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Research Article

DESIGN AND GENERATION OF A RECOMBINANT GAMMAHERPESVIRUS ENCODING shRNA FROM A NATIVE VIRAL tRNA PROMOTER

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Abstract: Gammaherpesviruses are associated with multiple types of tumor development and understanding the pathogenesis of these viruses has been the subject of various studies. Throughout the lytic and latent life cycle, these viruses utilize numerous virally encoded microRNAs (miRNAs) to regulate the key mechanisms of the infected cell in their favor. Therefore, it is important to understand the miRNA and their mRNA target interactions for developing better therapeutics. In this study, the strategy and design of a recombinant virus expressing a short hairpin RNA (shRNA) element targeting the host B-lymphocyte-induced maturation protein 1 (Blimp1) transcript was evaluated. Here we have shown that viral tRNA-driven expression of anti-Blimp1 shRNA is able to reduce the target gene expression at a statistically significant level as assessed by luciferase assay during virus infection. This proof-of-principle experiment provides a means to study important miRNA-mRNA interactions *in vivo*. Further, the very short promoter of the murine gammaherpesvirus 68 (MHV68) viral tRNA (vtRNA4) has the ability to generate two shRNAs from a ~180 nucleotide sequence. If there is a size limit for the shRNA construct, viral tRNA promoter provides an effective shRNA expression system.

Özet: Gammaherpesvirüsler çeşitli tiplerde kanserlerin gelişimi ile ilişkilidir ve bu virüslerin patogenezi birçok çalışmaya konu olmuştur. Bu virüsler litik ve latent adı verilen iki farklı yaşam döngüleriyle enfekte hücrenin anahtar mekanizmalarını kendi lehlerine düzenlemek için çok sayıda viral kökenli mikroRNA (miRNA) kodlarlar. Bu nedenle, daha iyi terapötik maddeler geliştirmek için miRNA ve bunların mRNA hedef etkileşimlerinin anlaşılması önemlidir. Burada, viral tRNA tarafından üretilen ve Blimp1 transkriptini hedefleyen, bir shRNA'nın viral enfeksiyon aşamasında hedef genin ifadesini istatistiksel açıdan anlamlı oranda azaltabildiğini lusiferaz deneyi ile gösterdik. Bu deney dizaynı önemli olan miRNA-mRNA etkileşimlerini test etmek açısından bir kavram ispatı sunmayı hedeflemektedir. Ayrıca oldukça kısa bir promotör boyutuna sahip olan Murine Gammaherpesvirus 68 viral tRNA4, yaklaşık 180 nukleotid uzunluğunda bir diziden iki adet shRNA üretebilmektedir. İstenilen shRNA'nın anlatımı için yalnızca sınırlı bir alan mevcutsa, viral tRNA promotrü etkin bir shRNA anlatımı sistemi sunmaktadır.

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Introduction

Gammaherpesvirus infections are highly prevalent in human populations and persistent for the whole life of hosts with a functional immune system (Arvin *et al.* 2007). The persistent infection is called latency, and is characterized by expression of a limited set of viral genes and noncoding RNAs, including microRNAs (miRNAs) (Speck & Ganem 2010). Chronic gammaherpesvirus infections are associated with the development of lymphoproliferative diseases and certain types of lymphomas in humans, particularly in individuals with immune deficiencies, including those with primary immunodeficiencies, HIV infection, and organ transplant patients under immune suppression (Barton *et al.* 2011). Thus, understanding the establishment and maintenance of latency programs is important for developing preventative strategies and treatments for gammaherpesvirus pathogenesis as well designing gene delivery vectors and vaccines.

One category of key regulators of herpesvirus latency is the virally encoded miRNAs (Cullen 2011). miRNAs are short, 20-22 nucleotide long RNA molecules that play a key role in post transcriptional gene regulation (Bartel

