Spring 5-1-2016

Characterization of Induced RNAi Silencing of Vaccinia Virus Essential Genes

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Honors/University Scholar Thesis

Submitted in Partial Fulfillment of the

Requirements for the

Honors Program at the

University of Connecticut

2016

APPROVAL PAGE

Honors/University Scholar Thesis

Characterization of Induced RNAi Silencing of Vaccinia Virus Essential Genes

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2016

Acknowledgements

I give many thanks to Dr. Paulo Verardi from the Department of Pathobiology and Veterinary Science as my project and thesis advisor for providing me with the opportunity, environment, and support to conduct this research project. I would also like to thank Dr. Antonio Garmendia and Dr. Joerg Graf, my Molecular and Cell Biology Honors Academics Advisor, for serving on my University Scholar Committee. Additionally, I would like to give incredible thanks to past and present Verardi Lab graduate students, Brittany Jasperse, Caitlin O'Connell, and Dr. Allison Titong for teaching me the majority of skills in designing and conducting experiments and for improving my scientific writing. They have always been extremely supportive and have always provided guidance for me since joining the lab, including this project. I would also like to thank former Verardi Lab undergraduate Peter Larson for his support and mentoring. Current Verardi Lab members, Zhixin Jing and Sarah Robbins, have also provided a lot of assistance for this project. Especially Jing, we have conducted many of our later experiments together. This project was also supported by the University Scholars program here at UConn and the Office of Undergraduate Research (OUR).

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Abstract

Vaccinia virus (VACV) is a large double-stranded DNA virus and the prototypical member of the family *Poxviridae* and is most notable for its use as the vaccine that eradicated smallpox (variola virus). More recently, VACV has been used to develop recombinant vaccines and immunotherapies. However, many of these processes require VACV replication to be tightly controlled. RNA interference (RNAi) is a powerful tool for *in vitro* silencing of mRNAs that are complimentary to 19-21 base pairs (bp) of double-stranded RNA (dsRNA). This project outlines the design and preliminary analysis of two inducible RNAi silencing constructs targeting multiple VACV essential genes during VACV replication. The two designs include one multihairpin structure expressing multiple shRNAs separated by 6 bp spacers and one head-to-tail design with multiple shRNAs separated by 6 bp loops. Plasmids pKJ252 and pKJ253 (containing the two respective RNAi construct designs, designed such that their transcription can be controlled by *tet* operon elements) were transfected into BS-C-1 cells previously infected with VACV to generate two new recombinant VACVs. The newly generated recombinant VACVs were analyzed via plaque assays. Results suggest that one of the designed RNAi silencing constructs (multihairpin), when induced with tetracyclines, shows a statistically significant decrease in plaque size, indicating reduced VACV replication.

Introduction

Vaccinia Virus

Vaccinia virus (VACV) is the prototype member of the family *Poxviridae*. In 1796, Edward Jenner introduced the idea of vaccination by inoculating individuals with vesicular fluid of cowpox pustules to provide immunity against smallpox. Over time, VACV replaced cowpox virus as the vaccine against smallpox and eventually led to the eradication of smallpox in 1980 by the World Health Organization (Yilma, 1994). Currently, VACV vectors can be engineered to express heterologous viral or non-viral antigens (human and animal vaccines) or therapeutic proteins (immunotherapies), or to selectively infect and cause lysis in cancer cells (oncolytic therapies). VACV has been widely used as a vector because it is thermally stable, easy to propagate, has a broad tropism for mammalian cells, and is able to elicit strong humoral and cell-mediated immune responses (Verardi et al., 2012).

VACV is a large double-stranded DNA virus with a linear genome 200 kilobases (kb) in length that encodes about 200 genes (Smith & Moss, 1983). Its genome is packaged in an enveloped virion containing its own transcriptional enzymes and machinery, allowing VACV to replicate exclusively in the cytoplasm of host cells (Smith & Moss, 1983). The genome of VACV is capable of accommodating up to 25 kb of foreign DNA (Smith & Moss, 1983), which makes recombinant VACVs (rVACVs) ideal vectors to stably express different genes of interest (GoI) to high levels (if desired) in mammalian cells.

RNA Interference

RNA interference (RNAi) is a RNA-dependent gene silencing process in which the expression of a gene can be silenced post-transcriptionally by interaction with double-stranded RNA (dsRNA). This pathway is naturally found in many eukaryotes and is thought to be a cellular defense mechanism against dsRNA viruses. In research, RNAi can be used to knock out expression of a GoI with high selectivity and specificity (Rao, Vorhies, Senzer, & Nemunaitis, 2009). Currently, RNAi is a commonly used tool in *vitro* and *in vivo* to silence gene expression (Zamore, Tuschl, Sharp, & Bartel, 2000). The two most common applications to mediate the RNAi effect involve small interfering RNA (siRNA) and short hairpin RNA (shRNA). siRNA is a class of dsRNA molecule that is capable of knocking down expression of specific GoIs via the RNAi pathway. On the other hand, shRNA is another class of dsRNA with a hairpin turn that also can be used to silence gene expression (Rao et al., 2009). Double-stranded siRNAs are typically 19 - 21 bp and produced when dsRNA or shRNA encounter the cellular enzyme Dicer in the cytoplasm. The resulting siRNA will then bind to the enzymatic portion of the RNA-induced silencing complex (RISC) and lead to the cleavage of mRNA that is complementary to the siRNA (Rao et al., 2009). Thus, RNAi is a powerful mechanism for silencing host and even viral genes. Since VACV replication occurs exclusively in the host cell cytoplasm (Smith et al., 1983), RNAi can be utilized to target genes that are essential to VACV replication. Viral siRNA constructs have been previously shown to inhibit

monkeypox virus (another virus that belongs to the *Poxviridae* family) replication by 65-95% *in vitro* (Alkhalil et al., 2009). Additionally, siRNAs that target several essential VACV genes (including B1R, D5R, and G7L) have been shown to inhibit VACV replication when each siRNA was introduced individually (Vigne et al., 2008). This suggests that VACV replication is sensitive to RNA silencing.

The use of siRNA as a mediator requires it to be introduced exogenously via transfection or other injection methods while an shRNA approach requires a transient expression, usually through a viral vector (McIntyre et al., 2011). The advantage of using siRNA-mediated RNAi is that the siRNA will interact directly with RISC without the need for the dsRNA to be processed by Dicer. siRNAs are chemically synthesized and easier to modify as well (Rao et al., 2009). Concentrations for effective siRNA-mediated gene knockdown are usually in low nM ranges. However siRNA-mediated RNA interference has a shorter duration than shRNA. Fluorescent-tagged siRNA exhibits high degradation after reaching its 24 hour peak for silencing and RNAi diminishes within 48 hours (Rao et al., 2009).

RNAi mediated by shRNA is usually vector-based and relies on the host's transcription machinery. When compared to siRNA, only low copy numbers (as low as 5) of shRNA are sufficient to provide a continuous gene knockdown effect since shRNA is constitutively synthesized in the host (Rao et al., 2009). More importantly, vector-based shRNA expression can also be regulated or induced by manipulating the expression strategy, such as altering the promoter or redesigning the shRNA structure (Rao et al., 2009). Additional studies have

shown that a net suppressive activity can be maintained from multiple simultaneous acting shRNAs (McIntyre et al., 2011). For example, studies regarding a combinatorial RNAi approach involving the expression of multiple shRNA combinations renders a near-complete coverage of all HIV-1 strains, providing a potential novel therapy that is protective against current strains of HIV (McIntyre et al., 2011). This means a combinatorial RNAi approach with multiple gene targets could potentially be used in knocking down expression of VACV essential genes with higher efficiency when compared to targeting one gene at a time.

