Title
Human Cytomegalovirus and Small RNAs

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Publication Date
2017

Peer reviewed|Thesis/dissertation

Human Cytomegalovirus and Small RNAs

By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Comparative Biochemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Fenyong Liu, Chair
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Summer 2017

Abstract

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Professor Fenyong Liu, Chair

With a genome size of 230–240 kb, human cytomegalovirus (HCMV) has the largest genome in the human herpes virus family. Once the hosts are infected with HCMV, HCMV remains latent throughout the lifetime, but the latent infection can be reactivated when the host is immunocompromised. The infection symptom is mild or subclinical diseases in immunocompetent adults, but HCMV infection leads to severe, life-threatening complications in people who are immunocompromised, including AIDS patients and transplant recipients. While there's no vaccine available for HCMV infection, understanding the nature of HCMV replication, latency, and transmission is crucial for preventing and curing the disease.

Here we discussed the relationship between various small RNAs and HCMV. In the first part of the study, we examined one viral encoded micro RNA, HCMV-mir-UL22A*, and its influence on HCMV viral infection. We investigated the mechanism of blunt-end HCMV-mir-UL22A* inhibition of HCMV viral replication. The rapid and long lasting suppression of viral replication by blunt-end HCMV-mir-UL22A* suggests blunt-end HCMV-mir-UL22A* a promising antiviral agent. Furthermore, we studied the stagger-end HCMV-mir-UL22A* in regulating host innate immune response. Stagger-end HCMV-mir-UL22A* targets innate immune sensors and facilitate HCMV evade host innate immune response during viral infection. Blunt-end HCMV-mir-UL22A* showed potential for treating CMV infection, while stagger-end HCMV-mir-UL22A* helped us understand HCMV viral innate immune evasion strategy.

In the second part of the study, we focused on applying RNA based precise gene editing technology to treat HCMV infection. Curing CMV infection requires disruption of the viral genome by novel gene editing technology. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system is a gene editing tool for the site-specific cleavage of DNA. We adapted CRISPR/Cas9 system in CMV infected cells. CRISPR/Cas9 could suppress HCMV and MCMV infection in vitro in cell model. Our study suggests CRISPR/Cas9 can potentially be applied to treat CMV infection.

To my parents

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Acknowledgments

I would like to express my sincerest gratitude to Professor Fenyong Liu. My graduate study has given me tremendous growth both in my academic and personal life. I would not be where I am today without his enormous support. I am also grateful to my thesis committee, Dr. Barry Shane, Dr. Qiang Zhou for their critical comments and tremendous support throughout my doctoral research.

I also would like to thank the past and present Liu lab members: Gong Hao, Rachael Burchfield, Michael Reeves, Marco Paliza-carre, Adam Smith, Momei Zhou. Paul Rider was my very first mentor and formed the foundation of my miRNA work. Many thanks to Phong Trang who always came to rescue in difficult situations. Special thanks to Gia Phong-Vu who taught me about MCMV and provided me with protocols and techniques that have benefited my graduate research.

Finally, I would like to express my appreciation to my parents for their unconditional love and support. You are my heroes, my inspiration, my roots, and the reason I devoted myself to science. Thank you for giving me the freedom to pursue my dream.

Last but not least, I want to say thank you to my boyfriend, Filippo. Thank you for always being able to crack a smile on my face even in my most difficult times. Life is never boring with you.

Chapter 1

Introduction

1.1 Background and significance

Herpesvirus introduction

Herpesvirus genomes sizes range from 125 to 240 kbp. They contain from about 70 to 165 genes. All herpesviruses identified to date, which include eight different types that are known to infect human, and more than 170 other viruses that are found in animals. Herpesviruses are a set of ancient viruses who have evolving with their host and developed unique set of viral proteins representing species specificity. However, a large number of herpesvirus common (core) gene products are evolutionarily conserved. These core proteins carry out functions that every herpesvirus relies upon. The core gene products include common virion structure, a core genome replication unit, and similar entry and egress related viral proteins. These herpesvirus common functions are most often recognized by protein sequence similarity among alpha-, beta-, and gammaherpesviruses. These herpesviruses exhibit conservation that suggests a shared common ancestor at least 50 million years ago. Taking distant herpesviruses into account, such as the herpesviruses infecting fish, amphibians, and invertebrates, evidence suggests a common evolutionary origin dating back over 150 million years. In the more distant relatives, a common virion structure, genome organization and similarity across a small subset herpesvirus-common gene products provide the evidence of a common origin.

Herpesvirus genome classification

As shown in **Error! Reference source not found.**, there are 3 subfamilies in Herpesvirus, alphaherpesvirus, betaherpesvirus, and gammaherpesvirus. The herpesvirus family tree is based on amino acid sequence. Herpesvirus genomes are linear, double-stranded. Herpesvirus genomes compose from 32 to 75% of G+C[1]. Larger herpesvirus genomes are accommodated in larger capsids, but the relationship is not proportional, as the packing density of the DNA varies between species[2]. In contrast to the Alpha and Betaherpesvirinae, the genomes of most Gammaherpesvirinae are generally deficient in the CG dinucleotide. CG depletion in herpesviruses, and associated enrichment in TG and CA, has been considered as an indicative of latency in dividing cell populations, in which the latent genome replicates as host cells divide[3]. Thus, HSV-1 whose latency is in non-dividing neurons, has a CG content consistent with its nucleotide composition. EBV whose latently is in dividing B cell populations, is depleted in CG content. Local CG

suppression of the major immediate early gene locus of HCMV has also been noted[3]. Herpesvirus genomes are thought to replicate by circularization, then production of concatemers and cleavage of unit-length genomes when packaging into virus capsids[4]. There are five classes of genome structure in herpesvirus family. The class A genome consists of a unique sequence flanked by a direct repeat. Class A genome is the unique genome structure presented in Betaherpesvirinae such as HHV-6[5], HHV, and one member in the Gammaherpesvirinae, EHV-2. The direct repeat in Class A genome is several kbp in size. Other members of the Betaherpesvirinae also have direct repeats, but shorter in length such as the ones in Murine Herpesvirus[6]. Class B genomes have directly repeated sequences with variable copy numbers of a tandemly repeated sequence of 0.8–2.3 kbp. This arrangement characterizes most Gammaherpesvirinae, such as HVS and HHV-8[7]. The repeated regions may comprise up to 30% of the DNA. The class C structure represents an internal set of direct repeats that is unrelated to the terminal repeat. EBV, a member in Gammaherpesvirinae, has this arrangement[8]. Segment inversion does not occur because the internal and terminal repeats are not related. Class D genomes contain Unique Long (UL) and Unique Short (US) regions, each flanked by inverted repeats. This structure is characteristic of Alphaherpesvirinae such as PRV and VZV. Segment inversion occurs in US, but UL is present predominantly in one orientation. Class E is the most complex genome structure. It is similar to class D, except that TRL/IRL is much larger and segment inversion gives four equimolar genome isomers[9]. The class E arrangement is characteristic of Alphaherpesvirinae in the Simplexvirus genus, and has evolved independently in members of the Betaherpesvirinae such as HCMV and CCMV[10]. Class F is a type of genome structure that lacks the types of inverted and direct repeats that characterize other herpesvirus genomes, presented by THV, a member in Betaherpesvirinae[11].

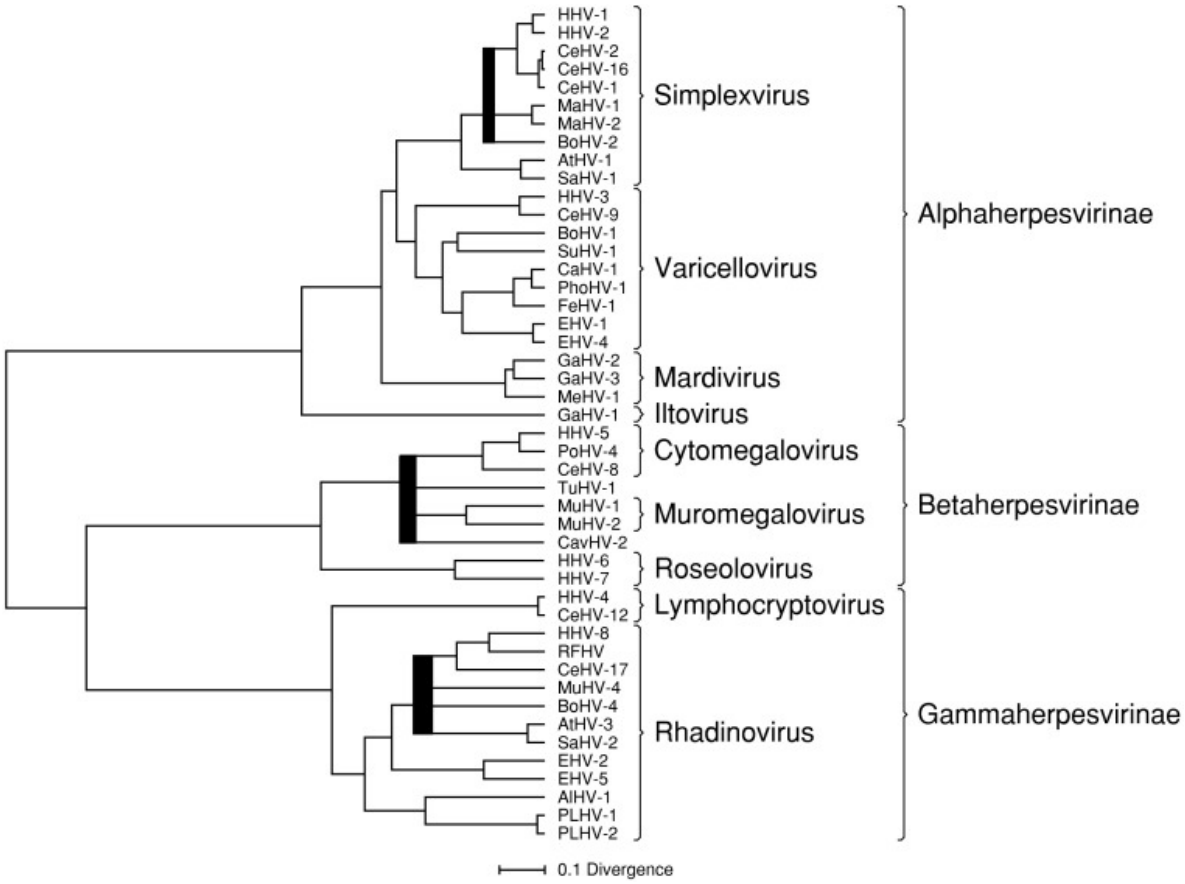

Figure 1 Herpesviridae family tree[12]. Composite phylogenetic tree for herpesviruses. The tree is based on amino acid sequence alignments of eight sets of homologous genes, constructed from maximum-likelihood trees for subsets of these genes, with molecular clock imposed. Thick lines designate regions of uncertain branching. Formal species abbreviations and designations for genera and subfamilies are given on the right.

Herpesvirus entry into host cells

As a prototype of all herpesviruses, HSV is the representative of alphaherpesvirus. The current model of HSV entry is that, the virus first attaches to cell membranes by the interaction of gC, and gB, to glycosaminoglycans (GAGs)[13]. gD facilitates penetration by interacting with one of the entry receptors, to signal receptor-recognition. This interaction triggers fusion by recruiting three additional glycoproteins – gB, gH, gL. The trio of gB, gH, gL, execute fusion with the plasma membrane of the target cell[14]. After fusion, the released tegumented nucleocapsid travels along microtubules to the nuclear pore, where the viral DNA is released into the nucleus.

Herpesvirus core glycoproteins - gB, gH: gL and gM: gN, appear to play the critical roles in entry in betaherpesvirus entry. HCMV as a prototype for betaherpesvirus, has been extensively studied. HCMV entry into fibroblasts is via direct fusion at the cell surface [15]and possibly via endocytosis in other cell types. Entry of betaherpesviruses appears to involve the interaction of viral glycoproteins with several cellular receptors, and is initiated by a common use of the cell surface proteoglycan heparan sulfate[16]. gB from both HCMV and roseolaviruses binds heparan sulfate facilitated by interactions with additional receptors[17]. HCMV gB has been shown to utilize the EGF receptor as well as integrins as entry mediators[18]. Signaling may play a critical role in conditioning the cell for viral replication following entry event.

gB gH, gL, gM, and gN are glycoproteins that have homologues in all herpesvirus studied to date and are now commonly referred to by the naming system originally developed for the prototype alphaherpesvirus, herpes simplex virus. However, there are several viral encoded proteins that are unique in gammaherpesvirus entry event. Glycoproteins gp350/220, gp42 and the BMRF 2 protein, together with the conserved glycoproteins gB, gH and gL, are all involved in virus entry as described below. It also is possible that complexes of gN and gM may play a role in this process although their major role is probably in envelopment and egress of newly made[19]. The phenotype of a virus that lacks gp150 slightly enhanced its ability to infect epithelial cells[20]. The BILF 1 gene product functions as a constitutively active G protein-coupled receptor.

Herpesvirus regulatory proteins

Followed by entry event, herpesvirus influences host cell events through a set of regulatory proteins. Most regulatory proteins encoded by herpesviruses are unique. Only one core protein is purely regulatory, acting as a multifunctional regulator of expression (MRE). MRE has been most extensively studied in HSV-1. MRE binds RNA and localizes to sites of transcription in the nucleus and interacts with components of the RNA polymerase II transcription machinery, the spliceosome complex and pre-mRNA export machinery. MRE stimulates late gene transcription and dictates the location of viral transcripts in infected[21]. MRE obstructs cellular mRNA splicing to allow the viral transcripts to be preferentially exported from the nucleus[22]. During the early stage of infection, MRE triggers splicing to stall by recruiting host cell kinases to the nucleus to inactivate splicing factors. In alphaherpesvirus and gammaherpesvirus systems, MRE recruits an export adaptor protein (Aly/REF) to sites of viral transcription to facilitate export. This inhibition is relieved during the late phase of infection when splicing and export of host and viral transcripts resumes. In betaherpesvirus such as HCMV, MRE interacts with an RNA helicase to promote cytoplasmic accumulation of viral mRNA[23]. The MRE may also influence shut-off of the host cell and transcriptional events, however the mechanism is still unclear.

Herpesvirus DNA synthesis

All herpesviruses encode a core set of six DNA synthesis enzymes that direct viral DNA synthesis. Herpesviruses initiate lytic DNA replication at defined sites on the viral genome. Some herpesviruses have a single origin of DNA replication (oriLyt), such as betaherpesviruses (HCMV, HHV-6A, HHV-6B, HHV-7). Others have either two (VZV, EBV, KSHV) or even more (HSV-1, HSV-2) oriLyt sites. The relative position of one copy of oriLyt adjacent to the single stranded DNA binding protein (SSB) gene is conserved in many alpha-, beta- and gammaherpesviruses, even though the primary DNA sequence of oriLyt is not conserved in all of these settings. All herpesviruses appear to rely on virus-encoded DNA binding proteins to control initiation at oriLyt. In alphaherpesviruses and the roseolaviruses subgroup of betaherpesviruses, a dedicated ori binding protein is essential for replication. Gammaherpesviruses and cytomegaloviruses in betaherpesvirus rely on DNA binding regulatory proteins that control gene expression and also function during initiation of replication. Herpesvirus DNA replication starts at nuclear domain 10. This process overwhelms the nucleus and proceeds to form replication units where viral replication proteins and DNA accumulate[24]. Viral DNA synthesis caused the

production of multi-genomic length concatemers that are the substrate for genome packaging using conserved viral gene products. Distinct DNA replication origins separate from oriLyt sites that are responsible for the synthesis of viral DNA during latent infection have been identified in gammaherpesviruses but not in other subfamilies.

Herpesvirus capsid assembly and maturation

Herpesvirus genomes are protected by a highly ordered icosahedral-shape nucleocapsid that is 125–130 nm in diameter. The capsid is surrounded by a partially ordered proteinaceous layer called tegument. Tegument is enclosed with a polymorphic lipid bilayer containing viral glycoproteins that are responsible for viral attachment and entry to host cells. Each of the herpesviruses encodes a specific set of capsid proteins that form virion. The assembly pathway of the virus particles is highly similar[25]. The nucleocapsid is formed in the nucleus and follows a pathway that assembles virion. First, a procapsid is assembled with the formation of the capsid shell and the internal scaffolding structure. Second, the procapsid is converted into mature nucleocapsid. At the same time, the internal scaffolding protein is released and replaced by the viral DNA genome, along with a major conformation change of the capsid shell[26]. The mature nucleocapsid exits the nucleus and acquires its tegument and envelope by detachment from nuclear membranes and fusion with other cellular membranous. Eventually, the mature virion particles are released into the extracellular through cellular secretory pathways.

Herpesvirus transmission and infection

In the alphaherpesvirus family, taking HSV as example, the transmission of HSV infection is dependent upon intimate, personal contact of a susceptible seronegative individual with someone excreting HSV. Virus need to come in contact with mucosal surfaces or abraded skin to host infection. With viral replication at the site of primary infection, the capsid is transported retrograde by neurons to the dorsal root ganglia where latency is established after round of replication. The more severe the primary infection, the more likely it is that recurrences will follow. After latency is established, a proper stimulus causes reactivation; virus travels back to mucocutaneous sites, appearing as skin vesicles or mucosal ulcers. Most patients who have serologic evidence of infection with HSV-1 and/or HSV-2 do not recognize that they have been infected. Most infections are asymptomatic or associated with non-specific signs and symptoms.

However, when symptoms do occur, they tend to be more severe with primary compared with recurrent infections. Also, whether accompanied by symptoms or not, viral excretion during primary infection is more prolonged than shedding during recurrent infection.