2009). Many viral miRNAs have been identified within different viral families including the gammaherpesvirus subfamily (Pfeffer *et al.* 2004, Samols *et al.* 2005, Carnero *et al*. 2011, Jurak *et al*. 2011). Due to the narrow host range of human gammaherpesviruses such as Kaposi's Sarcoma Associated Herpesvirus (KSHV) and Epstein Barr Virus (EBV), the murine gammaherpesvirus 68 (MHV68) offers a small animal model infection system to study viral pathogenesis *in vivo* (Simas & Efstathiou 1998). In recent years, several studies identified the "targetome" of viral miRNAs by utilizing Ago protein immunoprecipitation-linked sequencing based methods (Chi *et al*. 2009, Helwak *et al.* 2013, Bullard *et al.* 2019). Similar to the human gammaherpesviruses, MHV68 encodes multiple miRNAs (Reese *et al.* 2010, Zhu *et al.* 2010). Interestingly though, MHV68 miRNAs are transcribed from viral tRNA promoters as approximately 200 nucleotide long tRNA-miRNA linked elements called tRNA-miRNA Encoding RNAs (TMERs) and processed to become mature miRNAs through tRNase Z and Dicer (Bogerd *et al.* 2010).

The biological importance of a given viral miRNA and its target mRNA or Long non-coding RNA (lncRNA) is mostly understudied and requires new and applicable methods. In order to study specific viral miRNA-mRNA interactions in an animal model, we hypothesized that target mRNA-specific short hairpin RNAs (shRNA) could be used to understand the function of a specific miRNA and mRNA interaction *in vivo*. Since miRNAs can bind and repress more than one target gene, such a shRNA strategy will specifically pinpoint the function of a given miRNA-mRNA interaction.

Generally, shRNAs are designed to produce a 21-22 nucleotide long RNA molecule that is perfectly complementary to the target mRNA while miRNAs bind to their targets with imperfect complementarity. The shRNAs are typically transcribed by a U6 promoter, which is a RNA III dependent promoter, from a plasmid vector (Moore *et al.* 2010). The aim of this study is to generate a recombinant virus as a means to study specific miRNA-mRNA interactions by utilizing shRNA strategy since shRNAs can bind to their target in a much more specific manner as opposed to miRNAs. Therefore, we have tested whether a virally encoded tRNA, carrying a RNA polymerase III promoter, can drive shRNA expression and whether the heterologous shRNAs are functional in suppression of a specific mRNA target. Additionally, we have generated a mutant virus that expresses the designed shRNA from a neutral locus within the viral genome. The shRNAs used in this study were designed to target the mouse *Prdm1* mRNA. The *Prdm1* gene codes for B-lymphocyte-induced maturation protein 1 (Blimp1) protein, which is a transcription factor that drives the transition of B cells from a germinal center (GC) phenotype to a plasma cell phenotype by repressing the expression of GC-specific genes (Calame 2006, Kallies & Nutt 2007). Since MHV68 naturally establishes latency in B cells, this mutant virus is a valuable tool to investigate miRNA-mRNA interactions as a proof-of-concept.

Here, we have shown that two shRNA stem loop structures can be designed and expressed by a minimal viral tRNA promoter. This vtRNA-shRNA construct can functionally reduce the target gene expression at a statistically significant level in the context of viral infection.

Materials and Methods

Design and synthesis of tRNA-shRNAs

MHV68 viral tRNA4 naturally contains two downstream stem loops for miRNAs that we designed to replace with two shRNA hairpins. The first one of the Blimp1 shRNAs was designed using the Invivogen siWizard algorithm (https://www.invivogen.com/sirnawizard/construct.php) (*SiRNA Wizard - Design Hairpin Insert - InvivoGen*, n.d.) by using standard criteria offered by the website. The other shRNA sequence was designed by using the sequence information of a small, interfering RNA (siRNA) that is used and successfully knocked down Blimp1 in a previous study (Zhou *et al*. 2013). siWizard was also used to design the scrambled version of these shRNAs. These sequences were then inserted at nucleotide position 74 of viral tRNA-like (vtRNA4) based on where the naturally occurring miRNA starts as well as the *in silico* folding analysis of vtRNA4 and its miRNAs. The shRNA sequences were separated by a spacer sequence (TTACGT) and inserted in place of viral miRNAs encoded by TMER4. The *in silico* folding analysis of the end construct was performed using the mFOLD algorithm (http://www.unafold.org/RNA_form.php) (*RNA Folding Form for 88.230.102.221*, n.d.). The *in silico* designed sequences of vtRNA4.Blimp1.shRNA or the scrambled version of it were synthetically produced and inserted into EcoRI site of the pUC57 plasmid (Fig. 1a) by Genewiz and send to us as lyophilized. Primers and shRNA/scrRNA sequences are listed in Table 1.