Selecting Targets for RNAi

A number of different RNAi targets and strategies for the inducible silencing of VACV replication were identified through literature review. Three VACV late essential genes were chosen based on previous inhibition efficiency. Several siRNA sequences targeting the VACV late essential gene D5R have been shown to lead to 90% decreased viral replication when transfected at 100 nM and exhibit a prolonged antiviral effect for up to 72 hours (Vigne et al., 2008). One of the sequences had up to 93.2% inhibition of VACV replication at 100 nM while two others had 86.9% and 90.2% inhibition of replication at 100 nM, respectively (Vigne et al., 2008). Another siRNA targeting a late essential gene, G7L, has been shown to reduce VACV replication by 97% at 100 nM (Vigne et

al., 2009). A third siRNA targeting the late essential gene E8L (equivalent to D8L in VACV) in monkeypox virus has shown 95% inhibition efficiency in viral replication (Alkhalil et al., 2009). The D5R gene encodes the D5 protein, a DNA-independent nucleoside triphosphatase that is essential for viral DNA replication, and is highly conserved in the *Orthopoxvirus* genus (Vigne et al., 2008). The E8L gene encodes the E8 protein, which plays an important role in cell attachment and viral entry (Alkhalil et al., 2009). Knockdown of this gene has been shown to cause reduction in plaque numbers (Alkhalil et al., 2009). Another gene G7L encodes the G7 protein, which is required for virus morphogenesis (Vigne et al., 2009).

Tetracycline and Lactose Operons

Expression of RNAi silencing must be tightly controlled and only expressed when inhibition of VACV is necessary. Therefore RNAi silencing should be incorporated in a system where its expression could be turned on in the presence of a “switch” -- an inducer.

The tetracycline (*tet)* operon is a bacterial gene expression regulatory sequence discovered in *Escherichia coli,* which plays a role in bacterial resistance against tetracycline antibiotics. The operon sequence consists of the *tetA* resistance gene and the *tetR* repressor gene. In the absence of tetracyclines, such as doxycycline (DOX), the repressor protein TetR binds to the $tetO_2$ operator sequence, thus inhibiting the transcription of *tetA* (Hillen & Berens,

1994). When tetracyclines are present, TetR binds to tetracyclines and undergoes a conformational change, which prevents TetR from binding to the operator, allowing the transcription of the *tet* operon genes (Hillen & Berens, 1994). The *tet* operon has been utilized in VACV and has been used to regulate the expression of genes essential to VACV replication. In current tetracycline-based replication-inducible and replication-repressible VACVs in the Verardi Laboratory, only expression of late genes can be tightly regulated (Verardi et al. 2013).

The lactose (*lac)* operon is another bacterial gene regulatory sequence that has also been used extensively in research. The *lac* operon contains a promoter, terminator, regulator, operator, and three structural genes (Lewis, 2005). The three structural genes *lacZ, lacY* and *lacA* encode the proteins β-galactosidase, a permease, and a transferase respectively. These structural genes are located downstream of the operator sequence lacO to which the *lac* repressor LacI binds (Lewis, 2005). When LacI binds to the operator, transcription of the three structural genes is inhibited. In the presence of allolactose inducers (e.g. isopropyl-β-D-thiogalactoside, IPTG, an allolactose analog), LacI binds to the inducer and transcription of the structural genes occurs (Lewis, 2005).

Hypothesis

If we can incorporate RNAi silencing of a VACV essential gene into an inducible system, we could control VACV replication with the addition (or removal) of an inducer that allows transcription of a RNAi construct and prevent VACV replication through RNAi. It is necessary to have the RNAi silencing mechanism be inducible in order to selectively halt VACV replication. For example, when generating a rVACV, replication of the parental strain must be restricted in order to purify the rVACV. This inducible system will utilize *tet* operon elements and therefore can be controlled with the addition or removal of tetracyclines. However, only late VACV essential genes can be regulated by the current inducible system (Weber et al., 2007), therefore only late VACV essential genes will be targeted for inducible silencing, with preference to those for which siRNA target sequences have been previously validated by other studies.

The goal of this project is to design and characterize the efficiency of an inducible RNAi silencing mechanism controlling the expression of VACV essential genes. This will be done by creating a rVACV that incorporates features based on previous designs and studies that involve shRNA-mediated RNAi expression (Lee et al., 2012; McIntyre et al., 2011). In dsRNA, the sense strand is the strand of RNA that has the same sequence as the matching mRNA. In order to achieve the highest efficiency of gene silencing, five different sense strands along with their antisense strands targeting three late essential genes were chosen based on high RNAi silencing efficiency in previous studies (Alkhalil

et al., 2009; Vigne et al., 2008, 2009). Two different RNAi constructs were designed to carry out expression of multiple shRNAs when transcribed. These constructs were placed under the control of a strong VACV late promoter and *tet* operator sequence to allow high amounts of RNAi expression to be induced with the addition of a tetracycline inducer such as DOX. A plasmid was generated for homologous recombination with the parental VACV to generate a new rVACV. A reporter gene was included to distinguish the new rVACV from the parental strain during the purification process. In the absence of inducer, TetR will be expressed and bind to $tetO_2$, preventing the expression of the RNAi construct. In the presence of DOX, TetR will bind to the inducer and undergo a conformational change, decreasing its affinity for the $tetO_2$ operator sequence. The RNAi construct is transcribed and should halt the expression of the targeted VACV essential genes, thus preventing VACV replication (Figure 1).

Materials and Methods

Identification of Target Genes

Only genes that have been shown to be sensitive to RNAi-mediated gene silencing were selected, with the preference of sense strands that have a high inhibition efficiency (i.e. decrease of viral replication by 90% or greater). Only sequences of late VACV genes were selected. Five sense strands from siRNA sequences that target D5R, D8L, and G7L (Table 1) that were shown to be effective were chosen from literature and were incorporated into the RNAi construct as shRNAs instead of siRNAs with the addition of a 4 bp loop in each hairpin.

Design of RNAi Construct Plasmids

After identifying target genes and sense strand sequences, two RNAi constructs were designed and synthesized into a vector backbone such that transcription of the construct would result in multiple shRNAs. One design incorporated a multi-hairpin structure, similar to a RNAi-microsponge structure decribed by Lee et al. (2012), where multiple shRNA repeats are transcribed from a single plasmid. The five shRNAs that target the essential genes are linked together starting from the sense strand of one shRNA followed by a 4 bp loop and the antisense strand of the shRNA, followed by a dTdT overhang and a 6 bp spacer between the first shRNA and the next shRNA (Figure 2A). Another design

incorporated a head-to-tail structure described by McIntyre, et al. (2011) which involves the linking of all five sense strands separated by a 6 bp spacer, a 4 bp end loop, and all five antisense strands corresponding with the sense strands (Figure 2B).

In the multi-hairpin construct, two unique restriction enzyme sites were incorporated in the spacer to allow the possibility of subcloning a plasmid with only a single shRNA construct by removing the remaining 4 shRNAs after restriction enzyme digest to compare single-shRNA and multi-shRNA efficiency. Two restriction enzymes were added on 2 sites on both ends of the construct for future digestion, isolation and subcloning. In both designs, the RNAi constructs were placed under the control of the strong VACV late promoter P_{11} and the *tetO*$_2$ operator sequence to drive high expression in the presence of DOX. The two constructs were then synthesized into plasmids by a third party vendor (DNA 2.0, Menlo Park, CA.) The natural promoter for D6R was also included in the synthesized construct to simplify and reduce the steps of cloning. Vector pKJ241 was synthesized with the multi-hairpin RNAi construct shG7L1-shD8L1-shD5R3-shD5R1-shD5R2. Vector pKJ242 was synthesized with the head-to-tail RNAi construct shD8L1-shD5R3-shD5R1-shD5R2-shG7L1. A proposed secondary structure of the transcribed RNAi constructs is shown in Figure 3 using a web server for RNA secondary structure prediction developed by Reuter et al., 2010.