In the betaherpesvirus family, taking HCMV as example, virus is assumed to be transmitted through direct contact with body fluids from an infected person. Sources of virus include oropharyngeal secretions, urine, cervical and vaginal secretions, semen, breast milk, blood products, and allografts[27]. Exposure to saliva and other body fluids containing infectious virus is a primary mode of spread and infected infants typically excrete significant amounts of HCMV for months to years following infection. Even older children and adults shed virus for prolonged periods (>6 months) following a primary HCMV infection. In addition, a significant proportion of seropositive individuals continue to shed virus intermittently.

In the gammaherpesvirus family, transmission routes are similar to alpha- and betaherpesvirus. Primary infections are usually asymptomatic. However, in immunocompromised patients and in other special but poorly understood circumstances, tumors and other virus-associated diseases may manifest. Both EBV and KSHV were first identified in tumors and primary effusion lymphomas are typically dually infected[28].

Therapeutic small RNAs

Herpesvirus infection has been proven to cause various of diseases including cancer. However, the state of art treatment does not precisely target specific viral replication. Furthermore, some herpesvirus has already developed drug resistance against anti-viral therapies. Recent progress of biological drugs has expanded the scope of therapeutic targets for fighting viral infection. Nucleic acid based molecules represent promising therapeutic strategies for targeting of specific sequences. Several RNA based therapeutics have gone through clinical trials. The pioneering RNA related therapies include therapeutic ribozymes, aptamers, small interfering RNAs (siRNAs), and recently discovered CRISPR/Cas9 system demonstrated the versatility of RNA therapies.

Ribozymes

Ribonucleic acid enzyme, ribozyme, is an RNA molecule that is capable of performing specific biochemical reactions, similar to the action of protein enzymes. Ribozymes are RNAs with enzyme activities that use RNAs as substrate. In nature, ribozymes synthesis RNA as part of the transcription process. Ribozymes also synthesis RNA primers during DNA replication. There are many different types of ribozymes including group 1 and group 2 splicing ribozymes, RNase P, hammerhead ribozyme, hairpin ribozyme, and the ribosome.

Group 1 intron ribozymes are found in bacteria, lower eukaryotic and higher plants[29]. The group 1 splicing reaction requires a guanine residue cofactor, the 3' OH group of guanosines is used as a nucleophile. The 3' 3' OH group attacks the 5' phosphate of the intron; then a new phosphodiester bond is formed. The 3' OH now acts as the nucleophile in a similar reaction at the 3' end of the intron. Finally, the intron is precisely excised, and exons are connected. Group 2 intron splicing are found in bacteria and the mitochondrial and chloroplast genomes of fungi, plants, protists, and worm[30]. In group 2 intron splicing, the 2' OH of an adenosine act as a nucleophile and attacks the 5' splice site creating a separate intron branch. The 3' OH of the 5' exon attacks the 3' splice site, ligating the exons and releasing the intron as a lariat structure.

Ribonuclease P (RNase P), a ribonucleoprotein, is an essential tRNA processing enzyme found in all living creatures. The discovery of RNase P led to the discovery of the catalytic properties of RNA[31]. All RNase P enzymes are ribonucleoproteins containing an RNA and protein subunits depending on the organisms. The role of protein component in RNase P is the bond between RNase and tRNA substrate. Similar to protein enzyme, RNase P requires metal ions for its enzymatic activities. In nature, endo-ribonuclease generates 5' end of matured tRNA. Cleavage is made through nucleophilic attack on the phosphodiester bond, leaving a 5'- phosphate and 3' OH at the cleavage site.

Hammered ribozyme are small autocatalytic RNAs that cleave single stranded RNA[32]. They are found as part of the virusoids. Virusoids are virus-like elements using "rolling circle replication" to replicate their small, circular RNA genomes[33]. Cleavage mediated by hammerhead ribozymes occurs through nucleophilic attack by the 2' OH of a core nucleotide on its adjacent phosphodiester bond. The cut

creates 2', 3' cyclic phosphate and 5'OH. This happens during the rolling circle replication while a long strand of multiple copies of the virusoids RNA is created. Each copy contains a hammerhead motif that catalyzes strand breakage in itself. This catalytic activity allows virusoid create multiple individual copies in one replication cycle. Hairpin ribozyme is another RNA motif that catalyzes RNA processing reactions for satellite RNA replication[34]. This reaction is also self-processing like hammered ribozymes. However, hairpin ribozymes do not require metal cations.

The ribosome is a vast and complex molecular machine. It is found in all living organisms. The ribosome is the primary site of translation. It consists of two subunits, one large subunit with 5S and 23S ribosomal RNA (rRNA) as its core, one small subunit with 16S rRNA[35]. The large subunit binds to the tRNA, the amino acids, and the smaller subunit. The smaller subunit binds to the larger subunit and the mRNA pattern. Ribosomes are ribozymes because the catalytic peptidyl transferase activity that connects amino acids together is done by the ribosomal RNA. Ribosome uses mRNA as the template, translating each codon of the mRNA, pairing it with the correct amino acid presented by aminoacyl- tRNA. Aminoacyl-tRNA contains anticodon and appropriate amino acid. Ribosome accurately recognizes tRNA by conformational changes in itself. Translation starts from AUG codon binding to the small ribosomal subunit. The large subunit is then recruited to the site. There are three RNA binding sites in the ribosome: A, P, and E. The A site binds an aminoacyl-tRNA. The P site binds to a peptidyl- tRNA. The E site binds to a free tRNA before it exits the ribosome. Protein synthesis begins at AUG at the 5' end of the mRNA. First, mRNA binds to the P site of the ribosome, Next to it, a new codon is exposed in A site. tRNA will then carry the next amino acid bind to the A site. When a new peptide bond is created, mRNA shifts forward by a codon, exposing the A site again. A peptide chain is set up by rounds of reactions like this.

Riboswitches are regulatory elements within its mRNAs that regulate mRNA expression. Riboswitch functions as a ribozyme and cleaves itself if sufficient concentration of its metabolite is present[36]. The riboswitch can fold the mRNA to prevent translation from occurring. It can also affect the splicing of the pre-mRNA molecule. Depends on its particular function, riboswitch can either inhibit or activate protein translation. The riboswitch contains two domains: the aptamer domain and the expression platform. A switching sequence can be placed in either domain, dictating the expression outcome of mRNA. Riboswitches can affect

either transcription or translation. The expression platform acts during transcription usually involves the ligand dependent formation of an intrinsic terminator or anti-terminator structure. However, riboswitch that acts during translation often interacts with the ribosome binding site. The translation stalls when ribosome binding site is masked, vice versa. Because riboswitch is versatile tools for controlling gene expression, they have been used to reprogram bacterial genomes[37].

Antisense oligonucleotides

Antisense oligonucleotides are synthetic, chemically-modified small RNAs. Their sequence is from 3' to 5' as antisense, complementary to the sense sequence of their corresponding mRNA. Antisense oligonucleotides are synthesized in the hope that they can be used as therapeutic agents to block disease progress by regulating particular disease related protein[38]. This could be achieved by binding of the antisense oligonucleotide to the mRNA of that particular protein. Binding of the antisense and the mRNA could physically halt ribosome activity, therefore, suppress protein synthesis, could accelerate mRNA degradation rate and could prevent splicing error that might produce a defective protein. However, to be useful in clinical therapy, antisense must enter the cell efficiently, avoid degradation by nucleases, and prevent the off-target effect. Therefore, antisense oligonucleotides are usually chemically modified to resist nucleases digestion.

RNA interference

RNA interference (RNAi) is a mechanism that inhibits gene expression at the stage of translation or by suppressing the transcription of specific genes. There are two types of small RNAs working as RNA interference. Small RNAs can bind to specific mRNAs to either increase or decrease their translation. In nature, RNA interference plays crucial roles in defending cells against pathogens and transposons[39]. RNA interference also sculpts our gene expression. More than 30% of the genes in the human genome is regulated by small RNAs. RNAi can be found in many eukaryotes. Small RNA processing is initiated by the enzyme Dicer that cleaves dsRNA into short double-stranded fragments of ~20 nucleotide siRNAs. Each siRNA is unwound into two single-stranded RNAs (ssRNAs): the passenger strand and the guide strand. The passenger strand is degraded in the cytosol with no binding activity, and the guide strand is incorporated into the RNA-induced silencing complex (RISC). RISC complex then binds to the target mRNA by sequence homology, regulating protein translation by creating cleavage

at the target site by Argonaute 2 (Ago2), the catalytic component of the RISC complex. RNAi has become a valuable research tool both in vitro and in vivo. Synthetic dsRNA introduced into cells can specifically and robustly suppression expression in the gene of interest. As small dsRNAs are much more stable than single-stranded antisense oligonucleotides, RNAi is used as a practical tool in biotechnology and medicine.

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Chapter 2 Type 1 interferon production induced by Human Cytomegalovirus encoded miRNA mimic

2.1 Introduction

It was only 25 years ago since Rosalind C. Lee and his colleagues discovered a C. elegans miRNA named lin-4[40]. Lin-4 negatively regulates lin-14 protein by complimentary binding on the 3' UTR region in lin 14 mRNA. The discovery of novel RNA based inhibition was thought to be exclusive in C. elegans until the discovery of let-7 and lin-4, conserved in many spices including human and fly[41], in 2001 by Reinhart BJ et al[42]. They discovered that lin-4 and let-7 triggered transitions in the complement of heterochronic regulatory proteins to control the timing of stem-cell division and differentiation. Let- 7 was then known as the first human miRNA. Since the discovery of let-7, human genome encodes more than 2500 miRNAs which regulate more than 60% of our genes[43]. In 2004, Tuschl and colleagues discovered five EBV miRNAs originated from five different precursors that are clustered in two regions of the EBV genome. This opened a new field of study. Nowadays, more than 500 viral encoded miRNAs have been discovered. Herpesviridae encodes 399 miRNAs in total and accounts for more than 60% the virus encoded miRNAs discovered.

With a genome size of 230–240 kb, human cytomegalovirus (HCMV) has the largest genome in the human herpes virus family[44]. HCMV is spread from an infected person's urine or saliva, transplanted organs and blood transfusions. Once the hosts are infected with HCMV, HCMV remains latent throughout the lifetime, but the latent infection can be reactivated when the host is immunocompromised. The infection symptom is mild or subclinical diseases in immunocompetent adults, but HCMV infection leads to severe, life-threatening complications in people who are immunocompromised, including AIDS patients and transplant recipients[45]. The symptoms of HCMV infection in immunocompromised patients include esophagitis, gastroenteritis, retinitis, and pneumonia. HCMV can also be transmitted through the placenta and breast milk. Recurrent or primary novel HCMV infection during the pregnancy is one of the leading cause of congenital disease. 5% of the pregnant women encounter primary infection during pregnancy, 5% of the infection can be transmitted to the fetus. HCMV infection causes premature birth, liver, lung and spleen problems, small size at birth, small head size, and seizures, hearing loss, vision loss, intellectual disability. [46] While there's no vaccine available for HCMV infection, understanding the nature of HCMV replication, latency, and transmission is crucial for preventing and curing the disease.

To understand the nature of HCMV infection, we need to look at the relationship between the virus and the host. HCMV is an ancient virus which has been evolving along with its host- human for millions of years and developed effective immune evasion strategies. The immune evasion strategy includes host proteins translation machinery inhibition, host immune regulation protein analogous, and antigen presentation inhibition. miRNAs play crucial roles in regulating both viral and host protein translation during herpes virus infection(**Error! Reference source not found.**). miRNAs are key factors that regulate HCMV immune evasion. Unlike other herpes viruses, HCMV miRNAs are encoded throughout the virus genome. This phenomenon suggests that HCMV can be expressed at various time in virus life cycle. Therefore, HCMV miRNAs play wide range of roles in facilitating virus infection. Interestingly, HCMV miRNAs are ones of the very few transcripts that are expressed during latent infection. It is plausible that HCMV-encoded miRNA can be exploited to avoid host immune response and help to maintain latency. For instance, hcmv-miR-UL112 down-regulates MICB expression during viral infection. MICB is a stress-induced ligand of the natural killer (NK) cell activating receptor NKG2D and is critical for the NK cell killing of virus-infected cells and tumor cells. Down-regulation of MICB decreased binding of NKG2D, and reduced killing by NK cells that targets the ligand of the natural killer cell activating receptor NKG2D[47]. Another HCMV miRNA, hcmv-miR-US33-5p targets host gene Syntaxin3 (STX3) which inhibits HCMV DNA synthesis and of viral replication[48]. Hcmv-miR-US25-1 targets multiple cell cycle genes including cyclin E2, BRCC3, EID1, MAPRE2, and CD147, suggesting that hcmv-miR-US25-1 is targeting genes within a related pathway[49]. An overview of HCMV miRNAs is illustrated in the Table 2.

Virus	# of miRNA precursors	# of mature miRNAs
Bovine foamy virus	2 precursors	4 mature
Bovine herpesvirus 1	10 precursors	12 mature
Bovine herpesvirus 5	5 precursors	5 mature
BK polyomavirus	1 precursors	2 mature
Bovine leukemia virus	5 precursors	10 mature
Bandicoot papillomatosis carcinomatosis virus type 1	1 precursors	1 mature
Bandicoot papillomatosis carcinomatosis virus type 2	1 precursors	1 mature
Duck enteritis virus	24 precursors	33 mature
Epstein Barr virus	25 precursors	44 mature
Herpes B virus	12 precursors	15 mature
Human cytomegalovirus	15 precursors	26 mature
Human herpesvirus 6B	4 precursors	8 mature
Human immunodeficiency virus 1	3 precursors	4 mature
Herpes Simplex Virus 1	18 precursors	27 mature
Herpes Simplex Virus 2	18 precursors	24 mature
Herpesvirus saimiri strain A11	3 precursors	6 mature
Herpesvirus of turkeys	17 precursors	28 mature
Infectious laryngotracheitis virus	7 precursors	10 mature
JC polyomavirus	1 precursors	2 mature
Kaposi sarcoma-associated herpesvirus	13 precursors	25 mature
Mouse cytomegalovirus	18 precursors	29 mature
Merkel cell polyomavirus	1 precursors	2 mature
Mareks disease virus type 1	14 precursors	26 mature
Mareks disease virus type 2	18 precursors	36 mature
Mouse gammaherpesvirus 68	15 precursors	28 mature
Pseudorabies virus	13 precursors	13 mature
Rhesus lymphocryptovirus	36 precursors	68 mature
Rhesus monkey rhadinovirus	7 precursors	11 mature
Simian virus 40	1 precursors	2 mature

Table 1 Virus encoded miRNAs[43]. This table is based on the registry in miRBase as March 2017.

HCMV miRNA	Sequence 5' to 3'
hcmv-miR-US25-1-3p	UCCGAACGCUAGGUCGGUUCU
hcmv-miR-US25-2-3p	AUCCACUUGGAGAGCUCCCGCGGU
hcmv-miR-UL69	CCAGAGGCUAAGCCGAAACCG
hcmv-miR-UL36-3p	UUUCCAGGUGUUUUCAACGUGC
hcmv-miR-US22-3p	UCGCCGGCCGCGCUGUAACCAGG
hcmv-miR-UL70-3p	GGGGAUGGGCUGGCGCGCGG
hcmv-miR-US29-3p	CCCACGGUCCGGGCACAAUCA
hcmv-miR-US25-2-5p	AGCGGUCUGUUCAGGUGGAUGA
hcmv-miR-UL70-5p	UGCGUCUCGGCCUCGUCCAGA
hcmv-miR-US5-2-3p	UAUGAUAGGUGUGACGAUGUCU
hcmv-miR-UL112-5p	CCUCCGGAUCACAUGGUUACUCA
hcmv-miR-UL22A-5p	UAACUAGCCUUCCCGUGAGA
hcmv-miR-UL36-5p	UCGUUGAAGACACCUGGAAAGA
hcmv-miR-US25-1-5p	AACCGCUCAGUGGCUCGGACC
hcmv-miR-US33-5p	GAUUGUGCCCGGACCGUGGGCG
hcmv-miR-US5-2-5p	CUUUCGCCACACCUAUCCUGAAAG
hcmv-miR-US4-3p	UGACAGCCCGCUACACCUCU
hcmv-miR-US22-5p	UGUUUCAGCGUGUGUCCGCGGG
hcmv-miR-UL148D	UCGUCCUCCCCUUCUUCACCG
hcmv-miR-US29-5p	UGGAUGUGCUCGGACCGUGACG
hcmv-miR-UL22A-3p	UCACCAGAAUGCUAGUUUGUAG
hcmv-miR-US33-3p	UCACGGUCCGAGCACAUCCAA
hcmv-miR-UL59	GUUCUCUCGCUCGUCAUGCCGU
hcmv-miR-UL112-3p	AAGUGACGGUGAGAUCCAGGCU
hcmv-miR-US5-1	UGACAAGCCUGACGAGAGCGU
hcmv-miR-US4-5p	UGGACGUGCAGGGGGAUGUCUG

Table 2 HCMV encoded miRNAs. This table is based on the registry in miRBase[4].

One particular miRNA, hcmv-mir-UL22A*, is a viral miRNA expressed HCMV Unique Long region (UL) 22 open reading frame(ORF), oriented in the 5' to 3' direction. Hcmv-mir-UL22A* was first discovered in our lab, and its expression was confirmed by real-time PCR, next generation sequencing, and Northern blot[50,51,52]. Interestingly, hcmv-mir-UL22A* is the most abundantly expressed miRNA during 24 hours' post-infection[5051]. Varies in cell types, hcmv-mir-UL22A* expression is 500-1000 times higher than other HCMV miRNAs. Mesfin et al[51]. discovered that at 24 hours' post-infection, HCMV mir-UL22A3p (*) has the highest abundance in HFF infected with AD169, MOI=2, determined by real-time PCR. Shen et al[53]. determined that hcmv-mir-22A* expression increased gradually. At 48 hours post-infection, hcmv-mir-22A* expression reached its peak (> 30-fold changes).

