were intracranially implanted in the right forebrain with 10µl naïve U87 cells (2e7 cells/mL) via stereotactic needle. On day 5 post-implant, 3.5e4 IU RRV-yCD2 or RRV-yCD2-142PN was injected intracranially at the same location. On day 17 post-implant, 5-fluorocytosine (5-FC) dosing was initiated. Mice were given once daily intraperitoneal (IP) injections of either 5-FC (500mg/kg) or PBS for control groups. Each dosing cycle consisted of 7 days of treatment, followed by 7 days without treatment, and a total of 5 cycles were administered. Tissues were collected following euthanasia after tumor progression. Survival data were plotted using the Kaplan-Meier method and compared by the log-rank test. All analyses were performed with Prism 6 statistical software (Graphpad Software Inc).

**MTS Assay.** To evaluate RRV cytotoxicity in vitro, 2x10\textsuperscript{3} U87 cells, naïve or fully transduced with RRV-GFP, RRV-GFP-142PsiNhe, RRV-yCD2 or RRV-yCD2-PsiNhe, were plated on 96-well plates in 200µl of media. Cells were grown in the presence of 0-500µm 5-FC for 3, 6 or 9 days. On days 3, 6, and 9, media was aspirated and replaced with 120µl media containing 20µl MTS reagent (CellTiter 96 AQueous Assay, Promega). The OD\textsubscript{490} was measured every hour for 4 hours following addition of MTS.
Results

Selection of miRT insertion sites.

We created RRVs carrying a single copy of the miRT142 in either the Mlu site, at the 5’ end of the IRES (RRV-GFP-142Mlu), the NheI site, in the 3’ LTR (RRV-GFP-142Nhe), or in one of seven sites in the ψ packaging signal (RRV-GFP-142PsiA-G) (Supplementary Figure 1a). The regions of the MLV ψ that are required for viral replication have been well characterized. The seven insertion sites in the ψ were located in or near a 24bp region at the 5’ end where insertions or deletions can be made without significantly compromising viability of the virus. The target sequence was created by mutating the wild-type virus sequence, and insertion sites were selected so as to minimize nucleotide changes required (Supplementary Figure 1a). The 3’UTR and 3’LTR are known permissive sites in retroviral vectors for the addition of exogenous sequences, therefore we did not anticipate that insertion of miRT142 in the Mlu or Nhe site would negatively affect RRV replication. Indeed, in cells not expressing miR142-3p, the replication kinetics of RRV-GFP-142Mlu and RRV-GFP-142Nhe were indistinguishable from the parental vector RRV-GFP (Supplementary Figure 1b). The replication kinetics of RRV carrying miRT142 in the ψ varied significantly, however, depending on the specific site of insertion (Supplementary Figure 1c). RRV with miRT142 in the PsiB and PsiC site exhibited replication kinetics most similar to the parental RRV, therefore these vectors were selected for further study.

RRV with only one copy of miRT142 are not completely repressed in hematopoietic cell lines, and eventually evolve mutations in the miRT site that allow virus escape from miRNA restriction. We wondered whether insertion of miRT142 in either the PsiB or PsiC site would delay virus escape since random mutations in the miRT or surrounding ψ could impair viral replication. Replication of RRV-GFP-142PsiC was suppressed for approximately 15 days, while replication of RRV-GFP-142PsiB was more robustly restricted for approximately 35 days before
viral escape occurred, suggesting that the miRT142 inserted in the PsiB site is more resistant to mutation (Supplementary Figure 1d). Since insertion of miRT142 in the PsiB location resulted in prolonged restriction of RRV replication, we used the PsiB insertion site for all future studies, and any further reference to RRVs with miRT insertion in the Psi site refers to the PsiB location.

**RRVs with multiple copies of miRT142 in different genomic sites productively infect U87 cells, but the location of miRT insertions can impair viral replication.**

We, and others, have shown that the insertion of multiple copies of miRT142 results in more robust knockdown of viral replication\(^1\),\(^4\),\(^6\). Therefore, we combined the insertion sites detailed above to create RRVs with two or three copies of miRT142 in different genomic locations (Figure 1a). To investigate whether the insertion of multiple miRT sequences affected viral replication, we infected U87 glioma cells, which do not express detectable levels of miR142-3p\(^4\),\(^6\), with each of the miRT-RRVs. The replication kinetics of RRV-GFP-142MluNhe (MluNhe) and RRV-GFP-142PsiNhe (PsiNhe) were similar to the parental vector, with complete infection occurring within 7 days (Figure 1b). However, the replication of RRV-GFP-142PsiMlu (PsiMlu) and RRV-GFP-142PsiMluNhe (PsiMluNhe) was significantly delayed, with complete infection requiring 15 days to occur (Figure 1b). Consistent with this delay in replication, the mean fluorescence intensity (MFI) of the GFP positive cells was significantly lower for cells infected with PsiMlu and PsiMluNhe until 13 days post-infection, suggesting that less viral protein was expressed in these cells at earlier time points compared to cells infected with MluNhe, PsiNhe or parental vector (Supplementary Figure 2a). To further investigate viral replication, we used qPCR to quantify the proviral copy number per cell in the genomic DNA of U87 cells 13 days post-infection, and normalized to the parental vector. On average, MluNhe and PsiNhe produced 70% as much proviral DNA as the parental vector, while PsiMluNhe and PsiMlu produced only 50% and 25% proviral DNA, respectively (Figure 1c). We next quantified viral RNA expression per provirus by using qRT-PCR to measure cellular viral RNA and normalizing
to the number of proviral copies per cell. Consistent with our previous results, we found that viral RNA expression from MluNhe and PsiNhe was comparable to that from parental vector, while expression from PsiMlu and PsiMluNhe was significantly reduced (Figure 1d). Together, our results indicated that while RRVs were tolerant of insertion of multiple copies of miRT142, the placement of the target sites could affect the efficiency of viral reverse transcription and relative RNA expression levels.

Multiple copies of miRT142 robustly restrict RRV replication in Sup-T1 T-cells and human peripheral blood mononuclear cells.