Plasmid Construction and Gene Cloning

As an overview, RNAi constructs with compatible sticky ends were synthesized and then cloned into the backbone derived from pBJ207, an existing plasmid in the Verardi laboratory that contains a kanamycin resistance gene, *tet* operon elements, including *tetR* placed under the control of a strong constitutive promoter $P_{E/L}$, to control transcription of the VACV D6R gene, a fluorescent reporter gene *EGFP*, and two flanking regions that are homologous to the VACV D5R and D6R genes along with the natural promoter for D6R. The two homologous regions flanking the construct allow the possibility of homologous recombination with a parental VACV to generate a new rVACV, which could be screened using the fluorescent reporter gene *EGFP* (Figure 4).

pKJ241 and pKJ242 were double-digested with EagI-HF and SphI-HF to create compatible sticky ends to ligate into the pBJ207-derived backbone. The two restriction enzymes used allow the placement of the RNAi constructs without removing any genetic elements from pBJ207. The vector derived from pBJ207 was also dephosphorylated with Antarctic Phosphatase under appropriate incubating conditions in order to ensure that the vector does not re-circularize during ligation. Inserts and vectors were then ligated together with T4 DNA ligase and electroporated into NEB 5 α-electrocompetent *Escherichia coli* using the Gene Pulser Xcell Electroporation System from Bio-Rad (Hercules, CA) to create pKJ252 (Figure 5A) and pKJ253 (Figure 5B), respectively. The transformed *E.coli* were then plated on LB agar with 50μg/ml kanamycin and incubated at 37^{o}C overnight for 16 hours. Isolated colonies were amplified and the plasmids

were isolated. Plasmids obtained were verified by restriction enzyme digestions and viewed on an electrophoresis gel containing ethidium bromide under UV light. After confirming the identity of the plasmid, one isolated colony containing the correct plasmid was then further amplified by growing the transformed *E.coli* in LB containing kanamycin. The amplified plasmids were then isolated and purified using a commercial NucleoSpin® Plasmid kit (Macherey-Nagel, Inc., Bethlehem, PA). After a pure stock of each plasmid was obtained, restriction enzyme digests were performed to again verify the identity of the plasmid. All enzymes and cells used were obtained from New England Bio Labs (Ipswich, MA.)

Mammalian Cell Culture

African Green Monkey kidney epithelial cells (BS-C-1) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with amino acids and glucose (cDME; Invitrogen, Grand Island, NY) with 10% tetracycline-tested fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA). Cells were incubated at 37^{o}C with 5% CO_2.

Viruses

The IPTG-inducible virus viD6RlacR was used as the parental strain. It contains the inducible elements of the *lac* operon and has been amplified,

purified and titered by the Verardi laboratory. The *lacO* operator is placed downstream of the natural D6R promoter, which functions similarly to the *tet* operator but instead with the inducer IPTG that binds to the repressor LacI. It also contains a fluorescent reporter gene *DsRed* under the control of the synthetic VACV promoter $P_{E/L}$. This construct has been placed between the D5R and D6R genes, which allows the transcription of the VACV essential gene D6R and therefore replication of the virus in the presence of IPTG. viD6RlacR was chosen as the parental strain to create a new rVACV because it is replication-inducible in the presence of IPTG and contains a selection marker distinctive from the selection marker of the new rVACV containing the RNAi constructs that allows selection to be more straightforward. The IPTG-inducible system in viD6RlacR is also flanked by the D5R and D6R genes, which allows homologous recombination to occur with pKJ252 or pKJ253 to create new rVACVs to test the efficiency of RNAi-mediated gene silencing.

Recombinants viRNAi-1 and viRNAi-2 were obtained by homologous recombination between viD6RlacR and pKJ252 or pKJ253, respectively. BS-C-1 cells were seeded in 12-well cell culture plates and reached ~95% confluency at the time of the experiment. The parental stock viD6RlacR was sonicated four times with 30 second bursts and vortexed in between. viD6RlacR was then diluted to reach a multiplicity of infection (MOI) of 0.1 for infection. Cells were then washed with cDME and overlayed with the diluted virus. Plates were then placed on a rocking platform and incubated for 1 hour. 1 hour post infection (hpi) cells were washed again and overlayed with cDME supplemented with 2.5% FBS

with or without 100 ng/ml IPTG and transfected with the appropriate plasmids using FuGENE HD transfection reagent (Promega, Madison, WI). 2 days post infection (dpi), supernatant of the 12-well cell culture plate was then collected, centrifuged, and transferred to a 24-well cell culture plate, which was overlayed with cDME supplemented by 2.5% FBS without IPTG to prevent the IPTG-inducible parental strain from growing. 2 dpi, supernatant from single, isolated rVACV plaques (indicated by EGFP expression observed under a fluorescence microscope) were then collected, centrifuged, diluted and transferred to a new 24-well cell culture plate. After 4 dpi, wells with single, isolated rVACV were screened to see if any parental virus was still present. Plaques with no indication of the parental virus were allowed to grow until 100% cytopathic effect (CPE) was reached. Cell lysate and supernatant of the rVACV were then collected.

Infection and Measurement of RNAi Efficiency

BS-C-1 cells were seeded in 6-well cell culture plates in order to reach 80-90% confluency at the time of the experiment. viRNAi-1 or viRNAi-2 were sonicated four times with 30 second bursts and vortexed in between. The viruses were then diluted to achieve ~30 PFU/well. Cells were then washed with cDME and infected with the diluted virus. Plates were then placed on a rocking platform and incubated for 1 hour. Cells were washed again and overlayed with cDME supplemented with 2.5% FBS in the absence or presence of varying concentrations of DOX (0.001 µg/ml DOX, 0.01µg/ml DOX, 0.1 µg/ml DOX, 1

μg/ml DOX, or 10 μg/ml DOX). Cells were incubated at 37^{o}C with 5% CO_2. 2 dpi plates were stained and fixed with 0.5% crystal violet/20% ethanol. Individual plaques were imaged with an inverted microscope and representative plaques were analyzed using measure software (AxioVision, 4.8.1, Carl Zeiss). The mean plaque radius was calculated in different concentrations of DOX with a 95% standard error of the mean (SEM) and statistical significance was determined using a t-test comparing different concentrations of DOX to the absence of DOX usinf GraphPad Prism v. 6.0f (GraphPad Software, La Jolla, CA).

Results

Results obtained by the experiments described above are considered preliminary because only one plaque assay was performed for viRNAi-1 and two were performed for viRNAi-2 without obtaining the titer of each recombinant due to time constraints. All calculations for the amount of virus used in infections were estimations based on previous observations in plaque assays in the Verardi laboratory. viRNAi-2 was tested first for its efficiency in RNAi silencing since its recombinants were first obtained and amplified.

In the first plaque assay, BS-C-1 cells were infected with viRNAi-2 at ~30PFU/well based on an estimated titer of 10^7 PFU/ml in the absence or presence of either 0.001 µg/ml DOX, 0.01 µg/ml DOX, 0.1 µg/ml DOX, or 1 µg/ml DOX (Figure 6). About 15 PFUs were obtained in each well. No distinguishable difference in plaque number or size was observed with the naked eye even at the highest concentration of DOX. Therefore in the second plaque assay, DOX concentration was increased 10 fold to 10 µg/ml and the amount of viRNAi-2 used to infect the cell culture was doubled. Appropriate concentrations of DOX were also added during the 1 hour infection phase of the experiment in the hope to prevent any potential leaky expression of the targeted late essential genes. The same DOX concentrations and calculations to obtain ~30 PFU/well were applied to viRNAi-1 as well once it was obtained and amplified.