We observed that overexpression of blunt-end HCMV-miR-UL22A* mimic dramatically inhibited the production of functional virions, while cells transfected with stagger-end HCMV-miR-UL22A* with 3' 2nt overhang did not shut down virus replication. We concluded that the inhibition of HCMV replication was initiated by RIG-I recognition of blunt-end 22A*. To study the function of dsRNA and 22A*, we will illustrate our theory in two separate parts. The first part we will discuss the function of blunt-end HCMV-miR-UL22A* in triggering innate immune response. The second part we will discuss stagger-end HCMV-miR-UL22A* in inhibiting the production of innate immune response protein.

2.2 Materials and Methods

Cells and virus

HFF cells were obtained from Clonetics, cultured as adherent cells DMEM(Invitrogen), with 10% FBS, 1% Penicillin streptomycin (Pen-Strep) from Invitrogen. HCMV Towne BAC was previously constructed by inserting a BAC sequence into the genome of HCMV laboratory-adapted strain Towne. HCMV Towne BAC strain was propagated from virus stocks in HFFs. Incubate the HFFs with HCMV Towne BAC at MOI = 0.01. After 2 hours incubation, wash and change media for HFFs. We collected both supernatant and cell lysis. Cell lysis were first collected by cell scrapers in 10% non-fat dry milk, followed by sonication at 15% amplitude for 1 minute at 30 second intervals. Virus stocks were stored at -80C.

Plaque forming assay

Seed the HFFs to 24-wells at 18 hours before performing the plaque forming assay. The seeding density for 24 wells plates is 0.05 * 10^6 cells per well. 18 hours after seeding, HFFs were at 1.2 * 10^ 6 cells per well. Dilute virus sample from 10^-1 to 10^-6 in complete growth medium. Aspirate the culture medium and add 250ul of the diluted virus to HFFs. Incubate the cells along with the virus for two hours. After incubation, wash and change media twice for HFFs. Add the melted 2% agarose with 4% FBS 2X DMEM overlay to the HFFs. Incubate the plates at 37C tissue culture incubator for 10 days. Count the GFP positive plaques under fluorescence microscopy.

Transfection

HFFs were seeded to 24 wells plates without antibiotic 18 hours before transfection at 0.05 * 10^6 cells per well. HFFs reached to 1.2 * 10^ 6 cells per well and were ready for transfection the next day. All transfections were carried out using Lipofectamine™ 2000 (Life Technologies). 25pmol miRNA and 1 ul Lipofectamine™ were transfected to HFFs. Incubate transfected HFFs for 18 hours. Wash and change the media to complete HFF growth medium with 1% pen-strep. All Stars Negative Control siRNA (1027280) and miScript miRNA Mimics:

HCMV-miR-UL22A (MSY0001574), HCMV-miR-UL22A* (MSY0001575), were obtained from Qiagen.

Nucleic acid purification and quantification

Total DNA were purified from HFFs using DNasey Blood and Tissue kit (Qiagen) according to manufacturer's protocol. Total RNAs were purified from HFFs using Trizol (Life Technologies) and miRNasy kit (Qiagen) according to manufacturer's protocol. DNA and RNA were determined using by spectrophotometry.

Luciferase assays

Luciferase reporter construct was constructed by inserting the target gene sequence into the psiCHECK-2. Cells were co-transfected with the psiCHECK plasmid containing the gene of interest, the psiCHECK-2 vector to normalize levels of luciferase activity, and a miRNA. 48 hours post-transfection, cells were then treated with passive lysis buffer in a 48 – well plate format. Luciferase levels were then measured using a "Dual Luciferase Assay" with the SpectraMax L Luminescence Microplate Reader in conjunction with Firefly and Renilla luciferase reagents and analyzed using Microsoft Excel.

Real-Time PCR

Total RNA was subjected to DNase digestion using the RNase-free DNase set (Qiagen) and reverse transcription using Affinity script reverse transcriptase (Agilent) with oligo dT (15) primer (Fisher Scientific). The SYBER Green real-time PCR kit (Bio-Rad) was used to perform QRT-PCR on CFX™ Real-Time PCR Detection System (Bio Rad). In 20ul reaction contains 300nm primer shown in **Error! Reference source not found.**, 10ng template cDNA, 10ul SYBER Green supermix. Real-time PCR conditions were 1 cycle of 3 mins at 95°C, followed by 40 cycles of 10 sec at 95°C, 30 sec at 55-60°C (depending on the primer pairs according to Table 3), and 30 sec at 72°, and finishing with melt curve analysis. We checked Real-time PCR products amplification and standard gel electrophoresis.

Western blotting

Proteins of the cells were prepared by suspending cells in NP40 lysis buffer, followed by centrifugation at 14,000 RPM 10min, 4C. Collect the supernatant. Total proteins were quantified using DC™ Protein Assay(Bio-Rad). A total protein of 20ng was loaded in the SDS-PAGE gel. Protein was separated by 10% SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. Block the membrane with blocking buffer (4% non-fat dry milk and 1% BSA in DPBS). Incubate the membrane with primary antibody overnight. Wash membrane with Tween-DPBS for 15mins, wash 3 times. Incubate the membrane with secondary antibody for 30 mins. Wash the membrane with Tween-DPBS for 15min, wash 3 times. Incubate the immunoblots with ECL detection system. Visualize the blot using ChemiDoc Touch imaging system. Sample loading was normalized by quantities of GAPDH detected parallel.

Name	Sequence (5’ -> 3’)
Interferon-beta Forward	CTCTCCTGTTGTGCTTCTCCA
Interferon-beta Reverse	TAGTCTCATTCCAGCCAGTGC
IFI27 Forward	TGGCTCTGCCGTAGTTTTG
IFI27 Reserve	TCCTCCAATCACAACTGTAGCA
IFI44 Forward	AGCCGTCAGGGATGTACTATAAC
IFI44 Reverse	AGGGAATCATTTGGCTCTGTAGA
OAS1 Forward	AGCTTCGTACTGAGTTCGCTC
OAS1 Reverse	CCAGTCAACTGACCCAGGG
RSAD2 Forward	CAGCGTCAACTATCACTTCACT
RSAD2 Reverse	GGAAACAGAAGCCGCATTTGTA
IL-6 Forward	CCAGCTATGAACTCCTTCTC
IL-6 Reverse	GCTTGTTCCTCACATCTCTC
GAPDH Forward	ACCCACTCCTCCACCTTTGA
GAPDH Reverse	CTGTTGCTGTAGCCAAATTCGT

Table 3 Primers design. All primers were ordered from IDR in 25nm standard desalt.

2.3 Results

Inhibition of HCMV viral replication by blunt-end HCMV-miR-UL22A*

To study the function of blunt-end HCMV-miR-UL22A* during HCMV viral replication, we first infected the cells with HCMV Town BAC strain that is lacking the HCMV-miR-UL22A* coding region. We did not observe virus replication being disrupted. Interestingly, when we transfected the cells with HCMV-miR-UL22A* from Qiagen, we observed HCMV replication declined Figure 2. As shown in the fluorescent microscopy, cells transfected with blunt-end HCMV-miR-UL22A* showed little to no GFP positive colonies at 5 days post-infection (Figure 3). Whereas cells transfected with negative control blunt-end miRNA indicated 90% GFP positive cells. Cell culture supernatants were collected for titration assay at 3-day interval for 10 days. As shown in the plaque forming assay, we discovered that blunt-end HCMV-miR-UL22A* could inhibit HCMV Town BAC GFP viral growth completely. Furthermore, the inhibition of virus production was stable. No virus could be detected even at 10 days post-infection (Figure 3). In addition to our prior finding which no viral growth change was observed in HCMV lacking HCMV-miR-UL22A* coding region, we suspected that the viral growth inhibition by HCMV-miR-UL22A* might be related to innate immune response triggered by introducing blunt-end miRNA in HFFs.

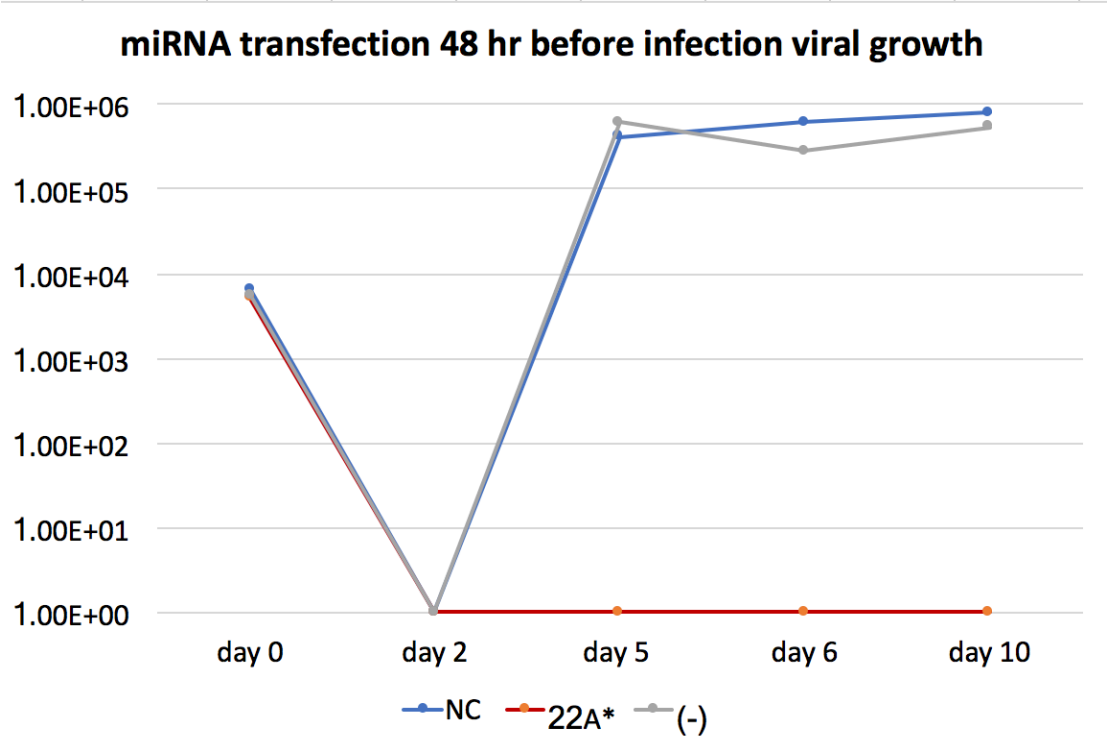


Figure 2 HCMV-miR-UL22A* inhibits viral replication in human foreskin fibroblasts(HFF). HFFs were transfected with the indicated RNAs or left untreated. 48 hours post-transfection cells were infected with HCMV Towne BAC at MOI of 0.01. Supernatants from infected cell cultures were harvested at the indicated times post-infection. Viral titers were determined using plaque forming assay.

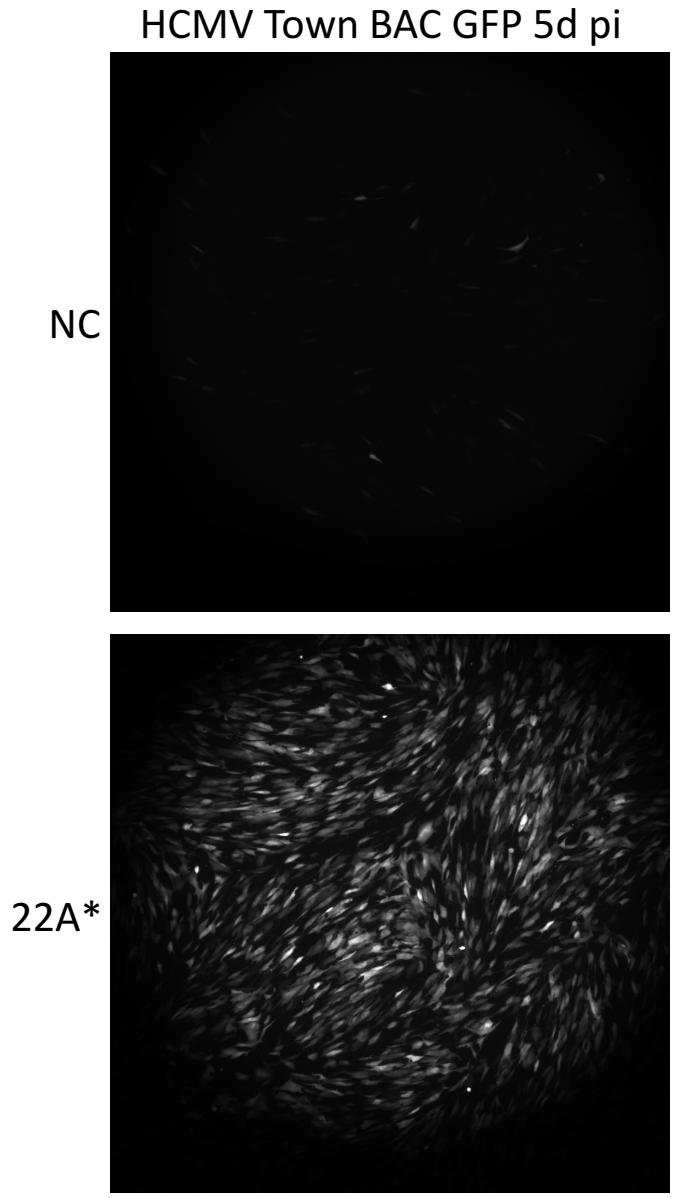


Figure 3 HCMV-miR-UL22A* inhibits viral replication in human foreskin fibroblasts(HFF). HFFs were transfected with the indicated RNAs or left untreated. 48 hours post-transfection cells were infected with HCMV Towne BAC at MOI of 0.01. HFFs were observed under fluorescence microscopy at 5 days post-infection.

Up-regulation of IFN-beta upon HCMV-miR-UL22A* transfection

To answer our question whether innate immunity was speculated upon blunt-end HCMV-miR-UL22A* transfection, we then examined Interferon Stimulated Genes (ISGs) expression changes in HFFs transfected with blunt-end HCMV-miR-UL22A* or negative control blunt-end miRNA. We first transfected the cells with blunt-end HCMV-miR-UL22A* or negative control blunt end miRNAs. Then at 48 hours' post-transfection, we harvested RNAs from HFFs to perform RT-qPCR. RT-qPCR results shown in (Figure 4)revealed that cells transected with blunt-end HCMV-miR-UL22A* expressed a significantly higher level of ISGs comparing to cells transfected with negative control miRNAs. Furthermore, the upregulation of ISGs is sequence specific, which is demonstrated in Figure 5 that cells transfected with 22A*, a miRNA contains 2 nucleotide variances comparing with 22A*, showed no elevated ISGs expression.

To determine if ISG induction was structural based, we transfected HFFs with various RNAs including the top and bottom strand of ul22A, and hybridized dsRNA. We harvested RNAs and performed RT-qPCR. We detected OSA1, one of the ISGs, expression. RT-qPCR results showed that OSA1 transcription was only elevated in HFFs transfected with blunt-end 22A*, proving that ISG stimulation is dependent on the structural basis of the double-stranded, blunt-end, short miRNA (Figure 4). Furthermore, to determine if the inhibition of HCMV replication was sequence specific, we transfected HFFs with HCMV-miR-UL22A* carrying various point mutations (Table 4). Among all mutated miRNAs, only mut2 could inhibit HCMV replication like wild-type 22A*. This indicates that inhibition of HCMV replication is sequence dependent. To conclude, blunt-end HCMV-miR-UL22A* stimulated ISGs expression based on sequence and structure specificity. Our next question is to ask which innate immune signaling pathways have been activated upon blunt-end HCMV-miR-UL22A* transfection.

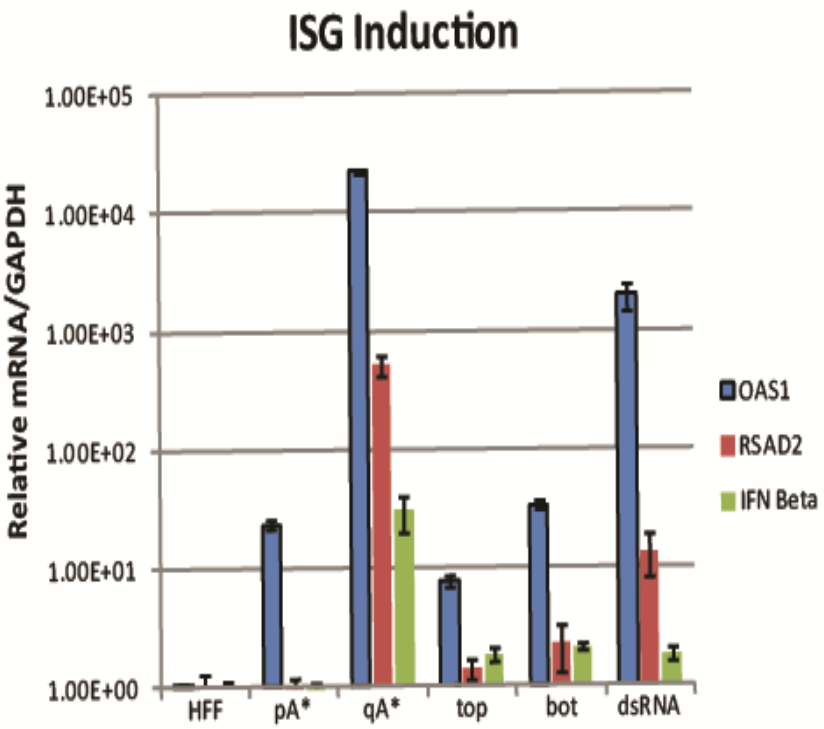


Figure 4 ISG could be induced by various types of RNAs. HFFs were transfected with the indicated RNAs or left untreated. 48 hours post-transfection, total RNAs from transfected cell cultures were extracted. OAS1, RSAD2, IFN Beta mRNA level were determined relative to GAPDH.