Generation of shRNA-Encoding Plasmids and Viruses

The PCR conditions used in the study are as follows: initial denaturing at 98°C for 30 seconds; 30 cycles of denaturation at 98°C for 10 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 60 seconds per kb; and final extension at 72°C for 5 minutes. An important note is that if longer than 50 nt primers are used for PCR reactions, 10-fold lower primer concentrations such as 1 picomoles per reaction are used to avoid self-annealing occurrences of the primers (Table 2). The ~200 base pair long shRNA and scrRNA sequences were PCR-amplified with primers number 1 and 2 (Table 1) and cloned with a Kanamycin resistance PCR fragment (primer number 3 and 4) into pUC19 vector by using GeneArt Seamless Cloning Assembly kit (Life Technologies) to generate the targeting construct (Fig. 1b, c).

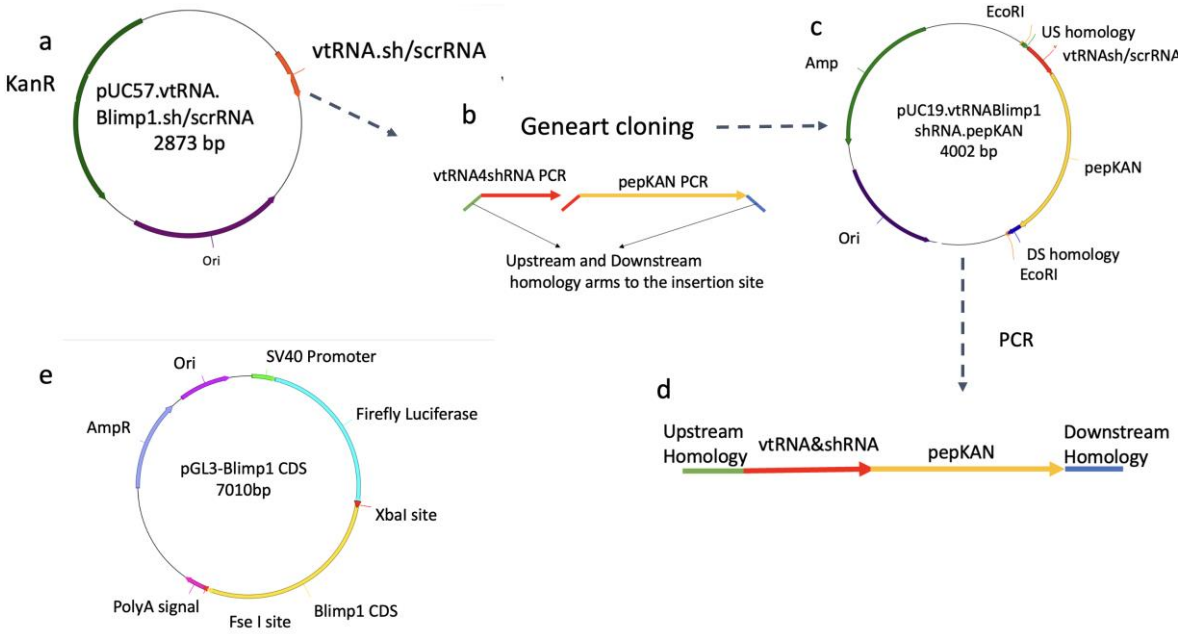

Fig. 1. The cloning strategy and plasmids used in the study. **a.** Viral tRNA and Blimp1shRNA or scrRNA plasmid map (plasmids are ordered from Genewiz), **b.** PCR amplified vtRNA.shRNA amplicon and pepKAN amplicon are used for Gibson cloning, **c.** plasmid map of the targeting amplicon in pUC19, **d.** Targeting construct used to generate shRNA encoding virus, **e.** pGL3-Blimp1 CDS (target region of shRNA) vector used in the luciferase assays.