After the second plaque assay, about 40 PFU were obtained in each well infected by viRNAi-1 with an observable difference in plaque size between 10

μg/ml DOX and no DOX (Figure 7A). About 20 PFU were obtained in each well infected by viRNAi-2. However, there was still no distinguishable difference observed in plaque number or size in cells infected by viRNAi-2, despite the increase in DOX concentration (Figure 7B). Therefore plaque size (radius) was only measured for viRNAi-1 under an inverted microscope (AxioVision, 4.8.1, Carl Zeiss) and analyzed for any statistical significance using GraphPad Prism v. 6.0f (GraphPad Software, La Jolla, CA).

The radius of plaques formed in the presence of DOX were distinguishable from plaques formed in the absence of DOX (Figure 8). In the presence of 1 μg/ml DOX there was a small but statistically significant difference ($P < 0.05$) between the sizes of plaques formed in the presence of 1 μg/ml DOX, compared to plaques formed in the absence of DOX. The average radius decreased by about 40 μm with the addition of 1 μg/ml DOX. A greater difference ($P < 0.0001$) was observed when comparing plaque sizes of no DOX versus 10 μg/ml DOX. Average plaque radius decreased by about 160 μm, an approximate 16% decrease in plaque size. This suggests that viRNAi-1 replication was inhibited with the increase of DOX concentrations while viRNAi-2 replication was unaffected. Taken together, it also suggests that a multihairpin RNAi design may be more effective than a head-to-tail RNAi design.

Discussion

In these preliminary experiments, we were not able to demonstrate efficient silencing of VACV essential genes through induced RNAi expression. In plaque assays of viRNAi-2 (head-to-tail), no distinguishable difference in VACV replication was observed in the presence or absence of DOX. Although there was a statistically significant decrease of viRNAi-1 (multihairpin) plaque sizes in the presence of 1 or 10 µg/ml, the decrease in plaque size was not enough to completely inhibit VACV replication. Therefore the implementation of this construct design may be limited. There is also a need to investigate the difference between targeting a single essential gene versus targeting multiple essential genes. Targeting multiple genes requires a more complex design and therefore may pose a greater number of genetic hurdles to overcome before the sense strand of the targeted sequence could be delivered to RISC and initiate the RNAi process. There is also the potential that the construct may have mutated either during the cloning or rVACV purification process. Sequencing may be required to confirm the identity of the plasmid before the infection/transfection process. It would have also been ideal if the titer of each recombinant was obtained before performing the plaque assay instead of relying on titer estimations.

Better implementation of inducible RNAi silencing of VACV essential genes may require construct designs that are more efficient. In this set of experiments, late essential genes were selected as targets. In other studies

(Vigne et al., 2009), early essential gene targets were also identified and have been shown to decrease VACV replication to 90% or higher. In this study, we used the *tet* repressor to control construct expression because it has been thoroughly characterized in VACV already. If both early and late essential genes could be targeted simultaneously, we may reduce VACV replication to a greater extent. The multihairpin design was derived from a multi-shRNA expression system that required multiple repeats of the same shRNA (Lee et al., 2012). In this study, different shRNAs that targeted multiple essential genes were used to form the multihairpin structure rather than the same shRNA, which only targets one gene at a time.

Eventually, successful designs could be implemented to control expression of GoIs in VACV for different purposes. For example, VACVs could be engineered to turn off essential genes for VACV replication upon addition of tetracyclines in response to adverse reactions in rVACV vaccines. Another potential implementation of induced RNAi silencing of VACV essential genes may include a more efficient way to generate rVACVs. The typical way to generate rVACVs is by transfecting cells infected with a parental VACV strain with a plasmid containing a desired genetic region flanked by sequences identical to the VACV genome (Falkner & Moss, 1990). Homologous recombination can occur between the identical sequences in the plasmid and parental VACV, resulting in the insertion of the desired genetic region into the VACV genome (Falkner & Moss, 1990). These infrequently-generated rVACVs must then be purified from the parental VACV, which usually takes months to

complete using the traditional transient dominant selection process (Falkner & Moss, 1990). If we can control VACV replication through RNAi during the purification process of creating an rVACV, the parental VACV can be rapidly eliminated from the parental/recombinant pool through silencing of essential VACV genes. This way we could purify rVACV in a much more efficient manner.

Although we have developed a prototype for inducible RNAi silencing for VACV, we did not succeed in inhibiting VACV replication to the extent where meaningful implementations could be applied. Time constraint was also another factor that prohibited further testing and troubleshooting for each construct and the possibility of including more RNAi construct designs for analysis. In the future, development of a more efficient inducible RNAi system would allow for better utility in VACV research.

References

Alkhalil, A., Strand, S., Mucker, E., Huggins, J. W., Jahrling, P. B., & Ibrahim, S. M. (2009). Inhibition of Monkeypox virus replication by RNA interference. Virology Journal, 6, 188. http://doi.org/10.1186/1743-422X-6-188

Beaud, G. (1995). Vaccinia virus DNA replication: a short review. Biochimie, 77(10), 774–779.

Broyles, S. S. (2003). Vaccinia virus transcription. The Journal of General Virology, 84(Pt 9), 2293–2303. http://doi.org/10.1099/vir.0.18942-0

Falkner, F. G., & Moss, B. (1990). Transient dominant selection of recombinant vaccinia viruses. Journal of Virology, 64(6), 3108–3111.

Hagen, C. J., Titong, A., Sarnoski, E. A., & Verardi, P. H. (2014). Antibiotic-dependent expression of early transcription factor subunits leads to stringent control of vaccinia virus replication. Virus Research, 181, 43–52. http://doi.org/10.1016/j.virusres.2013.12.033

Hillen, W., & Berens, C. (1994). Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annual Review of Microbiology, 48, 345–369. http://doi.org/10.1146/annurev.mi.48.100194.002021

Lee, J. B., Hong, J., Bonner, D. K., Poon, Z., & Hammond, P. T. (2012). Self-assembled RNA interference microsponges for efficient siRNA delivery. Nature Materials, 11(4), 316–322. http://doi.org/10.1038/nmat3253

Lewis, M. (2005). The lac repressor. Comptes Rendus Biologies, 328(6), 521–548. http://doi.org/10.1016/j.crvi.2005.04.004

Mcintyre, G. J., Arndt, A. J., Gillespie, K. M., Mak, W. M., & Fanning, G. C. (2011). A comparison of multiple shRNA expression methods for combinatorial RNAi. Genetic Vaccines and Therapy, 9, 9. http://doi.org/10.1186/1479-0556-9-9

Mcintyre, G. J., Groneman, J. L., Yu, Y.-H., Tran, A., & Applegate, T. L. (2011). Multiple shRNA combinations for near-complete coverage of all HIV-1 strains. AIDS Research and Therapy, 8, 1. http://doi.org/10.1186/1742-6405-8-1

Moss, B. (1991). Vaccinia virus: a tool for research and vaccine development. Science (New York, N.Y.), 252(5013), 1662–1667.

Moss, B. (1996). Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety. Proceedings of the National Academy of Sciences of the United States of America, 93(21), 11341–11348.