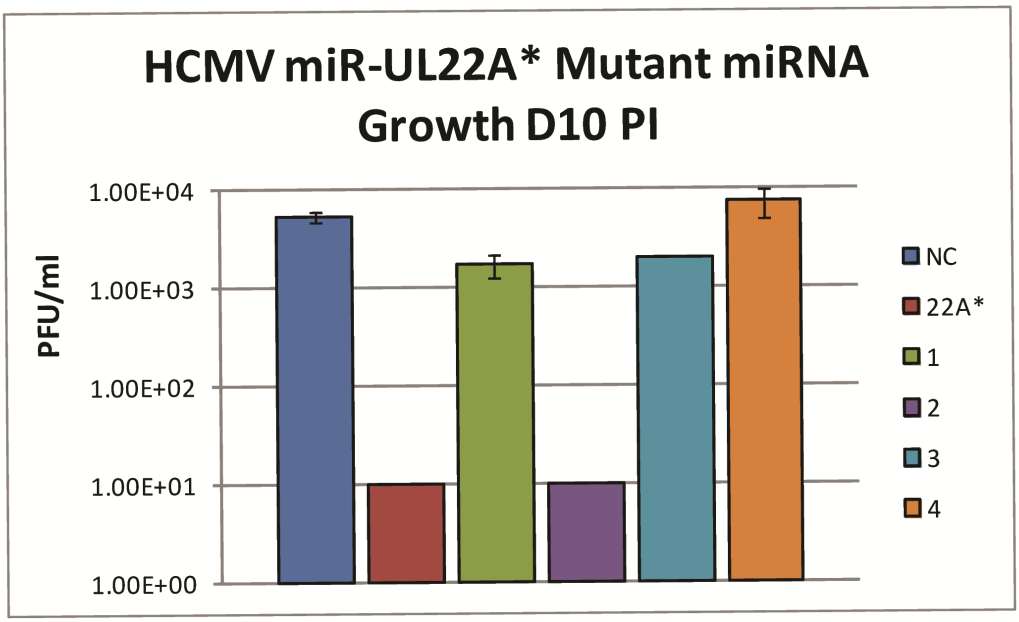


Figure 5 HCMV Town BAC GFP viral growth is inhibited by wild-type HCMV-miR-UL22A* and 22A*-mut2. HFFs were transfected with the indicated miRNAs. 48 hours post-transfection cells were infected with HCMV Towne BAC at MOI of 0.01. Supernatants from infected cell cultures were harvested at 10 days post-infection. Viral titers were determined.

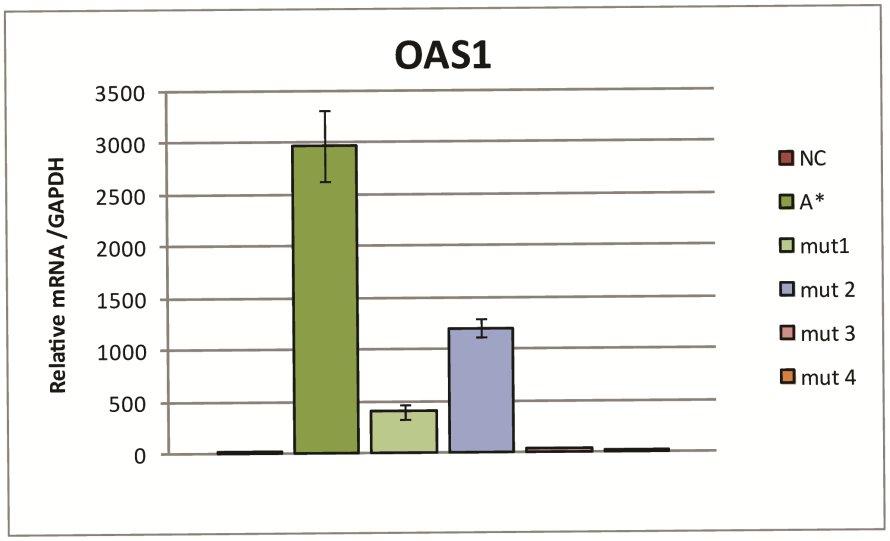


Figure 6 OAS1 transcription is upregulated by wild-type 22A*. HFFs were transfected with indicated miRNAs. Total RNAs were harvested 48 hours post-transfection. OAS1 mRNA level were determined by Quantitative RT-PCR.

MiRNA sequence 5' → 3'	name	Minimum Free Energy (MFE)
UCACCAGAAUGCUAGUUUGUAG	A*/22A*	-41.3 kcal/mol
UACACAGAAUGCUAGUUUGUAG	mut1	-39.3 kcal/mol
UCACCAGAAUGCUAGGGGGUAG	mut2	-48.4 kcal/mol
UCACCAGAAUGCUAGUUUGGCG	mut3	-44.8 kcal/mol
UCACCAGAAUUAGAGUUUGUAG	mut4	-39.1 kcal/mol

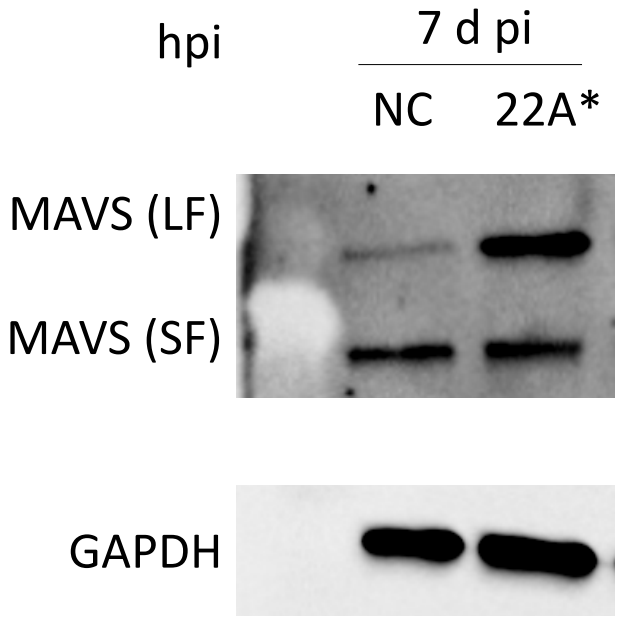
Table 4 Alignment of wild-type HCMV-miR-UL22A* and mutant HCMV-miR-UL22A* miRNAs.

Detection of HCMV-miR-UL22A* in cytosol by MAVS and RIG signaling pathway

To understand how ISG production was triggered after HFFs encountering blunt-end 22A*, we investigated the role of potential cellular RNA sensors that might be responsible for ISG production. There are several intracellular RNA sensors to our current understanding. RIG-I, MAVS and TLR3 detect short dsRNAs. PKR senses RNAs with hairpin structure. To study which cellular sensor was activated upon detecting 22A*, we generated HFFs depleting each sensor. Cells lacking different RNA sensor would allow us to study which knock-down could reverse the ISGs activation effect.

To establish cell lines lacking RNA sensors, HFFs were transfected siRNAs targeting TLR, PKR, RIG or MAVS. Only cells depleted of RIG and MAVS could reverse the ISG production after HCMV-miR-UL22A* transfection. In cells depleting TLR or PKR, ISG production was still elevated (Figure 7). This suggests that RIG-I and MAVS are essential responding to HCMV-miR-UL22A* transfection. We hypothesized that HCMV-miR-UL22A* could trigger RIG-I and MAVS mediated innate immune response. To examine our hypothesis, we further first tested if MAVS, the RNA sensor, expression was elevated upon transfection. We transfected the HFFs with 22A*. At 48 hours post-transfection, cells were infected with HCMV Town BAC strain. At 7 days post-infection, we harvest total protein from HFFs using NP40 lysis buffer. Western blotting using anti-MAVS was performed to evaluate MAVS protein production. There are two forms of MAVS in the cytosol. One form is truncated, the other form is full length. MAVS is cleaved when activated. We discovered that the truncated MAVS increased as the cells were transfected with 22A*, proving endogenous MAVS production was

activated when HFFs were transfected with blunt-end



22A*(

Figure 9). Furthermore, MAVS expression induction was detected at 7 days post-infection, suggesting that MAVS induction by HCMV-miR-UL22A* is stable, which is consistent with viral replication inhibition.

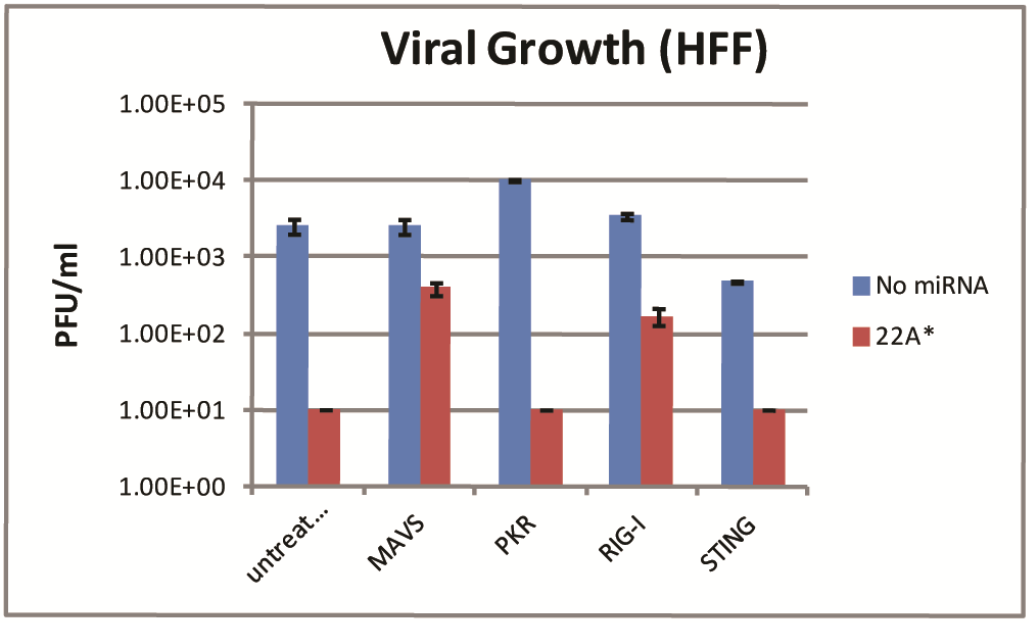


Figure 7 HCMV viral growth recovered in cells depleting MAVS and RIG-I. HFFs were treated with indicated siRNAs. Cells were subsequently transfected ul22A*. Supernatants from infected cell cultures were harvested at 4 days post-infection. Viral titers were determined.

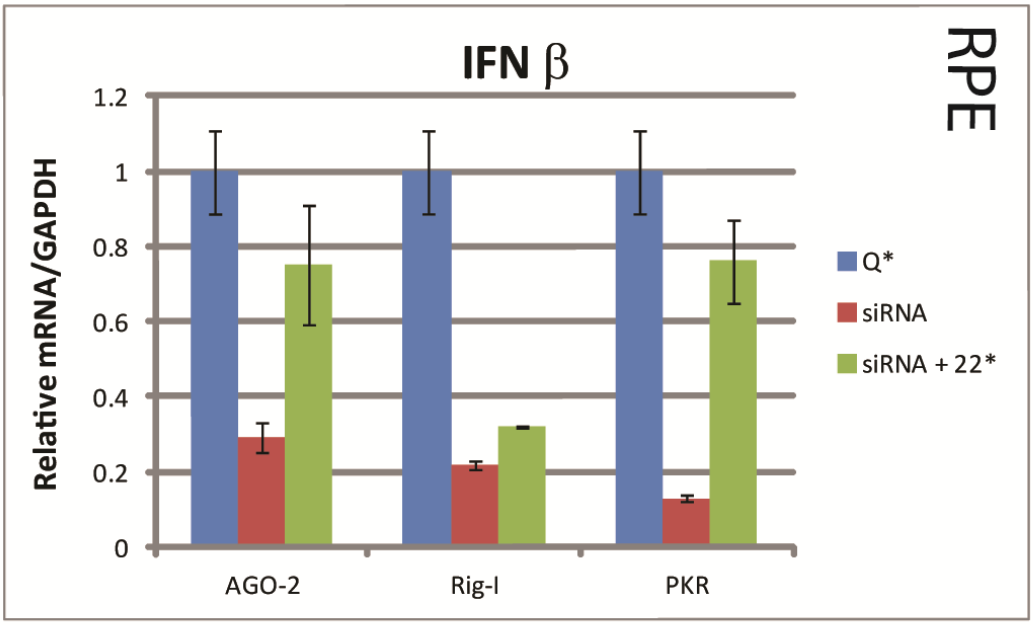


Figure 8 Maximal ISG induction and inhibition of HCMV replication by HCMV-miR-UL22A* is RIG-I dependent. Quantitative RT-PCR of IFN mRNA in cells treated with the indicated siRNAs and subsequently treated with 22A*.

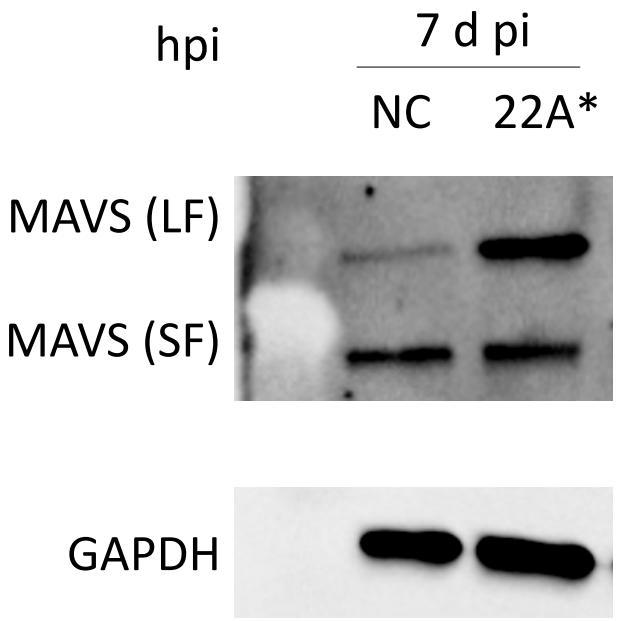


Figure 9 HCMV-miR-UL22A* activates MAVS expression. HFFs were transfected with HCMV-miR-UL22A* or NC miRNA. 48 hours post-transfection, HFFs were infected with HCMV Town BAC GFP at MOI= 0.01. The proteins were extracted at 7 days post-infection. MAVS protein was measured by immuno-blotting. GAPDH was measured as the loading control. Two forms of MAVS have been detected. Long form MAVS(LF) is the uncleaved form of MAVS. Short form MAVS (SF) is the cleaved form of MAVS.

2.4 Discussion

Pathogen Pattern Receptors is a set of innate immune sensors that could recognize foreign invaders. We utilize these PPRs to alert innate immune system to respond to pathogen attack. PPRs could be activated ranging from small genomic information to viral particles. PPR recognition could trigger the innate immune response that could suppress viral replication or alert nearby cells.

In this study, we accidentally discovered that innate immunity could be triggered by a viral miRNA mimic whose structure is blunt-end, lacking the 3' 2nt overhang. Also, we discovered the antiviral immune response triggered by this small RNA is not only structural related but also sequence specific. The antiviral response is also surprisingly durable- no active virion could be detected at even 10 days post-infection. To understand the mechanism of the viral replication inhibition, we investigated several intracellular RNA sensors that might be responsible for this antiviral effect. Eventually, we determined that MAVS and RIG-I have activated upon HCMV-miR-UL22A* transfection. Upon activation, MAVS were conjugated at mitochondria as detected by Immunofluorescent microscopy and DDS-page analysis.

We observed that overexpression of blunt-end HCMV-miR-UL22A* mimic dramatically inhibited the production of functional virions, while cells transfected with stagger-end HCMV-miR-UL22A* with 3' 2nt overhang did not shut down virus replication. We concluded that the inhibition of HCMV replication was initiated by RIG-I recognition of blunt-end 22A*. To our understanding, this is the first small RNA sensing mechanism that is both structural and sequence dependent. HCMV-miR-UL22A* transfection triggered a rigorous antiviral effect. Furthermore, the antiviral response is long lasting and non-toxic. As we could observe from the microscopy image, comparing to the non-transfected HFFs, HFFs treated with HCMV-miR-UL22A* showed no cytopathic effect caused by virus infection. Using small blunt-end HCMV-miR-UL22A* could be a new therapeutic approach for virus infection. However, we only studied the efficacy of blunt-end HCMV-miR-UL22A* in tissue culture; we should apply HCMV-miR-UL22A* in vivo in an animal model to test its antiviral effects. More importantly, finding a safe and efficient delivery method could also be challenging in the

animal model. Our future direction will be applying HCMV-miR-UL22A* in the animal model.

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Chapter 3 Human Cytomegalovirus encoded miRNA facilitates viral innate immune evasion

3.1 Introduction

MicroRNAs (miRNAs) are small (approximately 22 nt) RNAs that can regulate gene expression by a variety of mechanisms[54]. miRNA is first transcribed by RNA polymerase II (Pol II). Pol II binds to the promotor region upstream of the DNA sequence encoding what will become the hairpin loop of the pri-miRNA(primary-miRNA). Pri-miRNA is long (> 1kb) and contains stem loop structures. Transcribed by Pol II, pri-miRNA contains RNA post transcription modification such as 5' cap and 3' poly(A)tail[55]. In the nucleus, the double-stranded RNA (dsRNA) structure in a pri-miRNA is recognized by DiGeorge Syndrome Critical Region 8 (DGCR8). DGCR8 together with the RNase Drosha forms the microprocessor complex. In this complex, DGCR8 directs the catalytic domain of Drosha to cleave the dsRNA about eleven nucleotides from the hairpin base. The cleaved product (~70nt) is known as pre-miRNA (precursor-miRNA) that has two nucleotides overhang at its 3' end, 3' hydroxyl, and 5' phosphate groups. Pre-miRNA is exported from nucleus by exportin 5[56]. Exportin 5 recognizes the overhang at the 3' end of the pre-miRNA hairpin. Transportation to the cytoplasm is energy-dependent, using GTP bound to the Ran protein.