To evaluate the cell-type selectivity of the miRT-RRVs, we transduced Sup-T1 cells, and primary human peripheral blood mononuclear cells (PBMCs), and monitored RRV spread by flow cytometry. In Sup-T1 cells, GFP expression was undetectable eight weeks after infection for all miRT-RRVs, while the parental vector achieved complete infection within two weeks (Figure 1e). To validate our flow cytometry results, we collected gDNA at the end of each week and amplified the transgene region of the integrated provirus using primers specific to the viral envelope and the 3’ LTR. The full-length IRES-GFP transgene produces a 2.1kb PCR product, while any deletions within the transgene region result in smaller products. At each time point, there was a strong band at 2.1kb indicating the transgene remained intact throughout the entirety of the experiment, and therefore, spread of any escape mutants would have been evident by flow cytometry (Supplementary Figure 2b). Consistent with these results, we found that all miRT-RRVs exhibited significantly reduced integration frequency and viral RNA expression compared to the parental vector (Figure 2f, 2g). In primary PBMCs, replication of the miRT-RRVs was also significantly repressed, and the transgene was intact, for the duration of the in vitro culture life of these cells (Figure 2h, Supplementary Figure 2c). While the high multiplicity of infection (MOI) required to transduce PBMCs resulted in a low level of initial infection by each of the miRT-RRVs, this remained stable throughout the experiment, indicating
a repression of viral spread. Furthermore, the MFI of GFP positive cells infected with miRT-RRVs was less than 20% that of cells infected with RRV-GFP, indicating that the expression of viral proteins was significantly reduced by incorporation of miRT142 (Supplementary Figure 2d). This result was confirmed by qPCR and qRT-PCR, which again showed a reduction in proviral copy number and viral RNA expression in cells infected with miRT-RRVs compared to parental vector (Figure 2i, 2j). Together, these data demonstrated that inclusion of multiple copies of miRT142 in different sites in the RRV leads to robust knockdown of viral replication in hematopoietic lineage-derived cells.

**MicroRNA target sequence placement can reduce viral transgene stability and confer a selective advantage to escape mutants.**

To further characterize the miRT-RRVs, we investigated the miRT sequence integrity and viral transgene stability during serial passaging in U87 cells. Supernatant from infected cells was passaged each week onto naïve cells, replication kinetics were monitored by flow cytometry and gDNA was collected at the end of each week for analysis of transgene stability and miRT sequencing. To sequence the miRT insertion sites, we used primers spanning each miRT site, but specific for viral sequence, and then sequenced individual clones. After one week (passage 1), in cells infected with either MluNhe or PsiNhe, both sets of miRT sequences were fully intact in all clones sequenced (Supplementary Figure 3a). In cells infected with PsiMlu, sequencing revealed G-to-A point mutations in the Mlu miRT, although none of these were located in the critical seed region at the 3’ terminus of the target sequence. The Psi miRT site remained unchanged in all clones sequenced (Supplementary Figure 3a). Cells infected with PsiMluNhe contained A-to-T and C-to-T point mutations and multiple nucleotide deletions in 3/5 of the sequenced Psi sites, all of which localized to the seed sequence, and multiple nucleotide deletions in the Mlu miRT site in 3/5 clones, while the Nhel miRT site remained unchanged in all clones sequenced (Supplementary Figure 3a).
During passage 2, the delay in replication originally noted for PsiMlu and PsiMluNhe vectors was less pronounced and all vectors achieved complete infection by 7 days post-infection (Figure 2a). Sequencing across the miRT sites for PsiMlu and PsiMluNhe after passage 2 revealed point mutations and multi-nucleotide deletions in the Mlu miRT sites of both vectors, as well as dramatic deletions and point mutations in the Psi miRT sites of both vectors, specifically localized to the seed sequence. For PsiMluNhe, the Nhe miRT site also had a deletion in the seed sequence in 1/5 clones sequenced (Supplementary Figure 3b). The concomitant mutation of the miRT sites and restoration of replication efficiency suggests that the original delay to replication evident in passage 1 resulted from insertion of miRT in both the Mlu and Psi insertion sites of a single vector.

We continued passaging the virus and monitoring replication kinetics by flow cytometry. While MluNhe and PsiNhe maintained replication kinetics similar to the parental vector for the entirety of the experiment, by passage 5, the majority of the virus in PsiMlu and PsiMluNhe supernatants appeared by flow cytometry to have lost the GFP transgene (Figure 2a). This was confirmed by a PCR assay for transgene stability, which showed PCR products smaller than the 2.1kb full-length transgene as early as passage 1 for PsiMlu and PsiMluNhe, while transgene stability in MluNhe and PsiNhe was comparable to parental vector, with deletion mutants emerging by passage 6 (Figure 2b).

After passaging the viruses for eight weeks, we sequenced across the miRT insertion sites in cells infected with MluNhe and PsiNhe. MluNhe had accrued point mutations and point deletions in the Mlu miRT site in 4/5 clones sequenced, while there were no mutations identified in the Nhe miRT site. PsiNhe had single point mutations in the Nhe miRT site in 2/4 clones sequenced and no mutations in the Psi miRT site (Supplementary Figure 3c). To determine the functional consequences of the mutations to the miRT sites that occurred during passaging of the virus,
we used the supernatant from passage 8 to infect Sup-T1 cells and monitored vector spread by qPCR for integrated proviral DNA, as the GFP transgene had been deleted in a large proportion of the virus population for all vectors. MluNhe, PsiNhe, and PsiMluNhe remained restricted from spread within Sup-T1 cells, indicating that mutations to the miRT sequences that occurred during passaging in U87 cells did not block targeting of the viral RNA by endogenous miR142-3p in Sup-T1 cells. Conversely, PsiMlu was able to productively spread in Sup-T1 cells and by day 14 post-infection, the proviral copy number per cell was similar to that of the parental vector (Figure 2c). The reduced transgene stability and higher frequency of mutation of the miRT sites in PsiMlu and PsiMluNhe, together with their reduced replication efficiency in passage 1, suggests that these combinations of miRT insertion sites convey a selective disadvantage to the RRV, which promotes the emergence of escape mutants. We therefore excluded these RRVs from future studies.

**Spread of miRT-RRVs is restricted in the hematopoietic tissues of nude mice.**

To investigate the spread of miRT-RRVs in hematopoietic tissues *in vivo*, we injected athymic nude mice, via the tail vein, with 4e5 transducing units (TU) of MluNhe, PsiNhe or parental vector, and quantified the vector copy number in peripheral blood, bone marrow and spleen, 55 days after injection. Both vectors containing miRT142 were completely undetectable in blood and mostly below the lower limit of quantification (LLOQ) in bone marrow and spleen, while spread of the parental vector was evident in all tissues (Figure 3a-c). In the bone marrow and spleen, MluNhe was able to replicate slightly better than PsiNhe, a difference that was statistically significant in the spleen (p<0.01) but not in the bone marrow (p=0.06) (Figure 3b-c). Based on these results, as well as our *in vitro* data showing a slightly higher frequency of mutation of the Mlu miRT insertion site, we selected the PsiNhe vector for further *in vivo* investigation.
miRT-RRVs delivering yCD2 achieve efficient tumor cell killing \textit{in vitro} and \textit{in vivo}.