Moss, B. (2006). Poxvirus entry and membrane fusion. Virology, 344(1), 48–54. http://doi.org/10.1016/j.virol.2005.09.037

Popov, S., Mirshahidi, S., Essono, S., Song, R., Wang, X., & Ruprecht, R. M. (2009). Generation of Recombinant Vaccinia Viruses via Green Fluorescent Protein Selection. DNA and Cell Biology, 28(3), 103–108. http://doi.org/10.1089/dna.2008.0792

Reuter, J. S., & Mathews, D. H. (2010). RNAstructure: software for RNA secondary structure prediction and analysis. BMC Bioinformatics. 11,129.

Rao, D. D., Vorhies, J. S., Senzer, N., & Nemunaitis, J. (2009). siRNA vs. shRNA: similarities and differences. Advanced Drug Delivery Reviews, 61(9), 746–759. http://doi.org/10.1016/j.addr.2009.04.004

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., & Khvorova, A. (2004). Rational siRNA design for RNA interference. Nature Biotechnology, 22(3), 326–330. http://doi.org/10.1038/nbt936

Rintoul, J. L., Wang, J., Gammon, D. B., Buuren, N. J. van, Garson, K., Jardine, K., … Bell, J. C. (2011). A Selectable and Excisable Marker System for the Rapid Creation of Recombinant Poxviruses. PLOS ONE, 6(9), e24643. http://doi.org/10.1371/journal.pone.0024643

Smith, G. L., & Moss, B. (1983). Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. Gene, 25(1), 21–28.

Titong, A. (2014). Development of Replication-Repressible Vaccinia Virus Vectors for Vaccines and Therapeutics. Doctoral Dissertations. Retrieved from http://digitalcommons.uconn.edu/dissertations/622

Verardi, P. H., Titong, A., & Hagen, C. J. (2012). A vaccinia virus renaissance: new vaccine and immunotherapeutic uses after smallpox eradication. Human Vaccines & Immunotherapeutics, 8(7), 961–970. http://doi.org/10.4161/hv.21080

Verardi, P. H., Titong, A., Hagen, C. J., & Jasperse, B. (2013, July 4). Inducible and repressible vaccinia viruses with improved safety. Retrieved from http://www.google.com/patents/US20130171189

Vigne, S., Duraffour, S., Andrei, G., Snoeck, R., Garin, D., & Crance, J.-M. (2009). Inhibition of vaccinia virus replication by two small interfering RNAs targeting B1R and G7L genes and their synergistic combination with cidofovir. Antimicrobial Agents and Chemotherapy, 53(6), 2579–2588. http://doi.org/10.1128/AAC.01626-08

Vigne, S., Germi, R., Duraffour, S., Larrat, S., Andrei, G., Snoeck, R., … Crance, J.-M. (2008). Specific inhibition of orthopoxvirus replication by a small interfering RNA targeting the D5R gene. Antiviral Therapy, 13(3), 357–368.

Weber, P. G., L. A. Jones, T. D. Yilma, and P. H. Verardi. (2007). Tightly-regulated inducible promoters for vaccinia virus expression vectors, 26th Annual Meeting of the American Society for Virology, Oregon State University, Corvallis, OR.

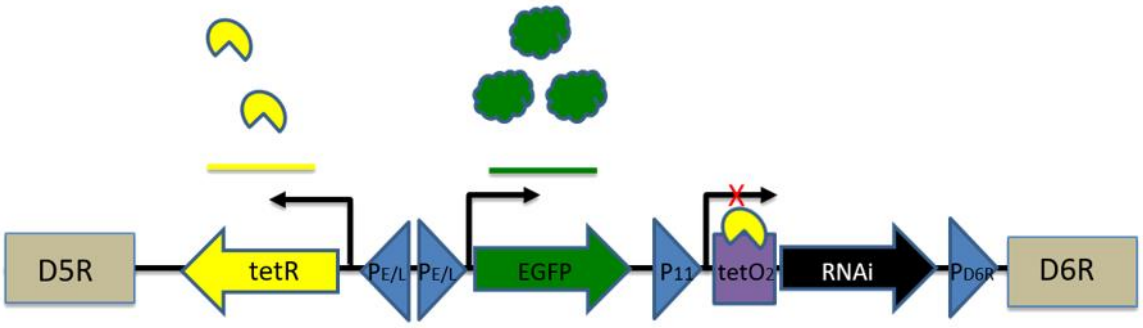
Yilma, T. (1994). Applications of recombinant vaccinia virus for veterinary vaccines. Developments in Biological Standardization, 82, 201–209.

Zamore, P. D., Tuschl, T., Sharp, P. A., & Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23

nucleotide intervals. Cell, 101(1), 25–33. http://doi.org/10.1016/S0092-8674(00)80620-0

Figure 1. Inducible RNAi mechanism. A: In the absence of DOX, TetR binds to the *tetO*$_2$ operator sequence. The RNAi construct is not transcribed and VACV replicates normally. **B:** In the presence of DOX, TetR binds to DOX and undergoes a conformational change. RNAi is transcribed and silences VACV essential genes, which prevents VACV replication.

A.


D5R
tetR
PE/L
PE/L
EGFP
P11
tetO2
RNAi
PD6R
D6R

B.

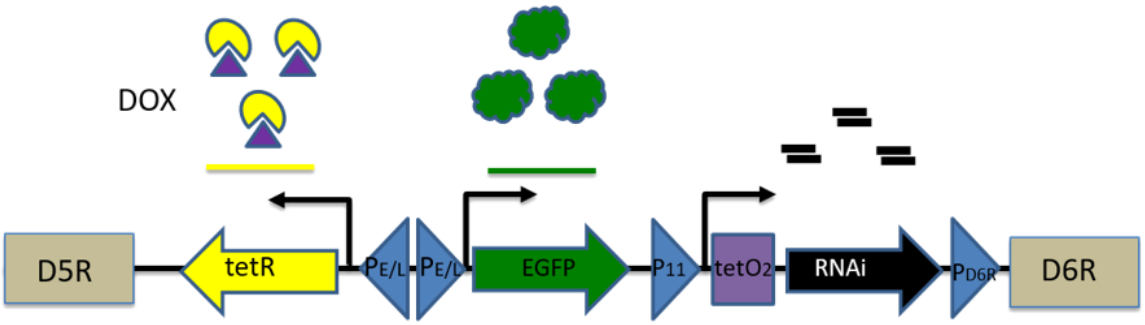
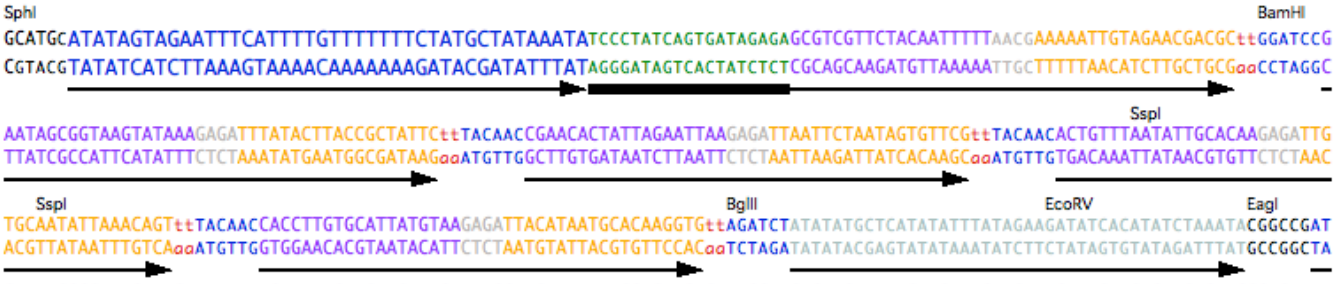

DOX
D5R
tetR
PE/L
PE/L
EGFP
P11
tetO2
RNAi
PD6R
D6R

Table 1. Sense strands chosen for targeting late essential genes based on literature review.