Following nucleus export, mature miRNAs are incorporated into RNA-induced Slicing Compounds (RISC), composed of Dicer, the double-stranded RNA binding protein TRBP, and Argonaute2, and bind to the target mRNA[57,58]. Only one strand of the dsRNA is incorporated to RISC complex, the other stand (star strand *) undergoes degradation. In the cytosol, animal miRNAs bind to the target mRNAs in the 3' UTR and 5' UTR region[59], whereas plant miRNAs bind to the coding region of the target mRNA[60]. Plant miRNAs require perfect complimentary with the target mRNAs for cleavage by the RISC complex. Animal miRNAs only require perfect complimentary in the "seed" region (nucleotide 2-7 in miRNA). Upon binding, targeted mRNAs are either cleaved or undergo translation inhibition. Translation initiation can be achieved by RISC complex targeting the 5' m7G-cap[61], following 3' poly(A) tail deadenylation and decay[62]. The recent discovery of virally encoded miRNAs, mostly in herpes viruses[63], suggests that miRNAs may function in regulation involving viral miRNAs and host genes by inhibiting mRNAs translation.

MAVS is an essential component of innate immune defense against viruses. Studies show that MAVS plays an important role in antiviral innate immune

response[64,65,66.] The retinoic acid-inducible gene I (RIG-I) encodes a cytosolic protein that detects both viral double-stranded RNA (dsRNA) and ssRNA inside the cells and initiates signaling pathways that produce type I IFNs and inflammatory cytokines. RIG-I can directly activate downstream signaling by adaptor MAVS, which leads to the activation of nuclear factor kappa B (NF-kappa B) and IFN regulatory factor 3 (IRF3). It has been reported that RIG-MAVS pathway can be targeted by various virus proteins leading to the cleavage of RIG-MAVS interaction, releasing MAVS from the mitochondrial, eventually blocking the activation of interferon factor 3[67686970]. In addition to blocking the host immune defense, some latent viruses favor blocking the RIG-MAVS signaling pathway in order to maintain latency. For example, Bovine herpesvirus 1 (BHV-1) abundantly expresses latency-related miRNAs, and its interaction with RIG-I enhances survival of infected cells, thus promoting the establishment and maintenance of latency[71]. HCMV protein viral mitochondria-localized inhibitor of apoptosis (vMIA) UL37 has shown to be capable of inhibiting infected cell apoptosis by disrupting mitochondrial[72], and also capable of hindering downstream signaling from MAVS[73].

NLRX1 (a member of NLR) is an intracellular viral RNA sensor that plays an important role in host immune defense viral infection. NLRX1 localizes in the mitochondrial outer membrane and interacts with the MAVS protein, and possibly negatively regulates RIG-MAVS signaling[74]. However, the role of NLRX1 is controversial in contemporary scientific literature. Some studies believe that NLRX1 functions as an inhibitor of the RIG-MAVS signaling pathway to attenuate inflammatory responses[7576]. Other studies conclude that NLRX1 has no inhibition on MAVS function[7778]. Despite the debates on whether NLRX1 works as an inhibitor of MAVS, increasing evidence suggests that NLRX1 is a viral RNA sensor that can be activated by viral infection or different stimuli. Hong et.al demonstrated that purified recombinant C-terminal fragment of NLRX1 can bind foreign RNAs (ssRNA, dsRNA) in vitro[79]. Still, the role of NLRX1 is critical for understanding the viral immune evasion strategy, although little has been studied regarding how HCMV escapes from NLRX1 recognition. Here, we examined the correlation between viral miRNAs and NLRX1, and then investigate the role of HCMV-mir_UL22A* on its potential target NLRX1. In our study, we investigated the function of HCMV-miR-UL22A*. We validated the mechanism of HCMV-miR-UL22A* targeting NLRX1 and MAVS.

3.2 Materials and Methods

Cells and virus

HFF cells were obtained from Clonetics, cultured as adherent cells DMEM(Invitrogen), with 10% FBS, 1% Penicillin streptomycin (Pen-Strep) from Invitrogen. HCMV Towne BAC was previously constructed by inserting a BAC sequence into the genome of HCMV laboratory-adapted strain Towne. HCMV Towne BAC strain was propagated from virus stocks in HFFs. Incubate the HFFs with HCMV Towne BAC at MOI = 0.01. After 2 hours incubation, wash and change media for HFFs. Both supernatant and cell lysis were collected. Cell lysis were first collected by cell scrapers, followed by sonication at 15% amplitude for 1 minute with 30 second intervals.

Plaque forming assay

Seed the HFFs to 24-wells at 18 hours before performing the plaque forming assay. The seeding density for 24 wells plates is 0.05 * 10^6 cells per well. The next day, HFFs were at 1.2 * 10^ 6 cells per well. Add 250ul of the diluted HCMV Town BAC GFP (from 10^-1 to 10^-6) to HFFs and incubate for two hours. After incubation, wash and change media for HFFs. Add the 2% agarose with 4% FBS 2X DMEM overlay to the HFFs. Incubate the plates at 37C tissue culture incubator for 10 days. Count the GFP positive plaques under fluorescent microscopy.

Transfection

HFFs were seeded to 24 wells plates without antibiotic 18 hours before transfection at 0.05 * 10^6 cells per well. HFFs reached to 1.2 * 10^ 6 cells per well and were ready for transfection the next day. All transfections were carried out using Lipofectamine™ 2000 (Life Technologies). 25pmol miRNA and 1 ul Lipofectamine™ were transfected to HFFs. Incubate miRNAs with HFFs for 18 hours, then changed the media with complete media. All Stars Negative Control siRNA (1027280) and miScript miRNA Mimics: HCMV-miR-UL22A (MSY0001574), HCMV-miR-UL22A* (MSY0001575), were obtained from Qiagen. To assess the transfection efficiency, cellular miRNAs were harvested and quantified transcription by QT-PCR.

Nucleic Acid purification and quantification

Total DNA were purified from HFFs using DNasey Blood and Tissue kit (Qiagen) according to manufacturer's protocol. Total RNAs were purified from HFFs using Trizol (Life Technologies) and miRNasy kit (Qiagen) according to manufacturer's protocol.

Real-Time PCR

Total RNA was subjected to DNase digestion using the RNase-free DNase set (Qiagen) and reverse transcription using Affinity script reverse transcriptase (Agilent) with oligo dT (15) primer (Fisher Scientific). The SYBER Green real-time PCR kit (Bio-Rad) was used to perform QRT-PCR on CFX™ Real-Time PCR Detection System (Bio Rad). In 20ul reaction contains 300nm primer shown in **Error! Reference source not found.**, 10ng template cDNA, 10ul SYBER Green supermix. Real-time PCR conditions were 1 cycle of 3 mins at 95°C, followed by 40 cycles of 10 sec at 95°C, 30 sec at 55-60°C (depending on the primer pairs according to Table 5), and 30 sec at 72°, and finishing with melt curve analysis. We checked Real-time PCR products amplification and standard gel electrophoresis.

Western Blotting

Proteins of the cells were prepared by suspending cells in NP40 lysis buffer, followed by centrifugation at 14,000 RPM 10min, 4C. Total protein were quantified using DC™ Protein Assay(Bio-Rad). Protein was separated by 10% SDS-PAGE electrophoresis and transferred onto a nitrocellulose membrane. Immunoblots were incubated with ECL detection system. Sample loading was normalized by quantities of GAPDH detected parallel.

Name	Sequence (5’ -> 3’)
Interferon-beta Forward	CTCTCCTGTTGTGCTTCTCCA
Interferon-beta Reverse	TAGTCTCATTCCAGCCAGTGC
IFI27 Forward	TGGCTCTGCCGTAGTTTTG
IFI27 Reserve	TCCTCCAATCACAACTGTAGCA
IFI44 Forward	AGCCGTCAGGGATGTACTATAAC
IFI44 Reverse	AGGGAATCATTTGGCTCTGTAGA
OAS1 Forward	AGCTTCGTACTGAGTTCGCTC
OAS1 Reverse	CCAGTCAACTGACCCAGGG
RSAD2 Forward	CAGCGTCAACTATCACTTCACT
RSAD2 Reverse	GGAAACAGAAGCCGCATTTGTA
IL-6 Forward	CCAGCTATGAACTCCTTCTC
IL-6 Reverse	GCTTGTTCCTCACATCTCTC
GAPDH Forward	ACCCACTCCTCCACCTTTGA
GAPDH Reverse	CTGTTGCTGTAGCCAAATTCGT

Table 5 Primers design. All primers were ordered from IDR in 25nm standard desalt.

3.3 Results

Overexpression and deletion of HCMV-miR-UL22A* does not affect viral growth

We overexpressed mir22A* to investigate the function of stagger-end 22A*. We first infected HFFs with HCMV Town BAC at MOI= 0.01. 24 hours post-infection, we transfected the HFFs with stagger-end 22A*. Culture supernatant was collected at 3-day interval until 10 days post-infection. Viral titer was determined by plaque forming assay. As we can tell from (Figure 12), HCMV virus titer was not affected in cells overexpressing 22A*. To study if HCMV lacking HCMV-miR-UL22A* would affect viral replication, we infected HFFs with HCMV lacking 22A coding region. HCMV mutant virus was generated using homologous recombination followed by antibiotic selection. In cells infected with mutant HCMV, no viral growth was affected.

Down-regulation of IFN-β and IL-6 by HCMV-miR-UL22A*

Previous research in our lab[80] shows that HCMV-miR-UL22A* is expressed as an immediate early gene. To examine if HCMV-miR-UL22A* has any effect in regulating innate immune response, we created a mutant virus lacking ul22A coding region. Total RNAs were harvested. RT-QPCR was performed to evaluate IFN-β and IL-6 production. In cells infected with HCMV Town BAC mutant virus, IFN-β and IL-6 were elevated at 4 hours post-infection(Figure 10), indicating HCMV-miR-UL22A* might interfere with the initial stage of the infection. In our previous study, we observed that in cells infected with HCMV first, then transfected with blunt-end HCMV-miR-UL22A* that proven to have antiviral effect, viral replication was not affected. Our hypothesis was that one viral factor encoded by HCMV had already downregulated the innate immune sensor. Therefore, cells infected with HCMV did not host blunt-end HCMV-miR-UL22A* antiviral activity. The up-regulated expression of IFN-β and IL-6 explained that phenomenon.

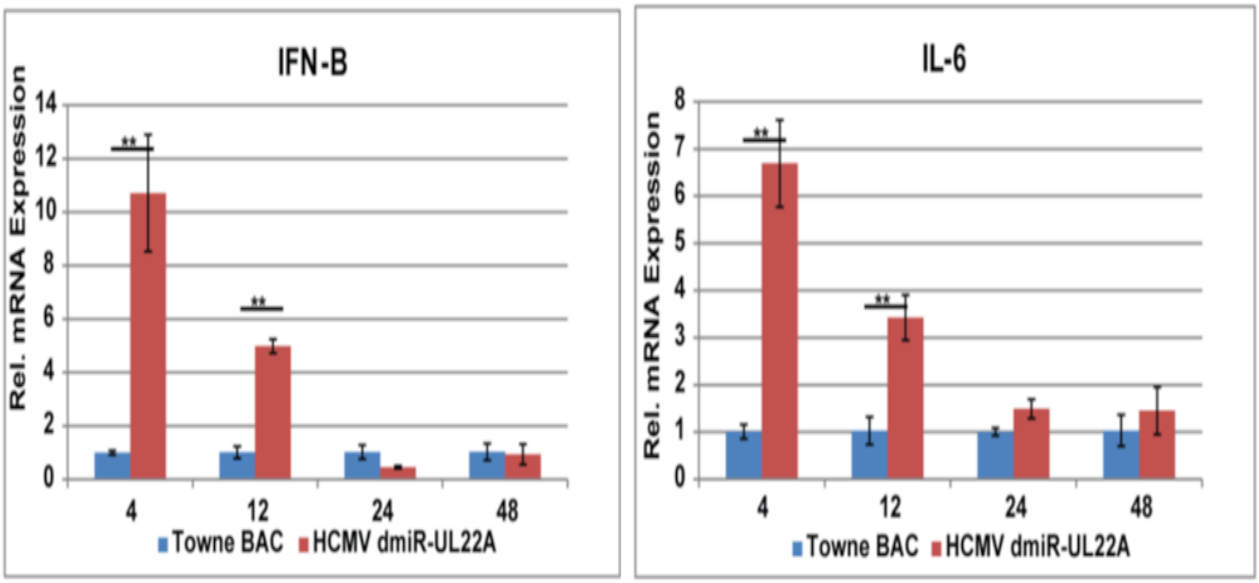


Figure 10 HCMV-miR-UL22A* downregulates IFN-B and IL-6 expression. HFFs were infected wild-type Towne BAC HCMV or the deletion mutant d22 HCMV. mRNA was measured with real time PCR to quantify levels of IFN-β and IL-6 transcripts.

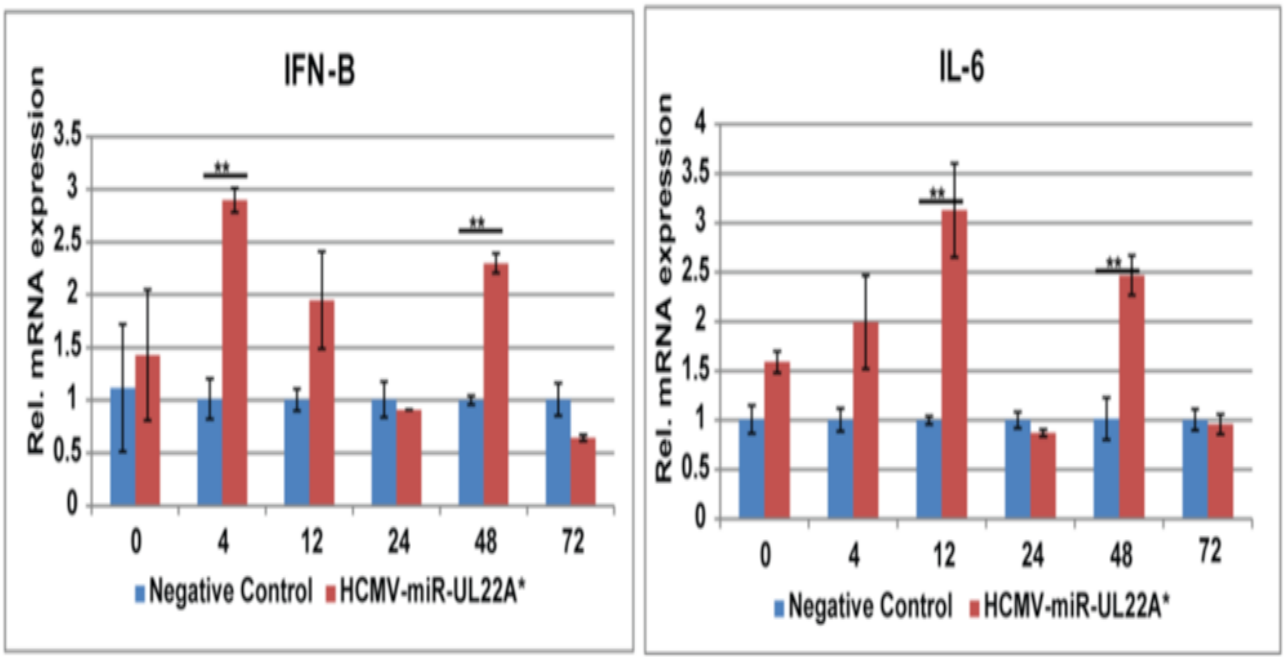


Figure 11 HCMV-miR-UL22A* regulates IFN-B and IL-6 expression. HFFs were first transfected with the negative control miRNA (NC) or HCMV miRNA UL22A* (22A*) and then infected with the UL22 deletion mutant HCMV. mRNA was measured with RT-PCR.

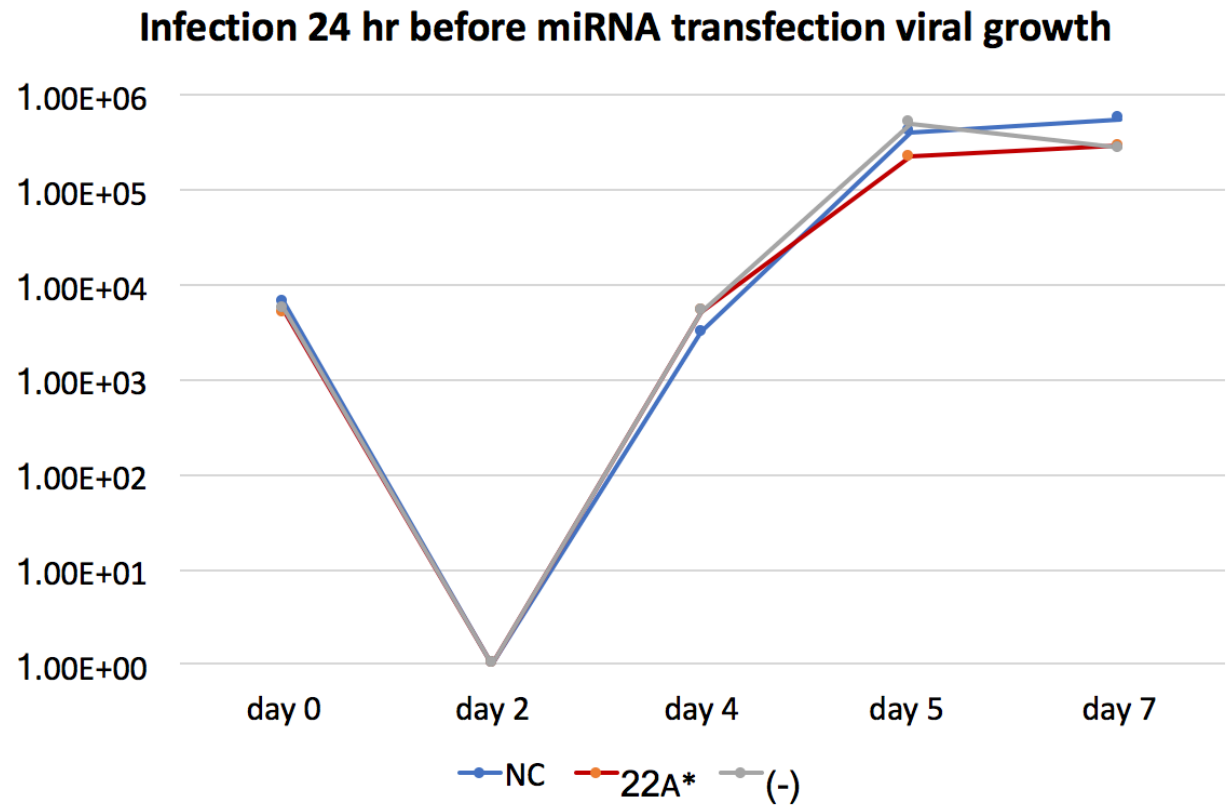


Figure 12 HCMV-miR-UL22A* did not affect viral replication in human foreskin fibroblasts (HFF). Cells were infected with HCMV Towne BAC at MOI of 0.01then transfected with the indicated RNAs or left untreated. Supernatants from infected cell cultures were harvested at the indicated times post-infection. Viral titers were determined using plaque forming assay.