To determine whether the cytotoxicity of RRVs expressing the yeast cytosine deaminase suicide gene (yCD2) was affected by insertion of miRT142, we replaced the GFP transgene with yCD2 and infected U87 cells \textit{in vitro}. Cells fully infected with RRVs expressing either GFP or yCD2 were grown in media containing between 0\(\mu\)m to 500\(\mu\)m 5-fluorocytosine (5-FC) for 9 days, at which point an MTS assay to measure cellular metabolism was performed. While there was no effect of even the highest concentrations of 5-FC on cells expressing GFP, there was significant cell death among cells expressing yCD2 at 5-FC concentrations greater than 5\(\mu\)m. Importantly, there was no significant difference in cell killing between PsiNhe and the parental vector, indicating that insertion of miRT142 does not affect yCD2 transgene function (Figure 4a).

To evaluate the \textit{in vivo} tumor toxicity of RRV-yCD2-142PsiNhe, we used the U87 xenograft model for glioma. Mice were implanted intracranially with U87 cells and given intratumoral injections of either RRV-yCD2 or RRV-yCD2-142PsiNhe, 7 days following tumor implant. The mice were then treated with 5 cycles of either 5-FC or PBS, and monitored for over 130 days. Control mice succumbed to tumor burden within 40 days of tumor implantation. Mice receiving either vector, and treated with 5-FC, survived for over 100 days, and there was no difference in overall survival between mice receiving RRV-yCD2 or those receiving RRV-yCD2-142PsiNhe (Figure 4b). After sacrifice, we harvested tumors, bone marrow, spleen and ovaries and analyzed the proviral copy number of RRV in extracted gDNA by qPCR. Consistent with our \textit{in vitro} results, there was no significant difference in vector copy number in the tumors isolated from treated animals (Figure 4c). In all other tissues examined, PsiNhe was undetectable by qPCR, while RRV-yCD2 spread effectively to these tissues, a difference that was statistically significant in bone marrow and spleen (p<0.005). Importantly, PsiNhe was undetectable even in the ovaries, demonstrating that inclusion of miRT142 in the RRV can restrict spread to non-hematopoietic tissues as well.
Discussion

The use of miRNA target sequences is emerging as a powerful method to restrict the replication of viral vectors in a tissue-specific manner, however the emergence of revertant viruses that have mutated or deleted the inserted miRTs is a barrier to long-term success of this strategy. Various approaches to address this issue have been used, including the addition of multiple target sites within a single vector, and the incorporation of target sequences into coding regions of the virus\textsuperscript{12,31,45,47}. While it is clear from our work, and that of other groups, that multiple target sequences affect more robust restriction of viral replication, the effect of the specific location of the miRNA target sequences in the viral vector has not been investigated. Here we examined the tissue-specific restriction of RRVs incorporating miR142-3p target sequences in the 3'UTR, 3'LTR and/or ψ packaging site. Our results demonstrated that durable, long-term restriction of RRV replication can be achieved, without a reduction in intratumoral spread or cytotoxic effect, and that this restriction was insertion site dependent.

Our data demonstrated the successful incorporation of miRNA target sequences into three novel locations in the RRV genome. While the majority of naturally occurring miRNA target sequences are localized to the 3'UTR of cellular mRNAs, many targets have also been identified in 5'UTRs, and in gene coding regions\textsuperscript{50,51}, and the mRNAs containing miRNA targets in these locations are repressed as effectively as those with targets in the 3'UTR\textsuperscript{52}. Therefore, the physical location of miRT in the RRV genome should not affect repression of the viral mRNA. However, the specific sequence context of the miRNA target can affect accessibility of the target for recognition and binding by the cognate miRNA\textsuperscript{53}. Both the IRES and ψ packaging site are highly structured regions, so recognition of miRTs in these areas may be affected by nearby secondary structures. We observed more robust repression, in Sup-T1 cells, of RRVs with miRT142 in the PsiB site compared to the PsiC site, which may be explained by differences in sequence context. Several miRNA target identification algorithms are available\textsuperscript{53–55}, which
could be applied to the selection of optimal insertion sites in viral vectors. In cases where target sequences are inserted into functional viral elements, however, maximum target recognition must be balanced with minimal disruption to viral replication in permissive cells. We observed significant variation in the replication kinetics in permissive cells of RRVs containing miRT142 in the ψ packaging signal. Creation of miRT142 in the PsiB site required the fewest overall nucleotide changes (13), and did not significantly affect viral replication in permissive cells, suggesting that these mutations did not inhibit site-specific protein recognition, or the formation of RNA secondary structures, required for viral replication.

On the other hand, combined insertion of miRT142 in the Mlu and Psi sites of a single vector resulted in delayed replication in permissive cells, reduced genomic stability and strong selective pressure for escape mutants. These escape mutants reverted to efficient replication kinetics subsequent to mutation of the miRT sequences during serial passaging, suggesting the presence of the miR142 target sequences conferred a selective disadvantage to the RRV. As we did not see evidence of impaired replication in vectors containing a single miR142-3p target in either the Psi or Mlu insertion site, the impaired replication was specific to the presence of both miR142-3p targets in the same vector. It is technically possible that recombination occurred between the identical miR142-3p targets, resulting in non-viable virus and selective pressure for viruses with mutations in the miRT sites. However, the probability of recombination for repeats of this size is extremely low\textsuperscript{56}, so this does not logically explain the rapid mutation that occurred during serial passaging. Inserting two different miRNA target sequences in these sites would further elucidate the contribution of sequence homology to the vector instability. Alternatively, miRT142 insertion may have resulted in independent small alterations to RNA secondary structures in the ψ and IRES, which in combination inhibited reverse transcription. Analysis of early and late reverse transcription products may reveal the mechanism for defective replication of the PsiMlu and PsiMluNhe vectors.
Despite any deficiencies in replication in permissive cells, spread of each of the miRT-RRVs was significantly suppressed in Sup-T1 cells and primary human PBMCs, in concordance with the hematopoietic-specific expression profile of miR142-3p\textsuperscript{29,37,38,46}. Similar to our previous results, the degree of repression of RRV spread was not fully explained by viral RNA degradation for any of the miRT-RRVs. This discrepancy suggests the involvement of other mechanisms of miRNA-mediated gene regulation besides RNA degradation, such as inhibition of translation, which is consistent with recent reports indicating that inhibition of translation is the primary mechanism of miRNA-mediated gene silencing, and is required for mRNA degradation\textsuperscript{57–59}. In Sup-T1 cells infected with miRT-RRVs, GFP expression was undetectable by flow cytometry, although there were still measurable amounts of viral RNA present. This is consistent with our previously published data in U937 cells\textsuperscript{46}, further supporting the idea that for vectors containing miR142-3p target sites, viral RNA degradation is not the only mechanism of viral protein repression. In PBMCs infected with miRT-RRVs, there were low levels of GFP expression as well as incomplete viral RNA degradation, however we did not see significant spread of the RRV. The low levels of viral RNA and proteins were likely not sufficient to achieve appreciable spread during the short culture life of these cells, as less than 1% of the virions produced by infected human T-lymphocytes are infectious\textsuperscript{41}.