Targeted Gene	siRNA	Sequence (sense, 5'-3')	Source
D5R	siD5R-1	ACTGTTTAATATTGCACAA (19nt)	(Vigne et al., 2008)
D5R	siD5R-2	CACCTTGTGCATTATGTAA (19nt)	(Vigne et al., 2008)
D5R	siD5R-3	CGAACACTATTAGAATTAA (19nt)	(Vigne et al., 2008)
D8L (E8L in MPV)	siD8L-1	GAATAGCGGTGAGTATAAA (19nt)	(Alkhalil et al., 2009)
G7L	siG7L-1	GCGTCGTTCTACAATTTTT (19nt)	(Vigne et al., 2009)

Figure 2. Inducible RNAi Construct Design Sequences. Both RNAi constructs were placed under the control of the strong VACV late promoter P_{11} (dark blue in larger font) and the *tet* operator sequence (green) to drive high expression in the presence of DOX. Purple represents the sense strand. Orange represent the antisense strand. The 4 bp gray sequence between the purple and orange sequences represent the loop. Red represents the dTdT overhang. Arrows represent a promoter or shRNA. Restriction enzyme sites are labelled. **A:** Multihairpin construct design containing shG7L1-shD8L1-shD5R3-shD5R1-shD5R2 (used to generate viRNAi-1). Each shRNA is separated by a 6 bp spacer (dark blue in smaller font). **B:** Head-to-tail construct design containing shD8L1-shD5R3-shD5R1-shD5R2-shG7L1 (used to generate viRNAi-2). Different sense strands and antisense strands are separated by a 6 bp spacer (gray for sense strands, cyan for antisense strands).

A.


SphI
BamHI
SspI
SspI
BglII
EcoRV
EagI

B.

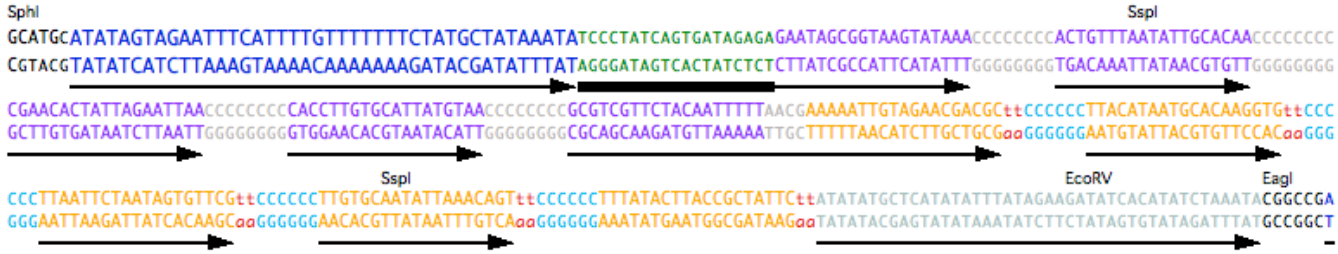
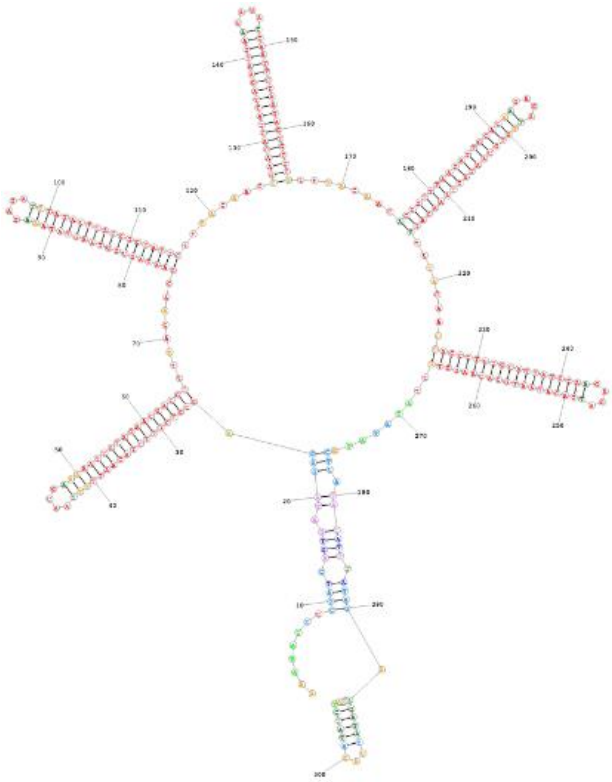

SphI
SspI
SspI
EcoRV
EagI

Figure 3. Proposed secondary structure of transcribed RNAi constructs. Structures were generated using RNAstructure: software for RNA secondary structure prediction and analysis (Reuter et al., 2010) **A:** Multihairpin construct design (viRNAi-1). **B:** Head-to-tail construct design (viRNAi-2).

A.



B.

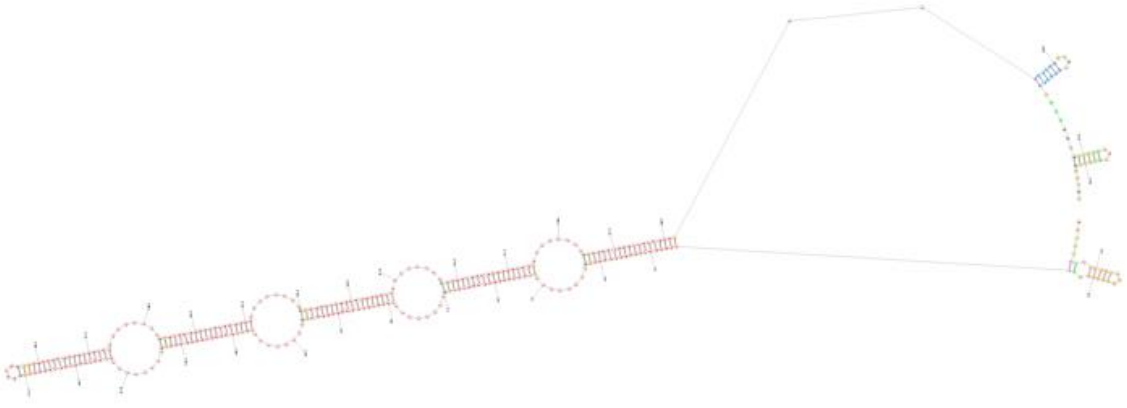


Figure 4. Features of pBJ207. Vector backbone for pKJ252 and pKJ253. RNAi constructs with compatible sticky ends (SphI and EagI) were inserted into pBJ207 by replacing the original sequence containing the D6R promoter and *tetO*$_2$ operator sequence with the sequences described in Figures 2A and 2B to generate pKJ252 and pKJ253, respectively.