HCMV-miR-UL22A* regulation on IFN-β and IL–6 upstream signaling

To study the potential target of 22A*, we used bioinformatics software to determine the potential targets of 22A*. We evaluated the potential targets for 22A*. IFN-β and IL–6 upstream signaling factors. Out of the numerous predictions, Mitochondrial antiviral signaling protein (MAVS) and Nucleotide-binding oligomerization domain, leucine rich repeat containing X1 (NLRX1) sequence both contained high levels of homology to the UL22A* miRNA sequence (Figure 13, Figure 14). MAVS and NLRX1 were chosen as the most likely candidates to be targeted by HCMV-miR-UL22A*.

To study whether Ul22A* could suppress MAVS and NLRX1 production, HFFs were infected with wild-type HCMV(wt.) or deletion mutant HCMV(d22). The proteins were extracted at a different time point after infection. MAVS protein was measured by Immuno-blotting. GAPDH was measured as the loading control. The immunoblotting images indicate that the translation inhibition of endogenously expressed MAVS(LF) is absent in UL22 deletion mutation HCMV. MAVS antibody two bands: The upper band represents full-length MAVS, whereas the lower band is a truncated form of MAVS, which lacks the N terminus but retains the C-terminal transmembrane domain. Interestingly, only the full-length MAVS formed a large complex capable of activating IRF3. Furthermore, almost all full-length MAVS shifted to the large complex in response to viral infection. This result indirectly indicates that MAVS is a potential target for HCMV-miR-UL22A* translation inhibition, although a direct relationship between HCMV-miR-UL22A* and MAVS mRNA needs further exploration and study.

The NLRX1 target specificity of NLRX1 was identified by with luciferase activity assay and immunoblotting (Figure 15, Figure 16). HFFs were infected with wild-type Towne BAC HCMV or a deletion mutant strain dUL22 HCMV. The proteins were extracted 72 hours post-infection. NLRX1 protein was measured by Immunoblotting. GAPDH was measured as loading control. We also co-transfected with different concentrations of FLAG- NLRX1 plasmid and HCMV-miR-UL22A* or a negative control miRNA. The proteins were extracted at 24 hours post-infection. FLAG-NLRX1 protein was measured by Immuno- blotting. GAPDH was measured as loading control. In the immunoblotting assay, we observed that NLRX1 production decreased in cells expressing UL22A*. In contrast, NLRX1 production was elevated in cells infected with mutant HCMV lacking UL22A coding region. This suggests that UL22A* could down regulate

NLRX1 production. Furthermore, we could detect the direct binding activity of HCMV-miR-UL22A* and NLRX1 target site from Luciferase assay. To conclude, UL22A* inhibits NLRX1 translation by binding to the sequence homology site in its mRNA.

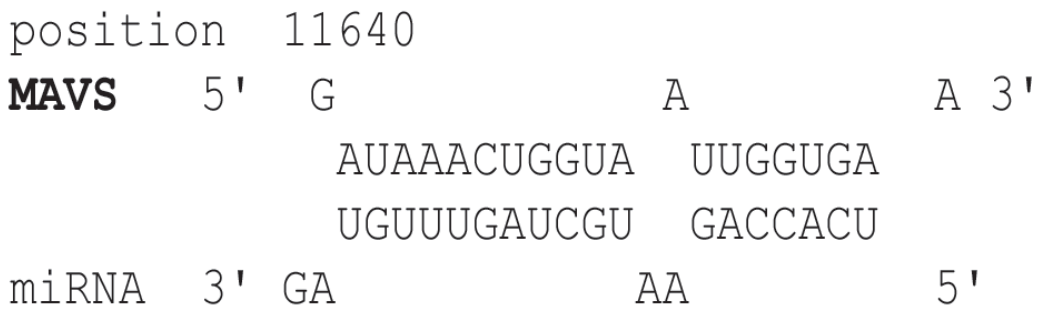


Figure 13 miRNA and MAVS sequence homology. The region of the NLRX1 mRNA transcript thought to be regulated by HCMV-miR-UL22A* determined by sequence homology.

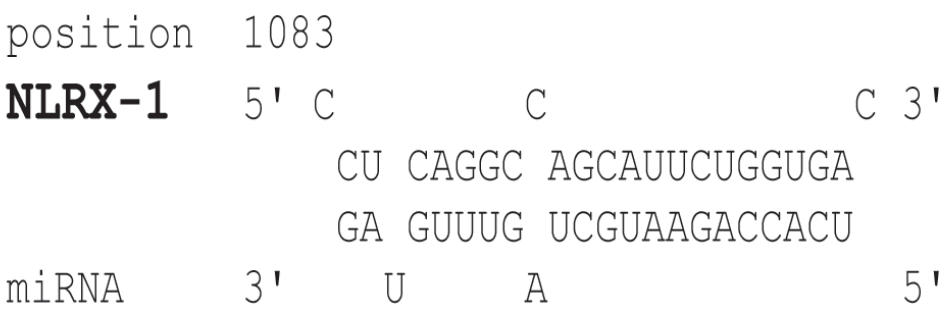


Figure 14 miRNA and NLRX1 sequence homology. The region of the NLRX1 mRNA transcript thought to be regulated by HCMV-miR-UL22A* determined by sequence homology.

A.

```
position  1083
NLRX-1
          5' C         C              C 3'
              CU CAGGC AGCAUUCUGGUGA
              GA GUUUG UCGUAAGACCACU
miRNA     3'    U     A               5'

NLRX-1.m1
          5' C         C              C 3'
              CU CAGGC AGCAUUCUGucuA
              GA GUUUG UCGUAAGAC   U
miRNA     3'    U     A          CAC   5'
```

B.

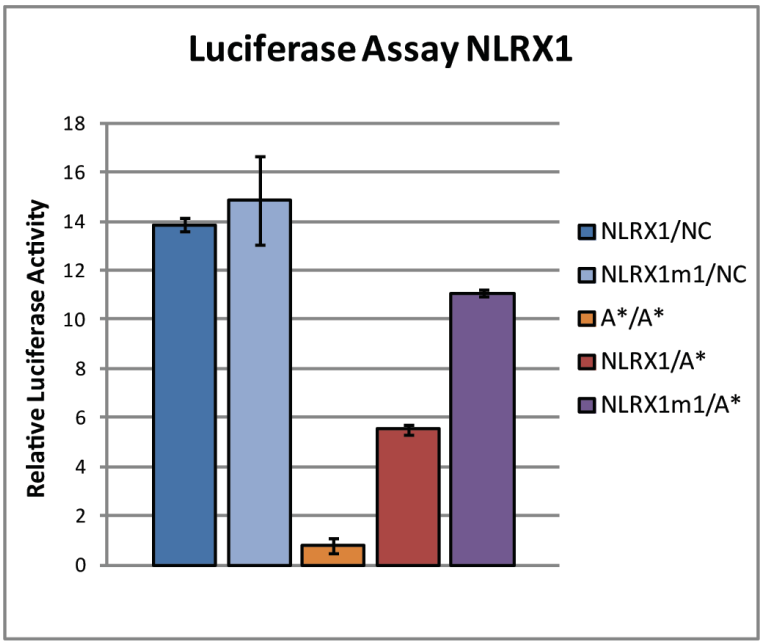
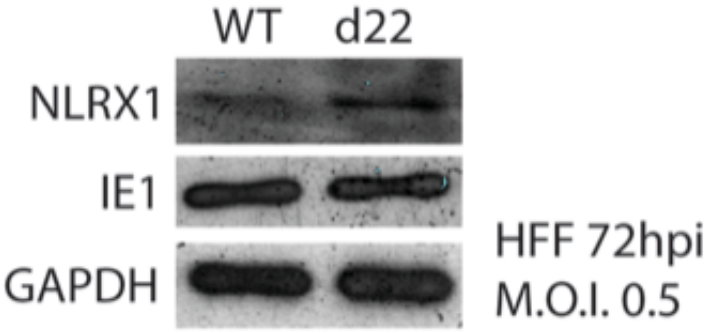


Figure 15 HCMV-miR-UL22A* specifically binds to NLRX1 target region. A. HCMV-miR-UL22A* specifically binds to NLRX1 targeting sequence. Luciferase assay NLRX1. HCMV-miR-UL22A* potential target region of NLRX1 3' UTR and a mutated sequence of NLRX1 changed by three base pairs near the 3' end (NLRX-.m1"). B. Hela cells were co-transfected with Luciferase expression

plasmid and indicated miRNAs. Luciferase assay was performed 48 hours post-transfection.

A.



B.

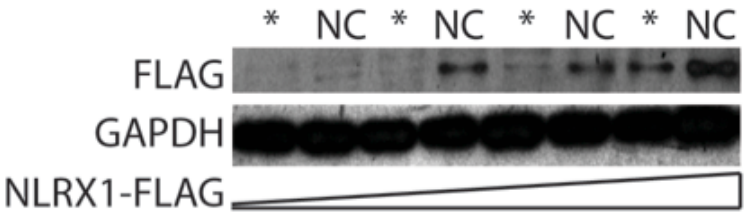


Figure 16 Western blot analysis of HCMV-miR-UL22A* regulation in NLRX1 translation A. HFFs were infected by wild-type Towne BAC HCMV or a deletion mutant strain dUL22 HCMV. The proteins were extracted 72 hours post-infection. NLRX1 protein was measured by Immunoblotting. GAPDH was measured as loading control. B. HFFs were co-transfected with different concentrations of FLAG- NLRX1 plasmid and HCMV-miR-UL22A* or a negative control miRNA. The proteins were extracted at 24 hours post-infection. FLAG-NLRX1 protein was measured by Immuno- blotting. GAPDH was measured as loading control.

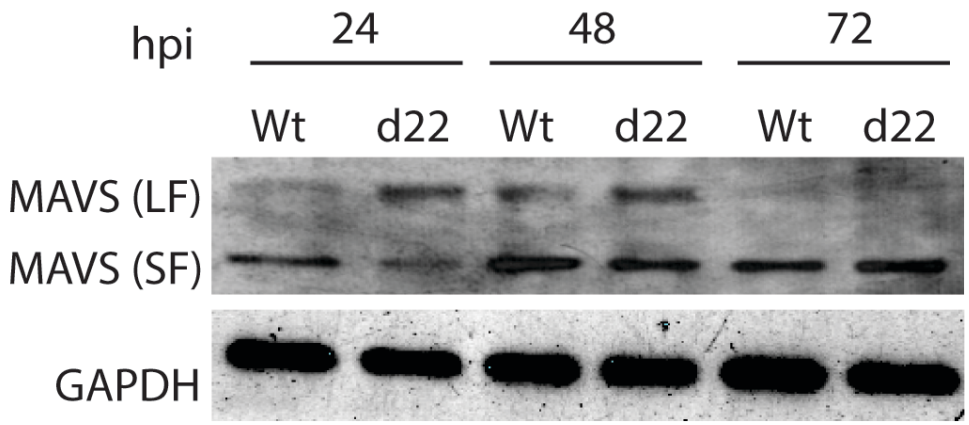


Figure 17 Western blot analysis of HCMV-miR-UL22A* regulation in MAVS translation. HFFs were infected with wild-type HCMV(wt.) or deletion mutant HCMV(d22). The proteins were extracted at a different time point after infection. MAVS protein was measured by Immuno-blotting. GAPDH was measured as the loading control. Two forms of MAVS have been detected. Long form MAVS(LF) is the uncleaved form of MAVS. Short form MAVS (SF) is the cleaved form of MAVS.

3.4 Discussion

Host innate immune surveillances and viral innate immune evasion has been a battle since the beginning of time. Various innate immune elements have evolved in the battle against pathogen invasion. Innate immune sensors have been ones of the first barrier facing viral infection. MAVS and NLRX1 are ones of the newly identified intracellular sensors facilitate innate immune surveillances.

MAVS is an essential component of innate immune defense against viruses. Studies show that MAVS plays an important role in antiviral innate immune response[81,82,83]. The retinoic acid-inducible gene I (RIG-I) encodes a cytosolic protein that detects both viral double-stranded RNA (dsRNA) and ssRNA inside the cells and initiates signaling pathways that produce type I IFNs and inflammatory cytokines. RIG-I can directly activate downstream signaling by adaptor MAVS, which leads to the activation of nuclear factor kappa B (NF-kappa B) and IFN regulatory factor 3 (IRF3). It has been reported that RIG-MAVS pathway can be targeted by various virus proteins leading to the cleavage of RIG-MAVS interaction, releasing MAVS from the mitochondrial, eventually blocking the activation of interferon factor 3[84858687.] In addition to blocking the host immune defense, some latent viruses favor blocking the RIG-MAVS signaling pathway in order to maintain latency. For example, Bovine herpesvirus 1 (BHV-1) abundantly expresses latency-related miRNAs, and its interaction with RIG-I enhances survival of infected cells, thus promoting the establishment and maintenance of latency[88]. HCMV protein viral mitochondria-localized inhibitor of apoptosis (vMIA) UL37 has shown to be capable of inhibiting infected cell apoptosis by disrupting mitochondrial[89], and also capable of hindering downstream signaling from MAVS[90]. NLRX1 (a member of NLR) is an intracellular viral RNA sensor that plays an important role in host immune defense viral infection. NLRX1 localizes in the mitochondrial outer membrane and interacts with the MAVS protein, and possibly negatively regulates RIG-MAVS signaling[91]. However, the role of NLRX1 is controversial in contemporary scientific literature. Some studies believe that NLRX1 functions as an inhibitor of the RIG-MAVS signaling pathway to attenuate inflammatory responses[9293]. Other studies conclude that NLRX1 has no inhibition on MAVS function[9495]. Despite the debates on whether NLRX1 works as an inhibitor of MAVS, increasing evidence suggests that NLRX1 is a viral RNA sensor that can be activated by viral infection or different stimuli. Hong et.al demonstrated that purified recombinant C-terminal fragment of NLRX1 can bind foreign RNAs (ssRNA, dsRNA) in vitro[96]. Still, the role of

NLRX1 is critical for understanding the viral immune evasion strategy, although little has been studied regarding how HCMV escapes from NLRX1 recognition.

While we study the function of blunt-end 22A*, we discovered that HCMV-miR-UL22A* failed to trigger the innate immune response in cells infected with HCMV Town BAC. We suspected that HCMV express innate immune modulator that could antagonize or block the expression of RNA sensor. We also discovered that interferon production spiked in cells infected with HCMV lacking the UL22A coding region. Also, HCMV-miR-UL22A* production could block the antiviral effect of blunt-end HCMV-miR-UL22A*. Based on in silico analysis, HCMV-miR-UL22A* has two potential targets MAVS and NLRX1. We validated HCMV-miR-UL22A* could bind to NLRX1 and MAVS through luciferase assay. Furthermore, we demonstrated NLRX1 and MAVS protein translation could be inhibited by HCMV-miR-UL22A* using immunoblotting. To conclude, HCMV-miR-UL22A* assists HCMV innate immune evasion by targeting MAVS and NLRX1.

In our study, we investigated the targeting activity of HCMV-miR-UL22A*. We now have a better understanding of how HCMV-miR-UL22A* involves the regulation of intracellular non-self RNA sensor activation. Viral encoded miRNAs processed into the target mRNA are crucial to the HCMV immune evasion strategy. The understanding of HCMV-miR-UL22A* and two intracellular viral RNA sensors, RIG-MAVS and NLRX1, reveals the immune evasion strategy of HCMV in early stages of infection. It is probable that we are far from revealing the last role of HCMV-mir_UL22A* in regulating the host immune antiviral response. This presumption may lead to interesting future work to reveal the total function of HCMV-mir_UL22A* in the HCMV immune evasion.

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3.5 Reference

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Chapter 4 Suppression of Human and Murine Cytomegalovirus replication and infection using CRISPR/Cas9 gene editing technology

4.1 Introduction

Human cytomegalovirus (HCMV) is the largest member of the Beta herpesvirus family. Like all herpes virus, once the hosts are infected with HCMV, it remains latent and can be reactivated at any time[97]. HCMV infection causes mild or subclinical diseases in immunocompetent adults but leads to severe, life-threatening complications in people who are immunocompromised, including AIDS patients and transplant recipients, as well as newborn infants[98]. Currently, there are four types of therapies for preventing CMV disease. High dosage of Acyclovir and Valacyclovir are recommended to reduce the risk of CMV infection[99100]. Valacyclovir has also been used to counteract the development of disease when a viral reactivation has arisen. Ganciclovir is an effective medication for CMV infection, but it is rarely used today due to significant toxicity[101102]. Furthermore, CMV has developed mutations that are associated with resistance to Ganciclovir, maybe even cross-resistance to other anti- CMV drugs. Drug resistance to current therapies makes it trickier to treat CMV infection[103]. Foscarnet or Cidovir are used when previously indicated medications have failed[104]. Nevertheless, Foscarnet has notable nephrotoxicity. With those being said, it is crucial to develop other treatments against CMV infection. As the state of art treatment is using nucleotide analogue to block viral polymerase replication, another difficulty in CMV or any other latently infected virus treatment is failing to control the reactivation. Treatment is only effective when virus genome is actively replicating; hence the remaining silenced viral genome can be reactivated at any time. We need to develop a treatment strategy that could disrupt the viral genome. To conclude, a gene therapy that can edit and eliminate the CMV virus is the only solution for curing CMV latent infection.