The PsiNhe vector, with miRT142 insertions in the 3’LTR and ψ, was the optimal vector configuration tested here. RRV incorporating miRT142 in the Psi and Nhe sites were stably restricted in hematopoietic-lineage derived cells \textit{in vitro} and \textit{in vivo}, while maintaining efficient intratumoral replication and prodrug-dependent tumor cell killing. Notably, the \textit{in vivo} spread of RRV-yCD2-PsiNhe was undetectable not only in hematopoietic tissues, but also in the ovaries, indicating that incorporation of miRT142 in these sites results in sufficient restriction of RRV.
replication, in circulating cells, as to prevent spread even to tissues where miR142-3p is not endogenously expressed.

Our results demonstrated the use of miRNA-targeting to improve the tumor specificity of RRVs, and illustrated how vector design can contribute to the efficacy of this strategy in restricting RRV replication. Initial patient data from the Toca511 clinical trials indicate that RRVs in the peripheral blood are well controlled by the innate immune system, however specific detargeting of RRVs from hematopoietic tissues, as demonstrated here, may improve the safety of RRV use in immune-compromised patients, thus expanding possible treatment options. Combined therapies using Toca511 with radiation or temozolomide (TMZ) have been investigated, and survival advantage is achieved in both cases\textsuperscript{25,26}, but the effect of radiation and TMZ on systemic biodistribution of RRV has not been evaluated. Additionally, a third clinical trial was recently initiated, employing IV injection of RRV. miRT-RRVs may be useful in this context if delivery of high titers of RRVs directly to the blood stream results in increased off-target toxicity or prolonged lymphoid infection. The effect of miRT142 incorporation on the host immune response to RRVs should be further investigated, however. While there was no observed reduction in anti-MLV antibody titers in the sera of immune competent mice implanted with RRV-infected subcutaneous tumors\textsuperscript{46}, incorporation of miRT142 into an AAV vector carrying the ovalbumin protein resulted in reduction of the transgene-directed immune response in muscle tissue\textsuperscript{60}. Limiting the host immune response against IV-injected RRV may allow higher titers of virus to ultimately reach the tumor site, but it may also contribute to greater systemic spread of the virus and the possibility of off-target effects. Further tumor specificity may also be achieved by incorporating multiple miRNA target sequences, specific for a variety of tissues, or by combining this miRNA targeting strategy with other vector targeting mechanisms, such as tissue-specific promoters.
Figure 2-1. a) Schematic of miRT142 insertion sites (indicated by striped bar) in the RRV genome. For PsiA-PsiG sites, the underlined sequence corresponds to the permissive region identified by Fisher and Goff\textsuperscript{18}, and lines indicate nucleotides that were unchanged during creation of the miRT142 sequence; b) replication kinetics, in PC3 cells, of RRV-GFP-142Mlu, RRV-GFP-142Nhe and parental vector, RRV-GFP; c) replication in PC3 cells of RRVs containing a single copy of miRT142 in the ψ packaging region; e) replication kinetics in Sup-T1 cells of RRV-GFP-142PsiB, RRV-GFP-142PsiC and parental vector, RRV-GFP. Data is presented as individual replicates.
**Figure 2-2.** a) Schematic of vectors containing multiple copies of miRT142 (striped bars); b) replication kinetics in U87 cells transduced at an MOI of 0.01; c) proviral vector copy number per cell relative parental vector, RRV-GFP, in U87 cells; d) viral RNA expression per proviral copy relative the parental vector, in U87 cells; e) replication kinetics in Sup-T1 cells transduced at an MOI of 0.1; f) proviral copies per cell relative parental vector, in Sup-T1 cells; g) viral RNA expression per proviral copy relative the parental vector, in Sup-T1 cells; h) replication kinetics in human PBMCs spinoculated twice at an MOI of 4; i) proviral copies per cell relative parental vector, in PBMCs; d) viral RNA expression per proviral copy relative the parental vector, in PBMCs.
Figure 2-3.  

a) Replication kinetics during passages 2, 5 and 7, of miRT-RRVs and parental vector in U87 cells;  
b) Transgene stability assay after each passage in U87 cells;  
c) Proviral vector copy number per cell, relative parental vector, RRV-GFP, in Sup-T1 cells infected with supernatant from U87 cells after eight serial passages.
Figure 2-4. a-c) Proviral vector copy number per 100,000 cells in blood (a), bone marrow (b), and spleen (c) of nude mice 55 days following IV-administration of RRV. One-way ANOVA was performed for statistical analysis *. p<0.05, **: p<0.01, ****: p<0.0001, ns: not significant.
Figure 2-5. a) MTS assay in fully transduced U87 cells after 9 days exposure to various concentrations of 5-FC; b) kaplan-meier survival curve of mice with implanted intracranial U87 xenograft tumors, injected with indicated RRV, and treated with five cycles of either PBS or 5-FC; c-f) proviral vector copy number per 100,000 cells in (c) tumor, (d) bone marrow, (e) spleen, and (f) ovaries of mice treated with 5-FC. One-way ANOVA was performed for statistical analysis ***: p<0.005, ****: p<0.0001
Supplementary Figure 2-1. a) Mean fluorescence intensity of GFP+ U87 cells; b) transgene stability assay in Sup-T1 cells for eight weeks following infection; c) transgene stability assay in PBMCs 10 days post-infection; d) mean fluorescence intensity of GFP+ PBMCs, 10 days post-infection.
Supplementary Figure 2-2. a) Sequencing across the miRT insertion sites of miRT-RRV after single passage in U87 cells; b) sequencing across the miRT insertion sites in RRV-GFP-PsiMlu and RRV-GFP-142PsiMluNhe after two passages in U87 cells; c) sequencing across the miRT insertion sites in RRV-GFP-142MluNhe and RRV-GFP-142PsiNhe after eight passages in U87 cells.
Chapter 3. Investigation of factors affecting the spread of retroviral replicating vectors in primary human glioblastoma cell lines.