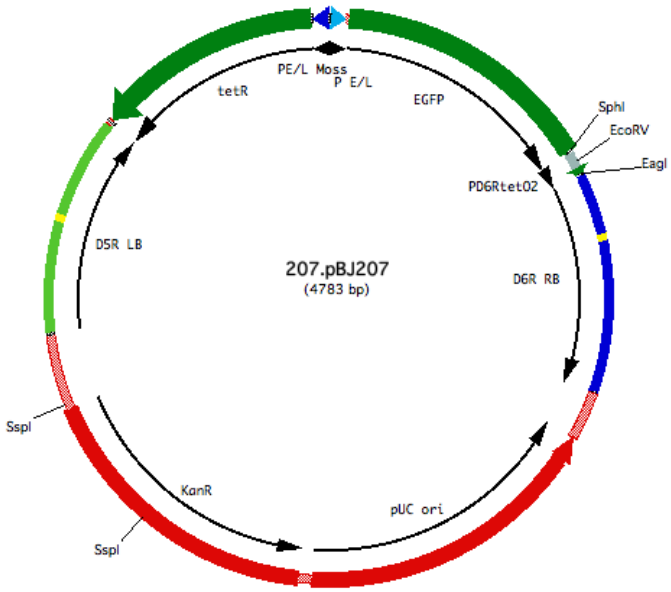
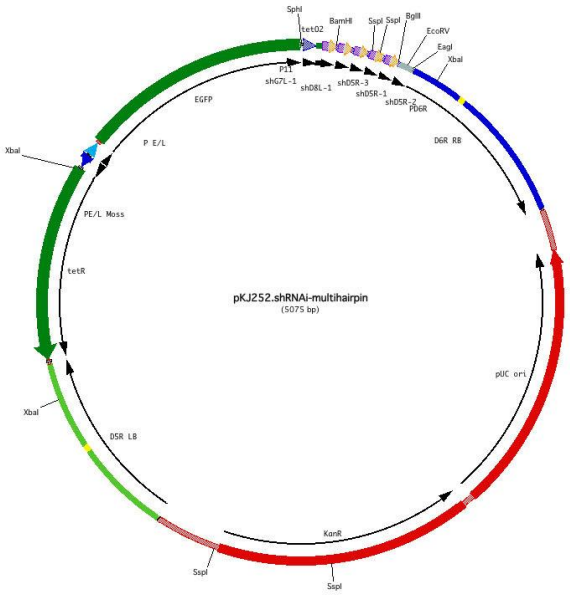

PE/L Moss
P E/L
tetR
EGFP
SphI
EcoRV
EagI
PD6RtetO2
D5R LB
207.pBJ207
(4783 bp)
D6R RB
SspI
KanR
pUC ori
SspI

Figure 5. Features of pKJ252 and pKJ253. Both plasmids contain a kanamycin resistance gene, elements of the *tet* operon to control transcription of the RNAi construct, *tetR* placed under the control of a strong constitutive promoter $P_{E/L}$, and a fluorescent reporter gene *EGFP* also under $P_{E/L}$. **A:** pKJ252 contains the multihairpin RNAi construct shG7L1-shD8L1-shD5R3-shD5R1-shD5R2 (viRNAi-1). **B:** pKJ253 contains the head-to-tail RNAi construct shG7L1-shD8L1-shD5R3-shD5R1-shD5R2 (viRNAi-2).

A.


SphI
tetO2
BamHI
SspI
SspI
BglII
EcoRV
EagI
XbaI
P11
shG7L-1
shD8L-1
shD5R-3
shD5R-1
shD5R-2
PD6R
D6R RB
EGFP
P E/L
XbaI
PE/L Moss
tetR
pKJ252.shRNAi-multihairpin
(5075 bp)
pUC ori
XbaI
D5R LB
KanR
SspI
SspI

B.

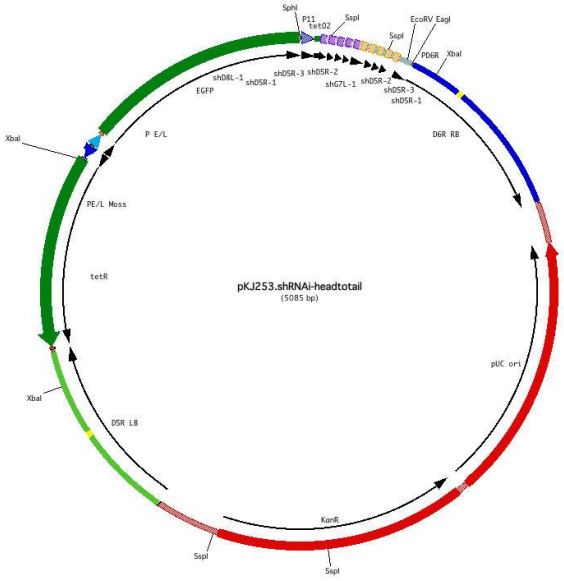
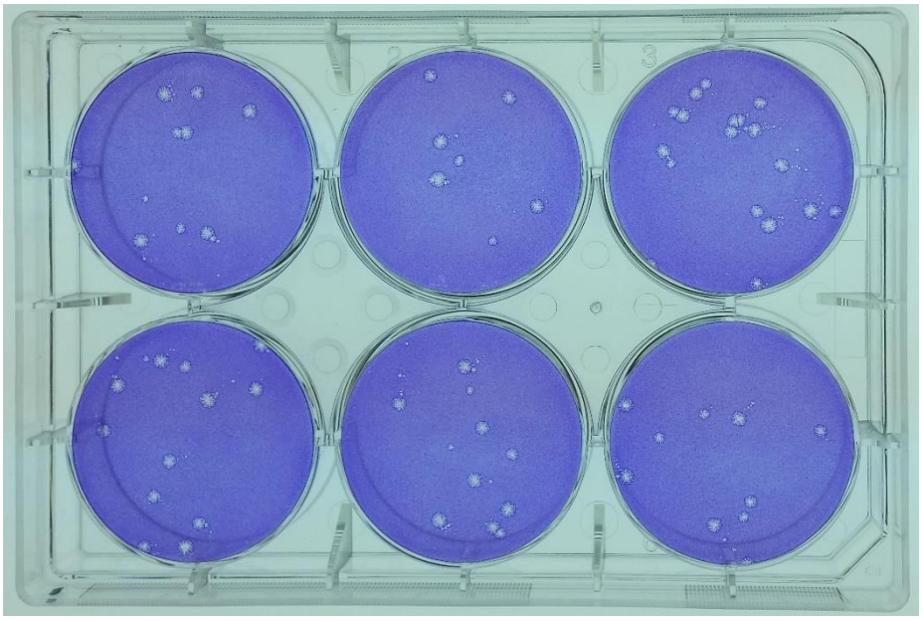

SphI
P11
tetO2
SspI
SspI
EcoRV EagI
PD6R
XbaI
shD8L-1
shD5R-1
shD5R-3
shD5R-2
shG7L-1
shD5R-2
shD5R-3
shD5R-1
EGFP
XbaI
P E/L
D6R RB
PE/L Moss
tetR
pKJ253.shRNAi-headtotail
(5085 bp)
pUC ori
XbaI
D5R LB
KanR
SspI
SspI

Figure 6. Plaque Assay of viRNAi-2 with varying DOX concentrations. BS-C-1 cells were infected with viRNAi-2 (head-to-tail) at ~30 PFU/well in the absence or presence of varying concentrations of DOX. At 48 hpi cells were stained and fixed with 0.5% crystal violet/20% ethanol and the entire well was imaged with a Samsung Galaxy S5 G900T.