The recombinant viruses MHV68.Blimp1-sh and MHV68.Blimp1-scr were generated in two stages using two-step Red-mediated recombination onto the wild-type MHV68.ORF73βla BAC backbone (Nealy *et al*. 2010). The shRNA-Kanamycin fragments were then amplified from the plasmids (primer number 5 and 6) with PCR amplicons (Fig. 1d) containing homologous regions of upstream and downstream of the insertion site, which are required for intramolecular recombination. The resulting amplicon was gel-purified, then electroporated into GS1783 *E. coli* cells containing the MHV68.ORF73βla BAC and Red recombinase machinery (Tischer *et al*. 2006). Transformed cells were incubated at 30°C overnight on Kan+ agar plates. DNA from the resulting colonies was isolated and digested with XhoI, then screened for the insertion genomic integrity by gel electrophoresis (GE). In selected positive clones, I-Sce was induced by incubation for 2 hours in 1% arabinose to induce Red recombinase expression for the second recombination. Resulting colonies were again screened by PCR and GE, then exact sequence was confirmed by Sanger sequencing. BAC DNA from the positive clones was isolated with a Qiagen large-construct plasmid purification kit, then transfected into NIH 3T12 fibroblast cells using a TransIT-3T3 transfection kit (MirusBio). A GFP cassette in the BAC DNA was used to visualize the transfection and propagation of the virus. To excise the BAC cassette, the resulting virus from the initial transfection was used to infect NIH 3T12 cells expressing Cre recombinase at an Multiplicity of Infection (MOI) of 0.05, and passaged twice. Titers of final viral BAC-minus virus stocks were determined by plaque assay on NIH 3T12 cells.

Luciferase Assay

The pGL3-promoter vector from Promega was used for firefly luciferase assay. pGL3-promoter vector was digested with Xba I. Putative mRNA target regions were PCR-amplified with primers number 7 and 8 (Table 1) to contain the Xba I site and homology to the pGL3-promoter, and then were incubated with linearized pGL3-promoter vector and the GeneArt Seamless Cloning Assembly kit (Life Technologies). Reactions were then transformed into competent Top10 *E. coli*. The transformants were then Sanger sequenced to validate insertion of the target sequence into the 3' UTR of the pGL3-promoter vector (Fig. 1e). For transfections, 10,000 NIH 3T12 fibroblasts were seeded onto 96-well plates the day before transfection. For shRNA and TMER plasmids, 1ng of pRL-SV40 renilla luciferase vector, 100ng of the pGL3-promoter containing the target sequence in the 3' UTR, and 100ng of the TMER or shRNA vector were transfected with Lipofectamine 2000 (Thermofisher). After 48 hours the efficiency was checked visually by GFP control plasmid transfection, then supernatant fluid was removed from wells, and 50µL of fresh DMEM plus 50 µL of Dual Glo Luciferase Reagent (Promega) was added back. Samples were incubated for 10 minutes, then transferred to 96-well NUNC plates, and firefly luciferase was measured using a plate luminometer. Next, 50 µL of Dual Glo Stop Reagent was added, and renilla luciferase activity was measured. Renilla luciferase activity was used to control the transfection efficiency.

Table 1. List of primers and shRNA sequences. The underlined sequences are the loop sequences of the stem loops in shRNA designs. Italic sequences in shRNA FWD and KAN REV are homologous to the Eco RI site in pUC19 vector. Italic sequences in Blimp1 FWD and REV are homologous to the Xba I site in pGL 3 Promoter vector.

No	Primer Name	Sequence
	Blimp1 scrRNA SL1	ATTGATTCGGGTCAGATCCTCTCAAGAGGAGGATCTGACCCGAATCAA
	Blimp1 scrRNA SL2	GGCGATCAGGAAACGTACGATCAAGAGTCGTACGTTTCCTGATCGC
	Blimp1 shRNA SL1	GAGGATCTGACCCGAATCAATTCAAGAGATTGATTCGGGTCAGATCCTC
	Blimp1 shRNA SL2	TGTTCCTGTTGCCACCGATCTTCAAGAGAGTACGGTGGCAACAGGAACTTTTTTC
1	shRNA FWD	GTAAAACGACGGCCAGTGCCAGGCTTTTATGTGCAACTTTCTATTTTTCTACTATGC ATCTGCTGATTGCCCCTCCGTCGGGGTAGCTCAATTGGTA
2	shRNA REV	TCGTCATCCTAAAAAAAGTTCCTGTTGCCACC
3	Kan FWD	GAACTTTTTTTAGGATGACGACGATAAGTAGGG
4	KAN REV	ATGCCGTACCGAGCTCGTTTGCATGAGGTTGCTAGACTCTCAATAATCAAGGGTCT TTAAAAAAAGTTCCTGTTGCCACCGTACTCTCTTGAAGATCG AACCAATTAACCAATTCTGATTAG
5	shRNA PCR out FWD	CCAGGCTTTTATGTGCAACTTT
6	shRNA PCR out REV	TTTGCATGAGGTTGCTAGAC
7	Blimp1 FWD	GATCGCCGTGTAATTGATTTTCAGAAAATAAGTGTT
8	Blimp1 REV	CCGCCCCGACTCTAGAAAGCATGAACATTTATATT