Despite the demonstrated ability of RRVs to spread effectively through implanted intracranial tumors in rodent models\textsuperscript{1,14,16–18}, the replication of RRVs in patient glioblastoma (glioma) tumors may be affected by aberrant and heterogeneous gene expression in the tumor microenvironment, which cannot be perfectly recapitulated \textit{in vitro} or in animal models. Changes in the expression levels of viral receptors or anti-viral response genes may limit RRV replication\textsuperscript{15}. For instance, the retroviral restriction factor, tetherin (aka BST2, CD317), was found to be highly upregulated in tumors from patients with glioblastoma multiforme (glioma)\textsuperscript{28}. Here we investigated the expression level of key factors, involved in promoting or restricting viral replication, in a panel of glioma cell lines derived from patient tumor isolates. Similar to previously published data, we observed an overexpression of tetherin in all cell lines tested, and correlated this with impaired cell-free transmission and reduced viral titers in glioma cells that were infected with RRVs. Further evaluation is necessary to confirm the role of tetherin in restricting RRV cell-free spread in glioma cells and may help develop clinical trial patient eligibility criteria, or inform the design of novel RRVs with increased tumor transduction efficiency.
Introduction

Efficient spread of oncolytic viruses through solid tumors is essential for the successful treatment of cancer, and achieving sufficient tumor infection before immune clearance of the virus remains a major obstacle to the field. The tumor specificity of many oncolytic viruses, is derived, in part, from the generally immune-suppressed environment of the tumor compared to normal cells. Strongly immunogenic oncolytic viruses, or those in which genes required for immune system avoidance have been removed, can be quickly cleared by the immune system in normal cells, and therefore replicate selectively in tumor cells, as reduced interferon (IFN) signaling is common to many cancer types, even in early stages of the disease. However, oncolytic viruses that retain the ability to avoid the immune system are capable of greater intratumoral spread, suggesting that IFN signaling is not completely absent from tumor cells, and may still limit viral replication.

Unlike many oncolytic viruses, RRVs do not induce, and even actively inhibit, induction of a cellular IFN response. The tumor selectivity of RRVs is thought to arise mostly from the absolute requirement for active cell division, as well as from high levels of antiviral proteins, such as APOBEC3G, and/or robust antibody responses in actively dividing normal cells. High levels of gene transfer from RRVs have been observed in both syngeneic and xenograft mouse models, however these models do not perfectly reproduce the heterogenous tumor microenvironment of glioma patients, and early clinical trial data suggests that intratumoral spread of RRVs may still be somewhat limited (unpublished data).

Multiple immune components could be involved in the progression or restriction of RRV replication in human gliomas. Efficiency of RRV cell entry is dependent on the availability of cell surface receptors that are required for viral fusion. Amphotropic MLV, and MLV pseudotyped with a gibbon ape leukemia virus (GALV) envelope, bind to the inorganic phosphate
transporters, PiT2 and PiT1, respectively\textsuperscript{63,64}, and the expression of these receptors on primary human glioma cells has not been investigated. Intratumoral spread of RRVs may be affected by the expression of antiviral immune factors, including proteins involved in double-stranded RNA sensing, such as Protein Kinase R, interferon regulatory genes, and retroviral restriction factors. At the time of the experiments presented in this section, there were three retroviral restriction factors known to be potent inhibitors of MLV replication: (1) members of the APOBEC3 family of cytosine deaminases, particularly APOBEC3F (A3F) and APOBEC3G (A3G), which inhibit viral replication by hypermutating the viral genome during the reverse transcription step, (2) tetherin (BST-2, CD317), a membrane protein that prevents budding of progeny virus from the cell surface, and (3) TRIM-5α, which interacts with the retroviral capsid protein and promotes premature uncoating of the virus, thus blocking replication before viral integration\textsuperscript{65}. Whether the expression levels of these factors within glioma tumors are high enough to significantly impair RRV replication is unknown, however, Wainwright \textit{et al} found that tetherin mRNA levels in human glioma tumor samples were 355\% higher than those in normal brain tissue, and tetherin protein levels were also increased\textsuperscript{28}.

Here we investigated the expression levels of several key antiviral response proteins and viral receptors in a panel of human glioma cell lines derived from patient tumor isolates, and examined the replication kinetics of RRVs in these same cells. Our data confirmed previous reports that tetherin is highly overexpressed in glioma cells and strongly suggested that this expression restricted the cell-free spread of MLV. We also identified a novel restriction factor, PKR, which may be overexpressed in glioma cells, and confirmed the general down regulation of IFN regulatory genes and IFN-stimulated proteins.
Materials and methods

Tissue microarray. Data analysis was performed by Ascia Eskin at the UCLA DNA Microarray Core Facility. We compared 284 glioma samples with 144 normal samples on the U133P2 and U133A arrays. Samples were compared for expression of the following genes: SLC20A1, SLC20A2, APOBEC3G, APOBEC3F, TRIM5, BST2, TMEM173, EIF2AK2, DDX58, IFNA1, IFNB1, IRF1, IRF2, and IRF7. All data was normalized using RMA to the same 50 files.

qRT-PCR. All qPCR experiments were carried out on the iQ5 thermal cycler (Biorad). RNA was isolated from cells using the Aurum Total RNA Mini Kit (Biorad). RT-PCR was performed using the High Capacity cDNA Reverse Transcription Kit (ABI). Expression of tetherin was measured by qPCR using primer Hs00171632_m1 (Applied Biosystems) and standardized to β-actin expression using the \(2^{-\Delta\Delta Ct}\) method.

Cell culture. Primary glioma cell lines were generously provided by Dr. Harley Kornblum’s lab at UCLA. Cells were maintained in non-serum media (250ml Neurobasal medium (Gibco), 250ml DMEM/F12 (Gibco), 5ml B27 supplement (Gibco), 2.5ml N2 supplement (Gibco), 0.5ml Poly-L-glutamine, 5ml Pen/Strep, and 3.4µl filtered β-mercaptoethanol), to prevent differentiation. For adherent cell culture, cells were grown on 6-well plates or 10cm dishes coated with 15µg/ml poly-L-ornithine (Sigma) and 5µg/ml laminin (BD Biosciences).

Plasmids. RRV-GFP, also known as pAC3-emd, was constructed as previously described. LV-CMV-hvpu-IRES-emd, was generously provided by Tocagen Inc.

Virus production. Virus stocks were produced by transient transfection of 293T cells using the calcium phosphate precipitation method. Briefly, \(5 \times 10^6\) 293T cells were seeded on 0.01% Poly-L-Lysine coated plates. The following day, cells were transfected with 22.5 µg of plasmid. Approximately fifteen hours post transfection, cells were washed with PBS and incubated with fresh DMEM medium containing 20mM HEPES and 10mM Sodium Butyrate. Approximately 24 hours post-transfection, cells were washed again with PBS and incubated with fresh DMEM with

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20mM HEPES. Viral supernatant was collected approximately 40 hours post transfection and filtered through a 0.45 µm syringe filter unit. Aliquots were stored at -80°C.