The idea of gene therapy is using engineered nucleases to specifically target viral genome, hoping to introduce breakage or mutation in the viral genome that can lead to failure in viral replication. There are currently three families of engineered nucleases being used: Zinc Finger Nucleases (ZFNs)[105], Transcription Activator-Like Effector Nucleases (TALENs)[106], and the CRISPR/Cas9 system. All of them have been applied successfully in anti-viral treatment. ZFN and TALENs are both nucleases relying on protein-DNA recognition.

ZFN pioneered in the field of gene editing. ZFNs are artificial restriction enzymes that are generated by combining specific ZFN proteins (ZFP)DNA binding domain and non- specific DNA cleavage domain of Fok1. ZFP region contains a tandem array of Cys2-His2 fingers, each recognizing approximately 3 bp of DNA[107]. Each ZFNs uses three fingers to bind a 9bp target. Therefore, ZFNs provide 18bp of DNA per cleavage in ZFN dimer. Gene editing with ZFN was initially tested in Drosophila melanogaster by Bibikova and her team in 2002. They designed a pair of ZFNs recognizing yellow gene of Drosophila. Since then, ZFNs have been adapted in a variety of organisms. Apart from the experimental purposes to study certain gene function, ZFN also created open doors for treating rare diseases and persistent viral infection. In 2014, Tebas et al. treated chronic HIV infected patients with autologous CD4 T cells whose CCR5 gene was rendered permanently dysfunctional by ZFN. CCR5 gene encoded protein is used by HIV to gain access to human T cells[108]. People who carry mutations in CCR5 are resistant to HIV. After researchers transfused the engineered CD4 T cells to the patients, all patients showed elevated T cell counts, meaning HIV was less capable of destroying T cells. Interestingly, the modified T cells proliferated faster than the wild-type T cells. Therefore, even after the patients got off from HAART therapy, HIV replication did not escalate. Sangamo Therapeutics started a clinical trial based on the is study in 2015. Currently, it is in Phase 2 clinical trial. In 2016, Sangamo Therapeutics launched a clinical trial to evaluate the safety, tolerability of ascending doses of an intravenously delivered ZFN that can insert a correct copy of the α-L-iduronidase (IDUA) gene in hepatocytes[109]. Patients lacking IDUA suffers from mucopolysaccharidoses (MPS I). The most severely affected children rarely live more than 10 years. 15 year after the invention of ZFNs in the field of gene editing, it already transformed laboratory discoveries into clinical application. Even though ZFN provides promising results in gene editing, its application is limited because the target sites can only be composed of triplets.

Transcription activator-like effector nucleases (TALENs) are similar to ZFNs. TALENs are a type of nucleases that comprise a non-specific Fok1 nuclease domain and a specific DNA-binding domain. The DNA-binding domain contains conserved repeats originated from transcription activator-like effectors(TALE), secreted from phytopathogenic bacteria. TALEs consists of 33-35 highly conserved amino acid repeats. Within each repeat, there are 2 hyper-variable amino acids at position 12-13. These variable residues consist of DNA specificity[110]. TALENs can be easier designed than ZFN because of the protein–DNA recognition code. TALENs target recognition only requires T at the 5' side, and it can be placed in virtually any location in the genome. Although TALENs

targeting is simple and straightforward, TALENs design can be challenging as it requires multiple excessive design and cloning efforts. As a breakthrough in the field of gene editing, TALENs have been used in multiple organisms, such as yeast, fruit fly, rat, human somatic cells, human pluripotent stem cells, etc. In 2015, Qasim et al. cured acute lymphocytic leukemia in two one-year-old infants using engineered non-matched donors' Chimeric antigen receptor (CAR) T cells[111, 112]. CAR T cell sharing can be promising in treating acute lymphocytic leukemia, but it can be challenging as CAR T cells need to be custom made to overcome the HLA barrier and the risk of graft-versus-host disease(GvHD). Engineered CAR T cells depleting CD52 and T cell receptor alpha constant chain locus could solve this problem. In patients who are on Alemtuzumab, an anti-CD52 lymph-depleting therapy, analogous CAR T cells lacking CD52 are safe from depletion. CAR T cells lacking constant chain locus could prevent GvHD. The engineered CAR T cells persisted in patients until a matched T cell donor is found. Even though engineered non-matched donor T cells are not the permeant solution to treat acute leukemia, they provided a bridge to keep the patients alive until finding a successful donor. Furthermore, the success in these two patients presented great potential in using gene editing technology to modify human cells in the clinical study.

ZFN and TALENs are useful in targeting the desired location within the genome while they can be technically challenging to design and assemble. Nevertheless, the high price of the commercial modules makes ZFNs and TALENs less accessible to researchers. The Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR associated protein 9 (Cas9) system recognizes the target regions based on RNA-DNA interaction. The CRISPR/Cas9 system can be easily designed by inserting the targeted sequence into the vector plasmid expressing gRNA and Cas9 nuclease. CRISPR/Cas9 system is a prokaryotic immune system. In this adaptive immune system of bacteria, the host can acquire short pieces of evaders' genome information and store it as spacer sequences in a CRISPR motif[113]. When encountering this specific phage, the host can recognize the foreign DNAs by complementation with the stored short spacer sequence from the host genome. After the binding event of CRISPR RNA, trans activating RNA and the phage genome, the host can recruit endonuclease Cas9 protein to cleave the foreign DNA, causing the elimination of foreign DNA from the host cells. Back in 2012, researchers adopted the CRISPR/Cas9 in the mammalian system. Since then, CRISPR/Cas9 application in mammalian systems has been a tremendous success. Many studies have also been completed to investigate the mechanism of CRISPR/Cas9 in mammalian system. For easier application in gene

editing, scientists developed plasmids that could express crRNA and tRNA together as guide RNA (gRNA). gRNA along with Cas9 protein can edit any spot in the genome as long as the target sequence is followed by a – NGG PAM sequence. When gRNA and Cas9 are expressed in the mammalian system, Cas9 protein can clamp on host genome, and actively scanning for PAM sequence. The GG dinucleotide is recognized through major-groove interactions with conserved arginine residues from the C-terminal domain of Cas9 protein. Interactions with the minor groove of the PAM duplex and the phosphodiester group at the +1 position in the target DNA strand leads to local strand separation immediately upstream of the PAM[114]. If the target DNA matches gRNA sequence, Cas9 HNH endonuclease domain cleaves the complementary strand, and the Cas9 Revco-like domain cleaves the non-complementary strand domain at 3 nt upstream of PAM[115]. If the host DNA does not match gRNA sequence, Cas9 will move on and eventually find its target. The double stranded break created by Cas9 can be repaired by either Non-Homologous End Joining(NHEJ) or Homologous Repair. Homologous repair can happen when a homologous donor DNA presents. Otherwise, NHEJ will repair the DSB. NHEJ will introduce a small insertion/deletion(indel) in the targeted DNA and will create in in-frame amino acid deletions, insertions, or frameshift mutations. This leads to premature stop codons within the open reading frame (ORF).

Depends on the gene editing purposes, either NHEJ or homologous repair can be utilized. In adaptation of treating viral infection, we aim to disturb viral replication by introducing mutation into the viral genome. The ideal end-result we expect to achieve is that the targeted genes loss of function due to in/del mutation at the targeted site. Also, DNA degradation by nucleases after double stranded break. Either event leads to suppression in viral replication. There are several studies[116,117,118,119,120,121] that have implemented CRISPR technology in treating DNA viral (HIV, EBV, HBV) infections, resulting in eradicating the viral genome from the hosts. Since the discovery of CRISPR application in mammalian system, scientists have been testing the efficacy of CRISPR/Cas9 in treating virus infection. In 2013, Ebina et al. applied CRISPR technology to treat latent HIV-1 provirus. While Highly active anti-retroviral therapy dramatically decreased AID mortality, no strategy has been proposed to treat latent HIV infection. Ebina et al. used Cas9 and gRNAs that specifically target Long terminal repeat(LTR) region in HIV-1 genome. LTR is the core promoter in transcription[122]. In addition to serving as a promoter, LTR also can act as enhancer that contains Nek binding region[123]. LTR is essential in regulating HIV reactivation from provirus. Ebina designed multiple gRNAs targeting NFkB and TAR protein binding sites. CRISPR/Cas9

system could not only inhibit the transcriptionally active provirus, but could also prevent the expression of latently integrated HIV provirus. Instead of treating HIV infection, Liao et al. engineered human-induced pluripotent stem cells stably expressing HIV-targeted CRISPR/Cas9[124]. These engineered stem cells can be efficiently differentiated into cell types that are HIV reservoirs. The engineered cells can maintain their resistance to HIV-1 infection. This provides possible strategy to immunize cells against HIV infection. Wang and Quake investigated the application of CRISPR/Cas9 in treating latent virus infection[125]. They designed several gRNAs that target EBV genome location responsible for viral structure, transformation, and latency. Patient-derived cells from a with latent EBV infection were used. Results showed a quarter of the cells completely eliminated EBV genome and half of the cells showed a decrease in viral load after CRISPR/Cas9 treatment. Roehm et al adapted CRISPR system in treating HSV-1 infection. Roehm and colleagues designed gRNAs targeting ICP0, a crucial viral encoded protein that can regulate viral gene expression and replication[126]. Although gRNA targeting Infected Cell Polypeptide 0 (ICP0), an immediate early gene that promotes transcription from viral gene[127], exhibited a significant decrease in viral production, a mixture of gRNAs targeting ICP0, ICP4, ICP27 eliminated HSV viral infection. These promising results suggest CRISPR as a possible solution for treating CMV infection. Seeger and Sohn deigned CRISPR/Cas9 system to target HBV cccDNA to suppress viral replication[128]. cccDNA is covalently closed circular DNA. HBV cccDNA resides in host nucleus as stable episome and acts as template for all viral RNAs. cccDNA can also replicate and create multiple episomes within one nucleus along with host cell DNA replication. Traditional nucleotide analogue targets viral reverse transcriptase in the cytosol, hence it is not possible to destroy viral cccDNA copy in the nucleus. HBV cccDNA in the nucleus causes chronic infection in the hepatocytes. Seeger et al. established HepG2 cell line stably expressing Cas9. gRNAs were designed to target regions that are important for transcription of pregenomic RNA encoding the HBV core antigen. After CRISPR treatment, infected cells showed 6-10 folds decrease in HBVcore antigen (HBcAg) expression comparing to untreated or Cas9 expression only cells. In a follow-up study by Seeger, they further determined the complete spectrum of CRISPR/Cas9 induced mutations on HBV cccDNA, proving CRISPR/Cas9 is the best model so far to inactivate and cure HBV infection[129]. These progress in adapting CRISPR/Cas9 in suppressing viral replication provides hope in finding a cure for HCMV infection, which brings us to use CRISPR curing HCMV infection.

To understand the efficacy of CRISPR in treating CMV latent and lytic infection, we need to select good model for both infection types. However, a good latent infection cell model has yet to be established. However, due to the species specificity of CMV, an animal model infection of HCMV cannot be performed unless on a humanized animal that transplants specific human tissue to the animal, such as a mouse. This is a very difficult model to work with and study. For decades, researchers rely on using Murine Cytomegalovirus infection in mouse to study the persistent infection, CMV pathogenesis, interaction between virus and the immune system, and congenital infection. MCMV recapitulates the lytic and latent infection in different tissue and organs. When infecting a healthy mouse using tissue culture proliferated MCMV as PFU of $10^{\wedge 5}$, the infection in asymptomatic. Virus particle can be detected in bone marrow, spleen, lungs, and salivary gland. The virus particle can also be cleared by mice immune system in different speed. At about 3 months post-infection, most virus particle can be cleared, except for those residing in salivary gland. It is a completely different story for virus genome since CMV establish latency after initial acute infection. Virus genome resides in host tissue for lifetime while they are cleared in intravascular leukocytes at 12 months post-infection. Since the discovery by McCordock and Smith reported on the distribution of MCMV in tissues of infected mice, numerous progress on understanding CMV pathogenesis and treating CMV infection has been made. Several CMV vaccines have been developed using Murine Cytomegalovirus model.[130,131] MCMV infection in mice is also a disease model for developing CMV infection treatment strategies.[132,133,134] In our study, we applied CRISPR/Cas9 treatment in MCMV infected mice as our animal model to study the efficacy of CRISPR/Cas9 in vivo.

There are several treatment strategies for treating CMV infection aiming at various stages of virus life cycle. Treatments can be designed to target CMV genome replication, gene expression, or virus production. We chose several ORFs that are critical members of genome replication and virion production. Genomic DNA replication is highly conserved across all herpesviruses and is the target for most of the current herpes viral infection therapeutic approaches[135, 136]. In current models of herpesvirus DNA synthesis, the viral DNA helicase unwinds the template at the replication fork, then primase primes lagging-strand synthesis[137]. HCMV-UL70 is believed to encode the primase for CMV DNA synthesis. HCMV-U105 is believed to encode the helicase during viral DNA replication event. Their functions were predicted based on the sequence and positional similarities to the HSV helicase and primase protein complex[138]. Evidence have suggested that UL70, UL105 along with UL105 can assemble a purifiable,

erotrimeric complex[139]. Both UL70 and UL105 are essential for HCMV growth. HCMV fails to replicate when lacking UL70 or UL105[140]. In MCMV, M70 and M105 encode the primase, helicase, respectively. Another proposed target for treating CMV infection is structural protein. CMV virus genome is enclosed within icosahedral capsid protein. UL86 encodes major capsid protein[141]. Major capsid protein composes more than 30% of the total viral protein. When cells are transfected with HCMV lacking UL86, cells cannot produce virions since the major capsid protein is missing. The detained virus genomes are degraded by nucleases. M86 encodes major capsid protein in MCMV.

We constructed all six gRNAs expression vectors. The efficiency of the gRNAs has been tested in an in-vitro model. The preliminary data show that the CMV virus titer is reduced by at least 90% after CRISPR/Cas9 treatment. To conclude, CRISPR/Cas9 system could disrupt HCMV and MCMV replication in cell model. CRISPR/Cas9 can be safely delivered into mice and has the potential to be studied as an animal model for CRISPR/Cas9 treatment in vivo.

4.2 Materials and Methods

Cells and virus

HFF cells were obtained from Clonetics, cultured as adherent cells DMEM(Invitrogen), with 10% FBS, 1% Penicillin streptomycin (Pen-Strep) from Invitrogen. HCMV Towne BAC was previously constructed by inserting a BAC sequence into the genome of HCMV laboratory-adapted strain Towne. HCMV Towne BAC strain was propagated from virus stocks in HFFs. Incubate the HFFs with HCMV Towne BAC at MOI = 0.01. After 2 hours incubation, wash and change media for HFFs. Both supernatant and cell lysis were collected. Cell lysis were first collected by cell scrapers, followed by sonication at 15% amplitude for 1 minute with 30 second intervals.

NIH 3T3 cells were obtained from ATCC, cultured as adherent cells DMEM(Invitrogen), with 10% Nu-serum, 1% Penicillin streptomycin (Pen-Strep) from Invitrogen. MCMV Smith strain was obtained from ATCC. MCMV Smith was propagated from virus stocks in NIH 3T3. Incubate the NIH 3T3 with MCMV Smith at MOI = 0.01. After 2 hours incubation, wash and change media for HFFs. Both supernatant and cell lysis were collected. Cell lysis were first collected by cell scrapers, followed by sonication at 15% amplitude for 1 minute with 30 second intervals.

Plaque forming assay

Seed the HFFs to 24-wells at 18 hours before performing the plaque forming assay. The seeding density for 24 wells plates is 0.05 * 10^6 cells per well. The next day, HFFs were at 1.2 * 10^ 6 cells per well. Add 250ul of the diluted HCMV Town BAC GFP (from 10^-1 to 10^-6) to HFFs and incubate for two hours. After incubation, wash and change media for HFFs. Add the 2% agarose with 4% FBS 2X DMEM overlay to the HFFs. Incubate the plates at 37C tissue culture incubator for 10 days. Count the GFP positive plaques under fluorescent microscopy

Seed the NIH 3T3s to 24-wells at 18 hours before performing the plaque forming assay. The seeding density for 24 wells plates is 0.05 * 10^6 cells per well. The

next day, NIH 3T3s were at 1.2 * 10^ 6 cells per well. Add 250ul of the diluted MCMV Smith (from 10^-1 to 10^-6) to NIH 3T3s and incubate for two hours. After incubation, wash and change medium. Add the 2% agarose with 4% Nu-serum 2X DMEM overlay to the HFFs. Incubate the plates at 37C tissue culture incubator for 5 days. Count the plaques.

Transfection

HFFs or NIH 3T3s were seeded to 24 wells plates without antibiotic 18 hours before transfection at 0.05 * 10^6 cells per well. Cells reached to 1.2 * 10^ 6 cells per well and were ready for transfection the next day. All transfections were carried out using Lipofectamine™ 2000 (Life Technologies). 25pmol miRNA and 1 ul Lipofectamine™ were transfected to the cells. Incubate plasmids with cells for 18 hours, then changed the media with complete media.