For the infection and transfection luciferase assay, virus transfections were performed in 12 well plates. 10^5 NIH 3T12 cells were plated into each well of a 12 well plate the day before infection/transfection. For infection first scheme, cells were washed with PBS and infected at multiplicity of infection (MOI) of 5 with 200 µL of media without FBS and incubated at 37°C for 1 hour with gentle mixing every 15 minutes. Infection media was then removed, washed once with PBS and incubated with 400 µL of plasmid lipofectamine mixture for 4 hours at 37°C. At the end of the incubation period, 1mL media was added to each well. If the cells were transfected first, transfection was conducted first and then the cells were infected as described above. 48 hours post infection cells were assayed for luciferase activity as described above. Data were analyzed by either Excel or the Graphpad Prism software.

Table 2. The list of PCR ingredients and concentrations.

Ingredient	Volume in µL
Primers	10 picomoles if primer <50 nt 1 picomole if primer > 50nt
Template	2-10 ng
NEB Q5 Mastermix Enzyme	15 µL
H_2O	Up to 30 µL

Results

The majority of the MHV68 encoded TMERs contain two stem loops which each are capable of generating two mature miRNAs. TMER4 expresses mghv- miR-M1-5-5p and mghv-miR- M1-6-3p from stem loops 1 and 2, respectively. Mghv- miR-M1-5-5p is expressed in both lytically-infected fibroblasts and latent cell lines, and in latently-infected mouse splenocytes *in vivo*. Mghv-miR-M1-6-3p is expressed at very low levels in tissue culture, but readily detected in latently infected mouse splenocytes *in vivo* (Feldman *et al*. 2014). For this reason, the TMER4 promoter element within vtRNA4 was chosen to drive expression of the *Blimp1* targeting-shRNAs. One of the shRNAs was designed by using an online algorithm SiWizard (Invivogen) and the other shRNA sequence was previously shown to inhibit Blimp1 expression *in vivo* (Zhou *et al*. 2013). In order to ensure the proper folding and structure of the construct, the sequences were folded *in silico* by mFOLD (Fig. 2a). The natural miRNA secondary RNA structure shows bulges in the hairpin region, whereas shRNA hairpins were perfectly complementary as expected, and there was no alteration in the predicted tRNA folding with the exchange of sequences (Fig. 2b). The Blimp1 targeting shRNA was tested by luciferase assay and compared to the scrambled RNA for the ability to bind and inhibit the 3' UTR of Blimp1 transcript cloned into the luciferase vector pGL3. The combination of two shRNAs showed a statistically significant (P value <0.001) reduction (approximately 65%) in luciferase assay (Fig. 2c).

Since the shRNA constructs worked efficiently, we then proceeded to generation of the shRNA encoding virus. Briefly, the targeting construct containing a kanamycin resistance cassette and the shRNA or scrambled version was prepared and inserted between TMER1 and TMER2 by Red mediated mutagenesis protocol (Fig. 3a). The resulting viral clones were screened in the gel electrophoresis for the viral BAC integrity (Fig. 3b). The intact clones were selected and viral stocks were prepared after transfecting BAC DNA into NIH 3T12 cells and passaging the virus through Cre recombinase expressing NIH 3T12 cells to remove the BAC cassette from the virus genome.