**RRV replication kinetics.** Replication kinetics of RRV-GFP were monitored by flow cytometry for the GFP transgene. Kinetics plots were obtained by plotting the number of GFP positive cells over time. All flow cytometric data were acquired on a BD FACSCanto II cytometer running BD FACSDiva (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (TreeStar, Ashland, OR). For the glioma cell kinetics experiment, 2e5 cells were plated on 6-well plates, then infected, 24 hours after plating, with 5µl RRV-GFP at a concentration of ~5e6 TU/mL (titer determined on U87 cells). For the cell-cell transmission experiment, 2e5 naïve 350 cells, mixed with 2e3 350 cells fully infected with RRV-GFP, or 5e4 U87 cells, mixed with 5e2 U87 cells fully transduced with RRV-GFP, were plated on 6-well plates. For the cell-free transmission experiment, 2e5 350 cells, or 5e4 U87 cells were plated on 6-well plates and incubated with 40µl supernatant from fully infected, confluent, 350 cells or U87 cells, respectively.
Results

Antiviral restriction factors tetherin (Bst2), and protein kinase R (PKR), are overexpressed in human glioblastoma samples.

To identify factors that may affect the ability of RRVs to infect primary glioma tumor cells, we compared the expression levels of key anti-viral response genes, viral restriction factors and viral receptors, in normal tissues and in tissues samples isolated from patient tumors (Figure 3-1a). Quantification of microarray data (Table 3-1) notably revealed significant upregulation of the retroviral restriction factor bone marrow stromal antigen 2 (Bst2, aka tetherin, CD317), which interacts with budding viruses and prevents their release from the cell surface\textsuperscript{66,67} as well as the double-stranded RNA response protein, protein Kinase R\textsuperscript{68}. Importantly, both inorganic phosphate transporters PiT1 and PiT2 were also significantly overexpressed, suggesting that cellular entry may not be a point of major replication restriction. Additionally, interferon alpha (IFNa) and beta (IFNb), and interferon regulatory factors (IRFs) were downregulated in glioma samples compared to normal tissues, in accordance with the general repression of immune factors in tumor cells\textsuperscript{62,69}.

RRV replication kinetics in primary glioma cell lines correlate with tetherin expression levels.

The upregulation of tetherin in the glioma samples analyzed by microarray was intriguing, as tetherin expression has been shown to increase during brain tumor progression\textsuperscript{28}, and has been correlated with poor clinical prognosis in high grade breast cancer cells and oral cavity cancer\textsuperscript{70,71}. Tetherin is a potent restrictor of MLV replication and is the major component of the interferon-activated anti-viral immune response in NIH/3T3 cells\textsuperscript{67}. We therefore investigated the tetherin expression levels and rate of RRV infection of a panel of glioma primary cell lines derived from human tumor isolates. Overall, the cell lines were moderately susceptible to infection by RRVs, requiring 9-20 days for complete infection (Figure 3-2a). We quantified
tetherin expression in naïve cells by qRT-PCR and normalized against the U87 glioma cell line. Tetherin expression was upregulated in all of our glioma cell lines, with expression ranging from 5-fold to over 1000-fold overexpressed compared to U87 cells (Figure 3-2b). Furthermore, the rate of RRV infection correlated loosely with tetherin expression levels, with the 296 cell line expressing the highest levels of tetherin and taking longest to infect, while the 206 and 350 cell lines expressed low levels of tetherin and were most readily infected by RRV (Figure 3-2a, b).

**RRV titers in the supernatants of primary glioma cell lines are low.**

Tetherin inhibits the replication of enveloped viruses by anchoring budding virions to the cell membrane, however cell-cell transmission can still occur, thus allowing spread of virus through cell culture. To measure the amount of RRV released into the supernatant of infected cells, we passaged supernatant at 2.5% from fully infected glioma cells onto U87s. Detectable levels of infectious virus were only observed in the supernatant of 350 cells, which had the lowest tetherin expression levels of the cells tested in this experiment (Figure 3-3a). To confirm the differences in cell-cell and cell-free transmission of RRV in the glioma cells, we measured replication kinetics of RRV-GFP in 350 cells after either co-incubation of naïve cells with 1% infected cells (cell-cell transmission), or passage of 2% supernatant from fully infected 350 cells onto naïve 350 cells (cell-free transmission). As a control, we performed the same experiment on U87 cells, which have qualitatively similar rates of RRV spread by both cell-cell and cell-free transmission. During cell-cell transmission, RRV kinetics in 350 cells were similar to those in U87s (Figure 3-3b). However, during cell-free transmission the replication kinetics in 350 cells were significantly delayed (Figure 3-3c), which is consistent with the phenotype associated with tetherin restriction. In an attempt to recreate, in U87 cells, the phenotype observed in our glioma cells, we created a non-replicating lentiviral vector expressing tetherin. Despite high transfection efficiency during viral production, no virus was recovered from the supernatant of transfected 293T cells, likely due to plasmid overexpression of tetherin preventing virus release.
Knockdown of tetherin expression may be toxic to glioma cells.

To further investigate the role of tetherin in the reduced viral production, and delayed cell-free transmission of RRV, in our glioma cells, we attempted to knockdown tetherin expression in these cells. The HIV accessory protein, Vpu, efficiently restricts tetherin function in HIV-infected cells by sequestering the protein to cellular compartments in the cytoplasm, thus preventing expression of tetherin on the surface of the cell\textsuperscript{73}. We therefore acquired LV-CMV-hVPU-IRES-emd (LV-VPU) a non-replicating lentiviral vector encoding Vpu, and transduced 350 cells. Transduction by LV-VPU resulted in significant cell death (Figure 3-4) that precluded the possibility of further experiments.
Discussion

Here we examined, for the first time, the replication of RRVs in primary human glioma cell lines, and investigated factors that may restrict the spread of RRVs in these cells. Our results corroborate the finding of Wainwright et al, that tetherin is overexpressed in glioma cells, and these studies lay the framework for further investigation into the effect of this overexpression on the ability of RRVs to completely transduce tumors.