No DOX **+ 0.01 µg/ml DOX** **+1 µg/ml DOX**

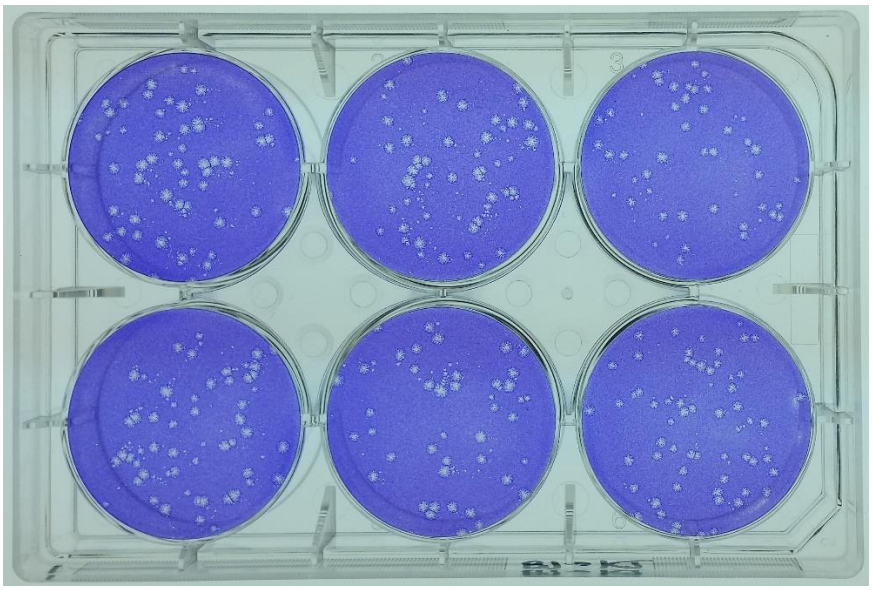


+ 0.001 µg/ml DOX **+ 0.1 µg/ml DOX** **+1 µg/ml DOX**

Figure 7. Plaque Assay of viRNAi-1 and viRNAi-2 with varying DOX concentrations. BS-C-1 cells were infected with either viRNAi-1 (multihairpin) or viRNAi-2 (head-to-tail) at ~30PFU/well in the absence or presence of varying concentrations of DOX. At 48 hpi cells were stained and fixed with 0.5% crystal violet/20% ethanol and the entire well was imaged with a Samsung Galaxy S5 G900T.. **A:** 6-well cell culture plate infected with viRNAi-1; **B:** 6-well cell culture plate infected with viRNAi-2.

A.

No DOX **+ 1 µg/ml DOX** **+10 µg/ml DOX**



B.

No DOX **+ 1 µg/ml DOX** **+10 µg/ml DOX**

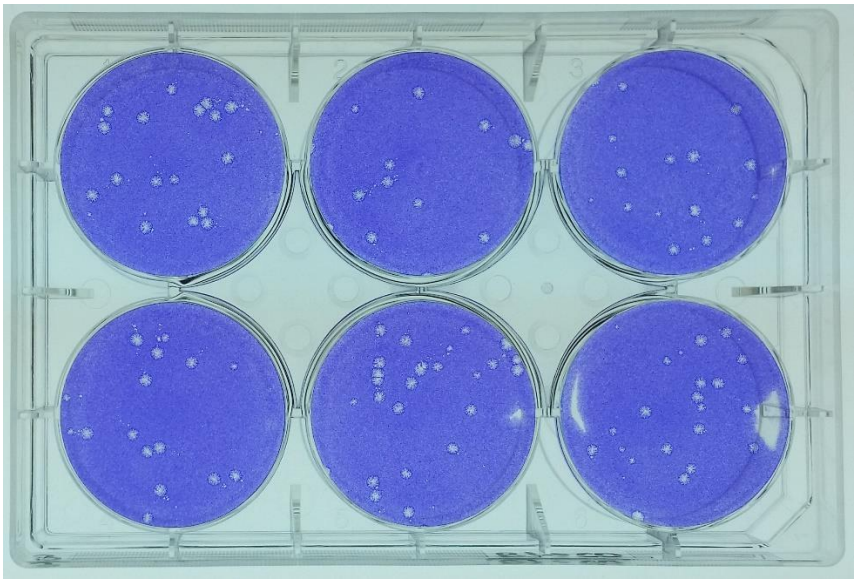
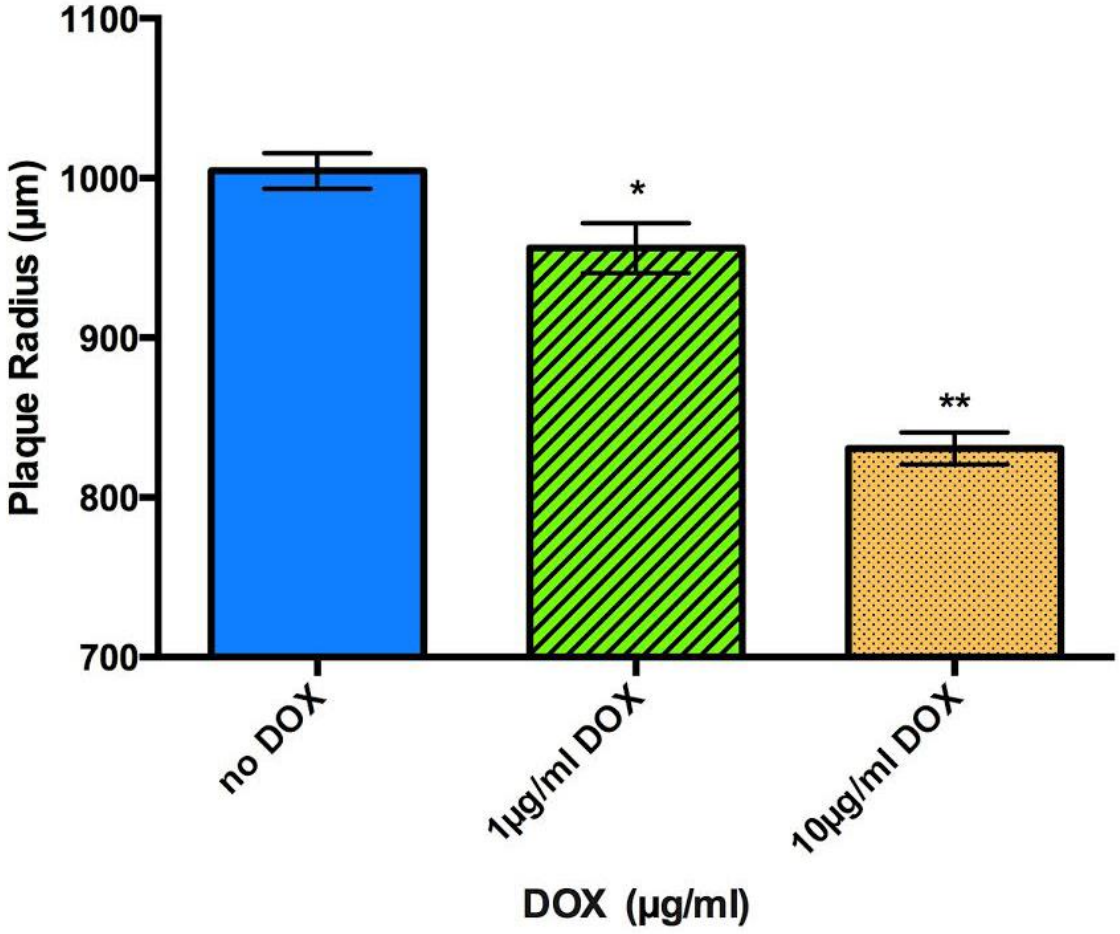


Figure 8. The effect of DOX on the plaque size (radius) of viRNAi-1 inducibly expressing the multihairpin RNAi construct. BS-C-1 cell s were infected with viRNAi-1 (multihairpin) at ~30 PFU/well in the absence or presence of multiple concentrations of DOX. At 48 hpi, cells were stained and fixed with 0.5% crystal violet/20% ethanol. Representative plaques were imaged under an inverted microscope and analyzed with measuring software (AxioVision, 4.8.1, Carl Zeiss). The symbol * indicates statistically significant differences ($P < 0.05$) when comparing no DOX vs. 1 µg/ml DOX. The symbol ** indicates statistically significant differences ($P < 0.0001$) when comparing no DOX vs. 10 µg/ml DOX. Error bars = SEM.


1100
1000
900
800
700
Plaque Radius (µm)
*
**
no DOX
1µg/ml DOX
10µg/ml DOX
DOX (µg/ml)