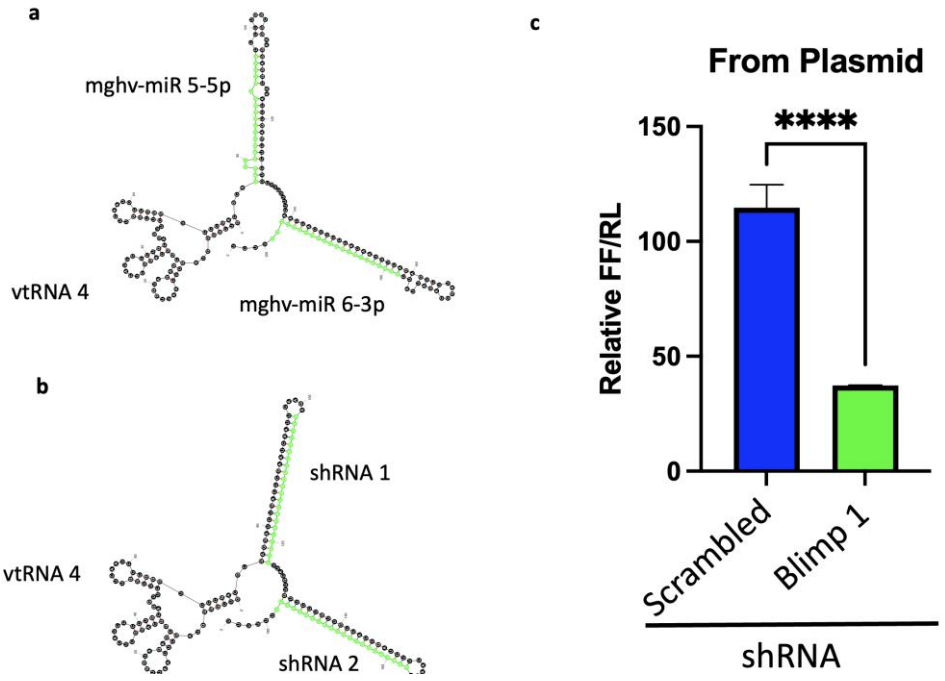

Fig. 2. Design and testing of Blimp1 shRNA and scrambled control. **a.** MHV68 vtRNA4 and corresponding miRNA stem-loop sequences in mFold secondary structure prediction, **b.** vtRNA4 plus two Blimp1-targeting shRNA sequences in mFold secondary structure prediction, **c.** luciferase assay. Vector pGL3 containing shRNA-targeted region downstream of firefly luciferase was transfected along with vector expressing scrambled (blue bar) or anti-Blimp1 shRNA (green bar) and renilla control plasmid into NIH 3T12 cells, and then assayed for luciferase activity. Relative values of firefly over renilla luciferase activity are shown. ****P* value <0.001. FF corresponds to firefly luciferase and RL corresponds to renilla luciferase.