The phenotype we observed in our glioma cell lines during RRV infection (replication kinetics correlating with tetherin expression levels, and reduced viral titers in supernatants of infected cells) is consistent with tetherin-mediated restriction of RRV replication, however, we have thus far been unable to prove this connection. The significant cell death we observed following treatment of 350 cells with LV-VPU suggested that tetherin expression may be required for the in vitro culture life of these cells. In support of this, Sayeed et al found that knockdown of tetherin expression in primary human breast cancer cells lines resulted in decreased cell proliferation and enhanced sensitivity to pro-apoptotic drugs. Conversely, Wainwright et al found no significant difference in overall survival between mice implanted with GL261 mouse glioma cells that had been treated either with anti-tetherin shRNA, or scrambled shRNA. While GL261 cells do overexpress tetherin, it is possible that this model does not adequately represent the level of expression or function of tetherin in human glioma. Therefore additional research into the effects of tetherin silencing in primary human glioma cells is warranted, as our observations suggested that tetherin may not only limit RRV replication, but may also be a valuable target for the treatment of glioma. Due to the extremely high levels of tetherin expression in some of our primary glioma cell lines, traditional methods of RNAi-mediated gene silencing, or even over-expression of Vpu, may not be sufficient to relieve the block to cell-free transmission of RRVs in these cells, therefore alternative methods of gene silencing, such as CRISPR-mediated gene editing, should be investigated.
While it is clear that tetherin is a potent inhibitor of cell-free transmission of enveloped viruses, its effect on cell-cell transmission is unclear. Enhanced cell-cell transmission of HIV-1 as a result of tetherin expression has been reported\textsuperscript{74}, however, reduction of tetherin expression did not have a significant impact on the cell-cell transmission of HTLV-1\textsuperscript{72}. The relative contribution of cell-cell and cell-free transmission to the efficiency of RRV replication in solid tumors is unknown. However, given the recent initiation of two additional clinical trial formats, in which RRV is injected either into the margins of a resected tumor, or intravenously, the ability of RRVs to spread to cells that are not in direct contact with infected cells may be of increased importance.

The biological rationale for the overexpression of tetherin in glioma and other cancers is unclear. The high levels of tetherin, which is strongly induced by IFN signaling\textsuperscript{66}, in our primary glioma cells was particularly intriguing in light of the fact that microarray analysis showed an overall downregulation of IFN activity, consistent with the idea that defects in IFN signaling are common in the tumor microenvironment\textsuperscript{62}. In addition to its induction by IFN, tetherin is involved in a negative feedback loop that modulates IFN expression levels; tetherin interacts with immunoglobulin-like transcript 7 (ILT7) on plasmacytoid dendritic cells (pDCs) resulting in a reduction in type I IFN release by these cells\textsuperscript{75}. Infiltrating pDCs have been found in multiple cancers, including breast cancer, where infiltrating pDCs have a reduced capacity for IFN-α production, and are associated with poor clinical prognosis\textsuperscript{76}. Therefore, overexpression of tetherin in cancer cells may reduce IFN signaling from infiltrating immune cells, thus contributing to tumor tolerance.

Microarray analysis also revealed upregulation of another IFN-activated protein, PKR, the overexpression of which in cancer cells is odd, given its proposed role as a tumor suppressor
and cell growth regulator. However, increased expression of PKR has also been reported in primary breast cancer cells and breast cancer cell lines, therefore, further investigation into the expression of PKR in primary glioma cells is warranted. In response to dsRNA, PKR phosphorylates eukaryotic initiation factor 2 (eIF-2α), which results in inhibition of protein translation, therefore it is likely that PKR present in cancer cells is in an inactive or repressed state. The effect of RRVs on the activation state of PKR is unknown, however we did not observe any changes in cell proliferation following RRV infection in our primary glioma cells, therefore any PKR expressed in those cells was presumably not activated by RRV infection. We believe it is unlikely that PKR overexpression contributes to the phenotype of reduced cell-free transmission of RRV that we observed in our primary glioma cells, however, our microarray results need to be corroborated by qPCR analysis in individual cell lines before conclusions can be drawn. Activation of latent PKR in glioma cells may represent a novel approach to glioma treatment, therefore this finding has relevance independent of our interest in factors affecting RRV replication.

Here we investigated the expression of several key factors involved in the progression, or restriction, of viral replication, and the affect of one of these factors, tetherin, on the replication of RRVs in primary human glioblastoma cells. It is likely that multiple cellular factors play roles in either restricting or promoting the spread of RRVs in glioma cells, and the limited analyses that we performed here do not presume to be exhaustive, however, our results showing reduced cell-free spread of RRV in our primary glioma cells, strongly point to the involvement of tetherin in restricting RRV budding. Further experiments are necessary to elucidate the effect of tetherin on both cell-free and cell-cell transmission of RRV, and to explore the implications of tetherin-restriction on RRV-mediated gene therapy in vivo. The results generated from these studies and other studies may ultimately be used to stratify patient enrollment in clinical trials, to tailor the
use of RRV-mediated gene therapy to patients with specific tumor expression profiles, or to inform the development of novel RRVs with enhanced tumor transduction capacities.
Figure 3-1. Differential expression in glioma cells of genes related to viral replication. (a) Heat map showing expression of genes (vertical) in 144 normal tissues and 284 glioma samples (horizontal). (b) Quantification of microarray data. P values were calculated using t-tests to compare the means of both data sets for each probe set.
Figure 3-2. RRV replication kinetics in primary glioma cell lines correlate with tetherin expression levels. (a) Spread of RRV-GFP in glioma cell lines as measured by flow cytometry. (b) Expression of tetherin in glioma cells as measured by qRT-PCR.
Figure 3-3. Cell-free transmission of RRV is impaired in glioma cells. (a) Supernatant from the indicated cell lines was passaged onto U87 cells, AZT was added 24 hours post-infection to halt viral replication, and then cells were analyzed by flow cytometry at 48 hours post-infection. GFP fluorescence is indicated on the x-axis. (b) Replication kinetics of RRV-GFP in 350 and U87 cells during cell-cell transmission (c) Replication kinetics of RRV-GFP in 350 and U87 cells during cell-free transmission
Figure 3-4. Transduction of 350 cells with lenti-Vpu is cytotoxic. (a) Naïve 350 cells (left) and 48 hours following transduction with lenti-Vpu.
Conclusions and Future Directions

Replicating retroviral vectors are an emerging technology that show great promise for the treatment of cancer. Interim data from Toca511 clinical trials show increased overall and progression-free survival at 6 months, compared to historical benchmarks for recurrent high grade glioma, and the initiation of phase II/III trials is expected in 2015. In light of the therapeutic potential of RRV-mediated pro-drug activator gene therapy, we are investigating mechanisms to improve both the tumor specificity and the tumor-specific infection efficiency of the virus. Here we developed novel RRVs whose replication is restricted in a tissue-specific manner, and identified cellular factors whose overexpression in glioma may limit the intratumoral spread of RRVs.

Improving RRV tumor-specificity

The use of miRNA “detargeting” to regulate the replication of RRVs has not been previously investigated. We showed that miR142-3p target sequences incorporated in the RRV genome effectively restricted RRV replication in hematopoietic lineage-derived cells in vitro and in vivo, without reducing intratumoral RRV spread or cytotoxic effect, or inhibiting the host anti-MLV immune response. Similar to other groups who have used this approach to regulate the spread of viral vectors based on RNA viruses, we observed mutation of the miRTs, after repeated replication cycles, which in some cases resulted in the emergence of escape mutants that evaded restriction. The insertion of miRTs into conserved regions of the viral genome has been proposed as a method to reduce the likelihood of miRT mutation, and here we showed the first direct comparison between vectors containing miRTs in different genomic locations, including in a novel site in the MLV ψ packaging signal. Our data revealed that the rate of miRT mutation, and the selective pressure for escape mutants, was affected by the location of the miRT142 insertions in the RRV genome, and that the effects of miRNA-mediated restriction on RRV replication could be prolonged by insertion of the miRTs into functional vector...
elements. Here we created miR142-3p target sequences in the ψ packaging signal by mutation of the wild-type sequence, which affected viral replication in some cases. Generating miRTs in coding regions of the virus may be an alternative approach to preventing the emergence of escape mutants, particularly if the miRT can be created without causing significant changes to the amino acid sequence. Although the exact miRT insertion sites investigated here cannot be directly applied to other viral vectors, careful vector design will likely improve the durability of miRNA-mediated restriction in other systems as well. Therefore similar evaluations of miRT insertion sites should be conducted in any context where the potential for escape mutants exists.

It was not within the scope of this work to determine the mechanism of escape mutant formation, or selection, in RRVs where revertant viruses emerged. However, further examination of miRT-RRVs with defective replication and reduced stability (those with miRT142 in the Psi and Mlu sites) may help guide future development of miRT-RRVs. For instance, RRVs with target sequences for two different miRNAs in the Psi and Mlu sites may not exhibit the same phenotype as those with miRT142 in both sites, which could then allow insertion of up to three miRTs in a single vector. The use of target sequences corresponding to different miRNAs may allow restriction of RRV replication in multiple tissues, or redundant regulation within a single tissue. Expression of miR-7 is downregulated in glioma compared to normal brain tissue, thus the target sequence for miR-7 may be a good candidate for incorporation into RRVs. However, before the inclusion of miRT sequences in vectors for clinical use, the expression of the cognate miRNAs in human primary tumors should be quantified to ensure that intratumoral spread of the vector is not inhibited.

As clinical trials of Toca511 progress, escalating dose levels, and new modes of RRV delivery, may increase the potential for systemic spread of the virus, and off-target toxicity. Additionally,
novel RRVs, designed for greater infectious potential, are currently in pre-clinical development. For instance, we are currently investigating the use of RRVs encoding the gibbon ape leukemia virus envelope, which infects human hematopoietic cells at much higher rates\textsuperscript{81}. Therefore, mechanisms to prevent or reduce the replication of RRVs in non-cancerous cells, such as the one presented here, are becoming increasingly important. As novel RRV configurations are developed, the efficacy of miRNA-mediated restriction will need to be reevaluated. We are also currently investigating alternative methods of improving the tumor-specificity of RRVs, such as the incorporation of tissue-specific promoters. Therefore, if in the future miRNA-mediated restriction is not sufficient to suppress extratumoral RRV replication, additional targeting methods can be incorporated.

**Investigation of factors affecting RRV intratumoral spread**

Despite the promising initial results from Toca511 clinical trials, there are still barriers to the effective delivery of RRVs to tumors. A better understanding of the tumor microenvironment and the effect of cellular factors on RRV replication is important for maximizing the efficiency of gene transfer to tumor cells. Here we presented the first investigation into RRV replication kinetics in primary cell lines generated from patient glioma tumors, and quantified the expression levels, in these cells, of factors that may play a role in limiting RRV spread. Our results demonstrated that the cell-free spread of RRVs is restricted in glioma cells, and implicated the overexpression of tetherin in creating this phenotype, however we were unable to confirm this connection. Previously published data from other groups showed that tetherin is highly overexpressed in glioma cells\textsuperscript{28} and that tetherin overexpression correlates with poor clinical outcomes\textsuperscript{70,71}, therefore further investigation into the effect of tetherin expression on RRV replication, and the role of tetherin in promoting growth of glioma tumors, is essential.
In our experiments, attempted knockdown of tetherin expression, by overexpression of Vpu, was extremely cytotoxic, which is consistent with previously published data demonstrating an anti-apoptotic effect of tetherin in primary breast cancer cells. If confirmed, this would make tetherin an exciting potential target for the treatment of multiple cancers in which tetherin overexpression has been observed. An important next step in this process will be investigating whether the cytotoxic effect we observed in vitro is reproducible in vivo. To this end, a method for the in vivo knock down of tetherin expression in intracranial xenograft tumors will need to be developed. Due to the extremely high levels of tetherin expression in some of our primary glioma cell lines, traditional methods of RNAi-mediated gene silencing, or even over-expression of Vpu, may not be sufficient to effect noticeable changes in tumor proliferation in vivo, therefore alternative methods of gene silencing, such as CRISPR-mediated gene editing, should be investigated as well.

Continued troubleshooting of methods to knockdown tetherin expression in vitro is necessary, as glioma cells that naturally overexpress tetherin are the best venue for investigating the effects of tetherin on RRV replication. As an alternative approach, tetherin could be overexpressed in the U87 glioma cell line in order to recreate the phenotype observed in our glioma cells. Attempts to create a lentiviral vector expressing tetherin failed, presumably due to tetherin expression preventing virus release from transfected cells. To address this issue, an inducible vector system, or a replication dependent tetherin gene could be employed. If the link between tetherin expression and cell-free spread of RRVs can be confirmed, it will be important to understand the relative contribution of cell-cell and cell-free spread of RRVs in tumor cells. Cell-cell spread appears to be a reasonably efficient mode of RRV movement between cells, as this was the major route of viral infection in our glioma cell lines. However, the inhibition of cell-free spread may be more detrimental to the spread of RRVs in vivo, particularly if there are multiple distant tumor sites involved. Generation of an in vivo model of intratumoral
tetherin knockdown, as discussed above, would also allow for further investigation into the effects of tetherin expression on RRV replication.

Understanding the role of tetherin in glioma cells, and its effects on RRV spread may inform the development of novel RRVs with enhanced tumor-infection capabilities. Inserting a Vpu expression cassette in the RRV could abrogate any tetherin-mediated blocks to RRV replication, or pro-tumor effects of tetherin expression. The consequences of this enhanced infectivity would need to be carefully considered however, as tetherin may play a significant role in the restriction of RRV replication in normal tissues. Alternatively, if tetherin proves to be a prognostic indicator of the efficacy of RRV-mediated gene therapy in patients, analysis of tetherin expression in tumor biopsies could serve as a selection criterion for enrollment in Toca511 clinical trials.
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