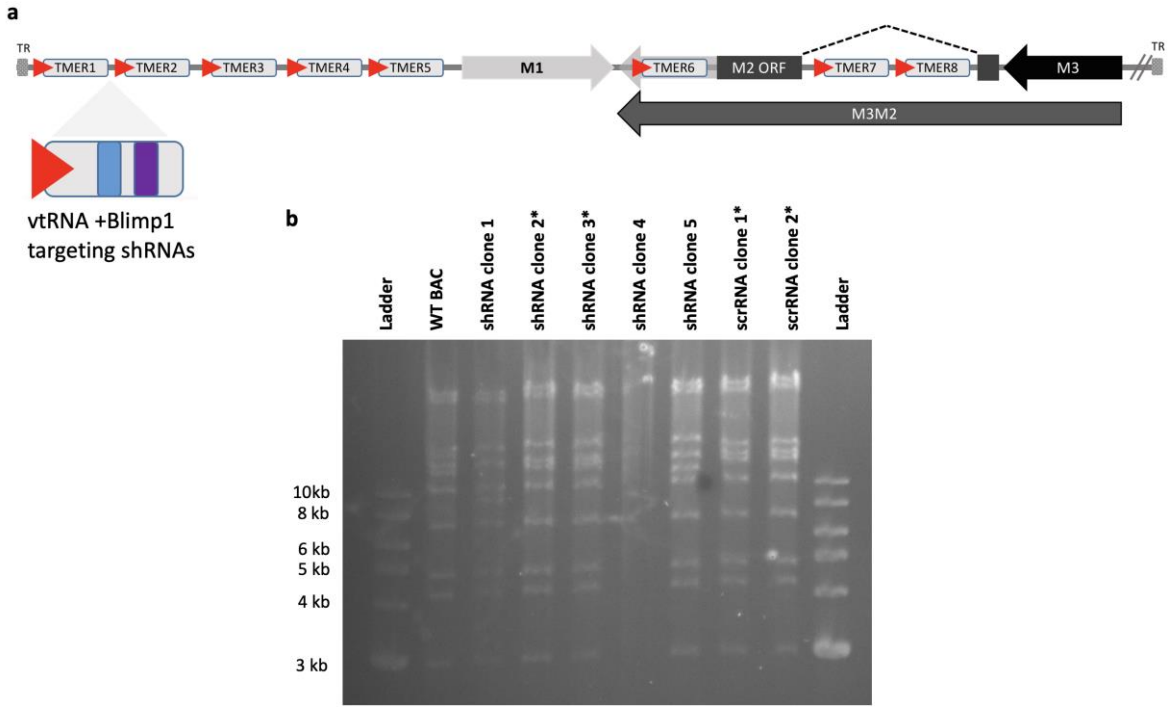

Fig. 3. Schematic representation of the left end of the viral genome and screening of recombinant clones. a. the relative insertion site for the shRNA or scrRNA constructs. vtRNA is viral tRNA. Red arrows are the promoter for tRNAs and blue and purple boxes are shRNA stem loops 1 and 2, respectively, b. after Red recombination, the mutant clones are screened for integrity of the virus and compared to wild type (WT). The ones with * indicate the intact clones which are selected to generate viral stocks.

After the virus stocks were prepared and tittered, we tested the shRNA construct in the context of virus infection. It is expected that the shRNAs are expressed from the virus. However, all other viral components produced from the virus must still be evaluated, because it is conceivable that cells may become stressed during simultaneous virus infection and transfection. Therefore, we tested two different options: infection first and then transfection, as well as the *vice versa* (Fig. 4a). Even though to a lesser degree when compared to the plasmid-produced shRNA, the virally encoded shRNA still showed a statistically significant (*P* value 0.0019) reduction about 50% in the luciferase activity (Fig.4b).

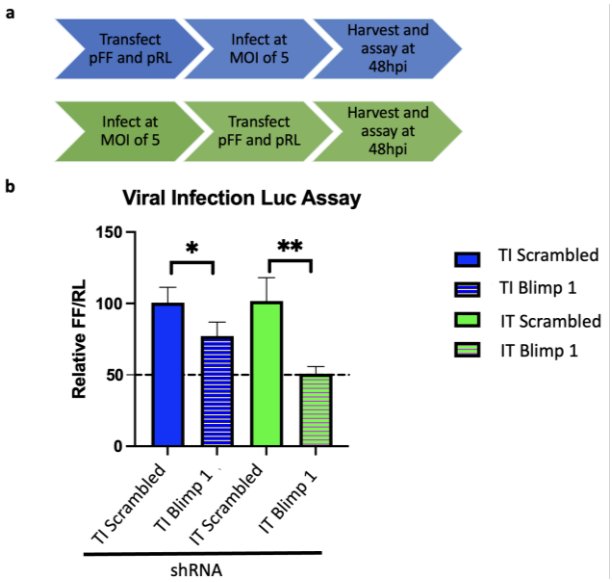

Fig. 4. Transfection and infection scheme and luciferase assay. **a.** Infection first followed by transfection second, or *vice versa* approach, was used to test shRNA constructs. pFF is firefly luciferase plasmid containing the shRNA target sequence and pRL corresponds to renilla luciferase plasmid. MOI indicates the multiplicity of infection, **b.** 5 plaque forming unit (pfu) virus used per cell in the assay and luciferase readings are recorded at 48 hours post infections (hpi). Y axis grids indicated the 100% and 50%. Data is displayed as relative values of firefly luciferase (FF) over renilla luciferase (RL) activity. TI is transfection infection, IT is infection transfection. *P* value * 0.0186, ** 0.0019.

Discussion and Conclusion

Defining the biological role of viral miRNA targeting of a transcript *in vivo* is one of the key aspects to study miRNA function in a relevant pathogenesis model. One means to examine the role of regulation of a specific target transcript (as opposed to other targets of the same miRNA) is to knock down that transcript more precisely using shRNAs, ideally expressed from the virus in place of the miRNA in question.

The aim of this study was to generate an experimental system using murine gammaherpesvirus 68 to test the hypotheses that i) a given shRNA can be produced by the viral tRNA promoter and ii) the shRNA can knock down the target transcript during viral infection. Here, we have designed shRNAs targeting the *Prdm1* gene that produces the Blimp1 protein. Blimp1 is a key regulator of the germinal center B cell transition to memory B cells (Calame 2006, Kallies & Nutt 2007). Therefore, in theory, *Prdm1*-targeting shRNAs encoded by the virus would result in down regulation of the Blimp1 protein, and preventing differentiation of the infected cell into an antibody-secreting plasma cell.

This construct containing the TMER4 promoter and two shRNA stem loops was then tested for its ability to knock down *Prdm1* expression in luciferase assay. The TMER4 promoter shRNA-encoding element was able to knock down the expression of luciferase carrying Blimp1 target sequence, showing that (a) functional shRNAs were produced under control of the vtRNA promoter, and (b) the shRNAs could successfully knock down the target transcript. Using two-step red recombination, we generated a shRNA-encoding virus in which the TMER 4 promoter shRNA cassette was inserted in a 'neutral' region between TMER1 and TMER2. This virus and its control were also tested in luciferase assay. The experimental efficiency of sequential plasmid transfection then virus infection versus virus infection then plasmid transfection was also compared. In both conditions, the shRNA-encoding virus displayed knock down of the target; however, infecting cells before transfection worked with better efficiency. The possible explanation for the difference in these two schemes is that the cells more readily take up plasmids after infection and the virus probably starts making shRNAs from the very early time points. However, if cells are transfected first, it is more likely that the infection ability of the virus is reduced because the cells are already stressed because of the transfection reagents and DNA and therefore less shRNAs are produced by the virus.

It is important to note that shRNA-encoding plasmids showed a greater reduction in the relative luciferase values when compared to shRNA encoding viruses (Figs 2c vs 4b). One possible explanation for the lesser reduction in the viral infection versus the plasmid transfection is that during viral infection many more miRNAs are present from the virus in the cell, therefore less RISC processing machinery is available for the shRNAs. Additionally, there are 8 more viral tRNA promoters present in the virus which reduces the amount of available transcriptional complex for the shRNA promoter.

One of the contributions of this study is the design of a small viral tRNA promoter for shRNA expression as an alternative to commonly used U6 and H1 RNA polymerase III promoters, which are around 250 to 350 nucleotides for the promoter sequence alone. Usage of the tRNA promoters have been tested before for the generation of shRNAs. $tRNA^{Lys3}$ promoter was used to deliver HIV tat/rev shRNA successfully (Scherer *et al.* 2007). Here, we utilized a similar strategy for one of the naturally occurring MHV68 vtRNAs, vtRNA4 which is only 74 nucleotides long. If the shRNA sequence is added before CCA addition site of this viral tRNA, a very

minimal RNA pol III promoter can be used to express shRNAs. In the context of MHV68 vtRNAs, most of the tRNA promoters can be utilized to deliver at least two hairpins if a stronger knockdown is desired. From a 180 nucleotide construct (promoter + two shRNA stem loops), a desired gene can be knocked down in the context of virus infection in a given specific cell (for example B cells for gammaherpesviruses) *in vivo.*

The oncogenic viruses human Kaposi's Sarcoma Associated Herpesvirus (KSHV) and avian Marek's Disease Virus (MDV) code for miRNAs, named KSHV-K-12-11 and MDV-miR-M4 respectively, that share a 100% sequence homology to a cellular miRNA miR-155 (Cullen 2011). miR-155 is expressed in immune lineage cell types and also associated with development of lymphomas in different animal types. Overexpression of these viral miRNAs induces lymphoproliferation in *in vivo* models (Boss *et al.* 2011). The targets of these miRNAs include important cellular mediators such as NFkB65, AID and STAT1 and many more (Parnas *et al.* 2014, Yang *et al.* 2014). It is not a trivial task to figure out whether biochemically or bioinformatically identified miRNA-mRNA interactions have biological consequences. Most of the published literature regarding viral miRNA functional analyses utilize miRNA knockout viruses. However, these strategies are not useful to tease out the relevance of a given miRNA-mRNA combination. Therefore, we think our study design here will help us to understand which specific miRNA-mRNA interaction is biologically important and we can experimentally test a given miRNA-mRNA arrangement in small animal pathogenesis model. The work described here provides the underlying mechanism to design an effective shRNA and to generate a shRNA encoding recombinant virus.

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Data Sharing Statement: All data are available within the study.

The authors confirm that the data supporting the findings of this study are available within the supplementary material of the article.

Author Contributions: Concept: M.K., S.T., Design: M.K., S.T., Execution: M.K., Material supplying: M.K., S.T., Data acquisition: M.K., Data analysis/interpretation: M.K., S.T., Writing: M.K., Critical review: M.K., S.T.

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