SURVEYOR nuclease assay

Harvesting cells for DNA extraction using DNasey Blood and Tissue kit (Qiagen) according to manufacturer's protocol. To amplify the targeted region in viral genome, 10ng genomic DNA was used as temple. Each 50ul PCR reaction contains 200 μM Phusion HF buffer, 500 μM each primer and 1 unit Phusion DNA polymerase (NEB). PCR conditions were 1 cycle of 30 sec at 98°, followed by 30 cycles of 10 sec at 98°, 30 sec at 72° or 58° (depending on the primer pairs according to Table 6), and 30 sec at 72°, and finishing with 10 mins extension at 72°. We checked PCR products amplification and concentration by spectrophotometry and standard gel electrophoresis. PCR products were purified using QIAquick Gel extraction kit. Purified PCR products were eluted in 22ul nuclease free water. Heteroduplex was formed using the following protocol. Each 20ul reaction contains 2ul Taq PCR buffer and 18ul purified PCR product. Annealing conditions: 95 °C, 10 min; 95–85 °C, –2 °C s –1 85°C,1min; 85–75 °C, –0.3 °C s–1 75°C,1min; 75–65 °C, –0.3 °C s–1 65°C,1min; 65–55 °C, –0.3 °C s–1 55°C,1min; 55–45 °C, –0.3 °C s–1 45°C,1min; 45–35 °C, –0.3 °C s–1 35°C,1min; 35–25 °C, –0.3 °C s–1 25°C,1min; 25–4 °C, –0.3 °C s–1 4 °C. Surveyor nuclease assay was performed using 44 μL of purified PCR products incubated with 1 μL of Surveyor nuclease (IDT SURVEYOR mutation detection kit for standard gel electrophoresis), 1 μL of Surveyor Enhancer, and 4 μL of 0.15 M MgCl2 in a 50-μL reaction. The mixture was incubated for 1 hr at 42° and. The reactions were subject to DNA gel electrophoresis.

DNA gel electrophoresis

Total DNA were purified from HFFs and NIH 3T3 using DNasey Blood and Tissue kit (Qiagen) according to manufacturer's protocol. Total RNAs were purified from HFFs using Trizol (Life Technologies) and miRNasy kit (Qiagen) according to manufacturer's protocol. DNA samples were resolved by 1-4% agarose gel electrophoresis depending on fragment size. Agarose gels were stained by Ethidium Bromide solution. Gel were analyzed under Bio-Rad Chemi-Dot Imager.

Name	Sequence (5’ -> 3’)
M70_1 Forward	CACCGACGTGAACAGGTTGCGGCCG
M70_1 Reverse	AAACCGGCCGCAACCTGTTCACGTC
M70_2 Forward	CACCGCGCGGGTAGCGGTGTGTTGG
M70_2 Reverse	AAACCGCGGGTAGCGGTGTGTTGGC
M70_3 Forward	CACCGGGTGGACACGTATTCGGACA
M70_3 Reverse	AAACTGTCCGAATACGTGTCCACCC
M70_4 Forward	CACCGTATGAAAACGGGGCGGACCC
M70_4 Reverse	AAACGGGTCCGCCCCGTTTTCATAC
M70_5 Forward	CACCGGGGTCGAATCCGGGCACCTC
M70_5 Reverse	AAACGAGGTGCCCGGATTCGACCCC
M86_1 Forward	CACCGGGTAACGCGTCAGCTGCCCG
M86_1 Reverse	AAACCGGGCAGCTGACGCGTTACCC
M86_2 Forward	CACCGACCGTCGTCTGTCGCGAGGT
M86_2 Reverse	AAACACCTCGCGACAGACGACGGTC
M86_3 Forward	CACCGCTGTCGCGAGGTGGGGATCC
M86_3 Reverse	AAACGGATCCCCACCTCGCGACAGC
M86_4 Forward	CACCGAGGTCCCTCGAGTGGCCACC
M86_4 Reverse	AAACGGTGGCCACTCGAGGGACCTC
M86_5 Forward	CACCGTTATATACAGGTCCCTCGAG
M86_5 Reverse	AAACCTCGAGGGACCTGTATATAAC
M105_1 Forward	CACCGGGATCTGCTGCGGTTGAGCG

M105_1 Reverse	AAACCGCTCAACCGCAGCAGATCCC
M105_2 Forward	CACCGGGGGAAGACCTCGAGCGTCC
M105_2 Reverse	AAACGGACGCTCGAGGTCTTCCCCC
M105_3 Forward	CACCGTGAGCGCGGAACTGAGCGCC
M105_3 Reverse	AAACGGCGCTCAGTTCCGCGCTCAC
M105_4 Forward	CACCGGCGCCACTGCTGCTGGCAGA
M105_4 Reverse	AAACTCTGCCAGCAGCAGTGGCGCC
M105_5 Forward	CACCGCGCCCTGCATCGTCTGCGTC
M105_5 Reverse	AAACGACGCAGACGATGCAGGGCGC
M105 Detect Forward	GTACGACAACATCTTCGTCCTGAATA
M105 Detect Reverse	CTTGTTGTTGATGAACATGACCCAGTT
UL70 Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACG AAACACCGCGGTGTCTGCGAGAGTACGT
UL70 Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACACGTACTCTCGCAGACACCG
UL86 Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACG AAACACCGGAGGCCTTACGTATCTACTA
UL86 Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACTAGTAGATACGTAAGGCCTC
UL105 Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACG AAACACCGATCAAGGCGTCGTCGTACTT
UL105 Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACAAGTACGACGACGCCTTGAT
M70 Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACG AAACACCGGCATATTGACTACGATATTT
M70 Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACAAATATCGTAGTCAATATGC
M86 Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACG AAACACCGCGATGCTGTAGTTGCCGTAG
M86 Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACCTACGGCAACTACAGCATCG

M105 Forward	TTTCTTGGCTTTATATATCTTGTGGAAAGGACG AAACACCGCGTCCGCCTCGAAGATCGAG
M105 Reverse	GACTAGCCTTATTTTAACTTGCTATTTCTAGCTC TAAAACCTCGATCTTCGAGGCGGACG

Table 6 Primers design. All primers were ordered from IDR in 25nm standard desalt.

4.3 Results

The design and construction of gRNAs.

Since our aim is to disrupt the viral protein coding capacity, it is optimal to choose a target that is closer to the N' terminus of a protein coding region because it is more likely to introduce a true null allele. Following these principles, we designed gRNAs that target near the 5' end of an open reading frame(ORF). To avoid off-target effects, we used CRISPRdirect[142], a bioinformatics tool based on selecting efficient CRISPR/Cas9 target sites with a reduced number of potential off-target effect candidates. To prevent disruption of the host genome, the candidate targets have been compared with the host genome, searching whether there are perfect matches for seed sequences (12 to 8 mer flanking the PAM). Designed targets for CRISPR treatment is shown in Table 7. Each gRNA sequence selected has no perfect complimentary targets in the host genome. Additionally, all gRNAs contain no complimentary targets in the 8mer region close to PAM (NGG) sequence. gRNAs are synthesized by cloning U6 promoter, target sequence, guide RNA scaffolding terminal signal into pCR-Blunt II-TOPO. Cas9 expression plasmid was constructed by inserting Cas9 gene into pcDNA3.3-TOPO plasmid. gRNA and Cas9 expression plasmids were obtained from George Church lab. Primers used for cloning the gRNA is shown in Table 6

ORF	Target Site (5’ – 3’)
UL70	CGGTGTCTGCGAGAGTACGT
UL86	GAGGCCTTACGTATCTACTA
UL105	ATCAAGGCGTCGTCGTACTT
M70	GCATATTGACTACGATATTT
M86	CGATGCTGTAGTTGCCGTAG
M105	CGTCCGCCTCGAAGATCGAG
M70_1	ACGTGAACAGGTTGCGGCCG
M70_2	CGCGGGTAGCGGTGTGTTGG
M70_3	GGTGGACACGTATTCGGACA
M70_4	TATGAAAACGGGGCGGACCC
M70_5	GGGTCGAATCCGGGCACCTC
M86_1	GGTAACGCGTCAGCTGCCCG
M86_2	ACCGTCGTCTGTCGCGAGGT
M86_3	CTGTCGCGAGGTGGGGATCC
M86_4	AGGTCCCTCGAGTGGCCACC
M86_5	TTATATACAGGTCCCTCGAG
M105_1	GGATCTGCTGCGGTTGAGCG
M105_2	GGGGAAGACCTCGAGCGTCC
M105_3	TGAGCGCGGAACTGAGCGCC
M105_4	GCGCCACTGCTGCTGGCAGA
M105_5	CGCCCTGCATCGTCTGCGTC

Table 7 CRISPR/Cas9 target sites in CMV genome.

Inhibition of viral growth in vitro in cultured HFFs by CRISPR/Cas9 gene editing tool

To assess the efficiency of CRISPR/Cas9 on suppressing CMV infection, we first investigated whether CRISPR/Cas9 treatment inhibits viral growth. Human Foreskin Fibroblast (HFF) cells were transfected with one of the following gRNAs/Cas9 expression plasmids: gRNA_UL70/Cas9, gRNA_UL86/Cas9, gRNA_UL105/Cas9, gRNA, GFP/Cas9, Cas9 only. To study inhibition of MCMV, NIH3T3 cells were transfected with one of the following gRNAs/Cas9 expression plasmids: gRNA_M70/Cas9, gRNA_M86/Cas9, gRNA_M105/Cas9, gRNA, GFP/Cas9, Cas9 only. HFFs were infected with HCMV strain Toledo at a multiplicity of infection (MOI) 0.01 at one day post-transfection. NIH 3T3 cells were infected with MCMV strain Smith at MOI 0.01 at one day post-transfection. Both HCMV and MCMV establish lytic infection once infected. Both viruses are commonly used in the field, with Toledo is a low passaged HCMV strain while Smith is an ACTT strain widely used in the field. DNA was extracted at one day post-infection. HCMV virus from HFFs was harvested at 7 days post-infection. We harvested the MCMV virus from HFFs at 3 days post-infection. We observed that CRISPR treatment could somewhat protect cells against cytopathic effects caused by CMV infection, perhaps viral growth was inhibited by CRISPR treatment. To test our hypothesis, we performed a virus plaque assay to determine the virus titer. The HCMV titer was determined by plaque forming unit count in HFF. The MCMV HCMV titer was determined by plaque forming unit count in NIH 3T3.

In HCMV studies, at 7 days post-infection, a reduction about 330 folds in viral yield was observed in the cells that were transfected by specific CRISPR treatment, compared to a 20 fold reduction that was found in cells that were subjected to non-specific CRISPR treatment(Figure 18). In MCMV studies, at 3 days post-infection, there is a decrease in virus titer from cells that were transfected with gRNAs/Cas9. Five days post-infection, a reduction about 1032 folds in viral yield was observed in the cells that were transfected by a specific CRISPR treatment, whereas only 8 folds reduction was found in those that were subjected to non-specific CRISPR treatment(Figure 19).

The preliminary data shows that our current CRISPR/Cas9 system can inhibit the growth of CMV virus. One of the critical aspects of improving the efficacy of CRISPR/Cas9 is the accessibility of the target DNA. To construct highly active CRISPR/Cas9 system, we designed 6 different gRNAs for each ORF in MCMV as shown in

Table 7. CMV genome is chromatinized inside the nucleus, introducing more gRNAs to target one open reading frame will help us increase the possibility of gRNA targeting into the accessible region in the viral genome. To improve the CRISPR/Cas9 delivery and expression, we chose to use a chimeric CRISPR/Cas9 plasmid, PX458. PX458 contains both sgRNA and Cas9 expression unit along with GFP fusion protein for evaluating transfection efficiency. A chimeric CRISPR/Cas9 expression plasmid allows us to deliver CRISPR/Cas9 effectively. We obtained CRISPR/Cas9 chimeric plasmid from Feng Zhang lab from MIT. The efficacy of gRNAs was tested later in the following experiments.

We first investigated whether the new CRISPR/Cas9 treatment inhibits viral growth. NIH3T3 cells were transfected with one of the following gRNAs/Cas9 expression plasmids: gRNA/Cas9 targeting MCMV genomes/ Cas9, gRNA/Cas9 cocktail, gRNA/Cas9 with no targets only, GFP expression plasmid, mock treatments. NIH 3T3 cells were infected with MCMV strain Smith at MOI 0.01 at one day post-transfection. MCMV established lytic infection once infected. Genomic DNA was extracted at two days post-infection. We harvested the MCMV virus from NIH3T3s at five days post-infection. Plaque forming assay was performed to assess the virus titer. The MCMV titer was be determined by PFU count in NIH 3T3. As shown in Figure 23, in cells transfected with CRISPR/Cas9 targeting viral protein M105 exhibited the best anti-viral effect.

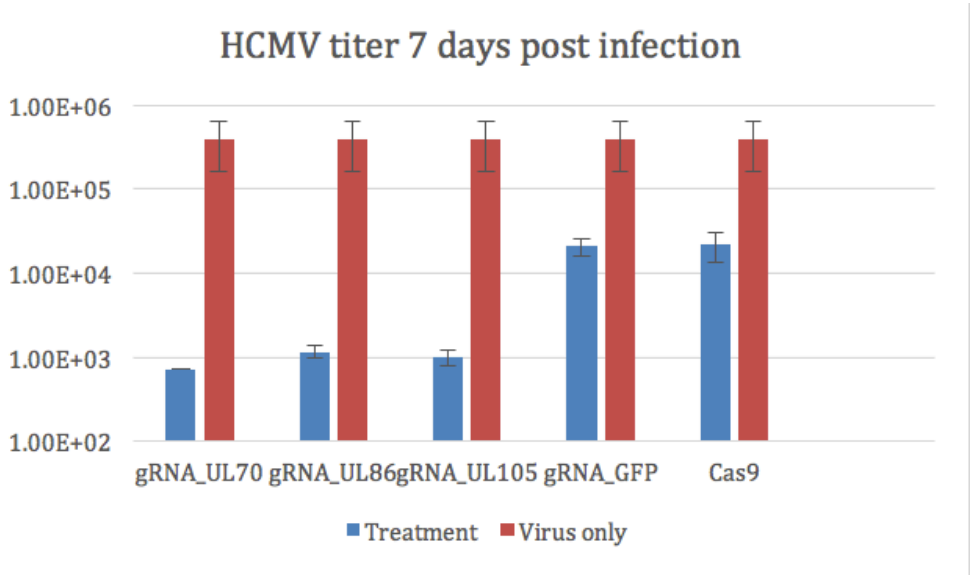

Figure 18 HCMV titer at 7 days post-infection after CRISPR treatment. HCMV-infected HFFs were either mock treated or transfected with different gRNAs and Cas9. HFFs were then incubated at 37 °C. HCMV virus was harvested 7 days post-infection. Virus titer was determined by plaque forming assay.

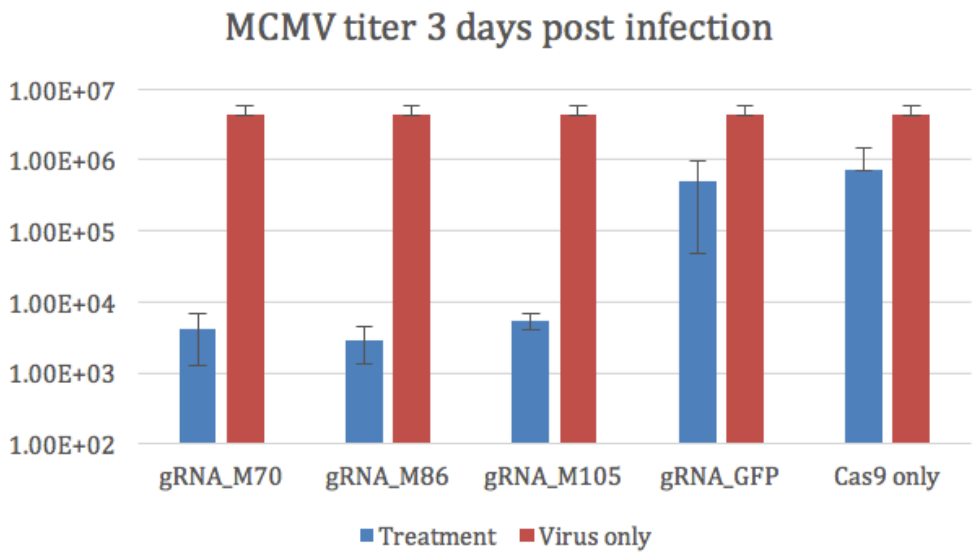

Figure 19 MCMV titer at 3 days post-infection after CRISPR treatment. MCMV infected NIH3T3s were either mock treated or transfected with different gRNAs and Cas9. NIH3T3s were then incubated at 37 °C. MCMV virus was harvested at 3 days post-infection. Virus titer was determined by plaque forming assay.

Detecting region: 151124- 152035

TGTCGAGGTACGACAACATCTTCGTCCTGAATATGTCGTCCGCCTCGAAGATCGAGCGGATCG
TTGACCG

CGTCAAGTCGCTGGCCTTGAAGCGGTTCTCCCGCGAATCTCTGTACAAGGACTGGTTCCGCCA
CATGTTG

GATCCCTGCGCGGGCCTGGTGGCGCCCGAGCTCGGCGACGACGGCAGCAGCGAAGGCGGCGG
CAACGCCG

CGATGATCGTCGGCGACCGCGAGCTGGCGCGCCGGCCGCCCTTCCTACCCTTCTCCTGCCTCCT
GATCAC

CGGTACCGCCGGCGCGGGGAAGACCTCGAGCGTCCAGGTCCTGGCCGCTAACCTCGACTGCG
TGATCACG

GGCAGCACCGTGATCTCCTCCCAGGCGCTCAGTTCCGCGCTCAACCGCAGCAGATCCGCTCAG
ATCAAGA

CCATCTTCAGGACTTTTGGGTTTAATAGTCGGCACGTCGCCCTCGCCGACCGCGTTCACCTCAG
GCGGCG

CGACGACGTCGCGTTCGACGGCGACGTCGACCCCATCTGCCAGCAGCAGTGGCGCGATCTCTC
GACCTAC

TGGCCGGTCGTCTCCGACATCGCCATCCGCGCCCTGGACGGCGGCAAGGGCCGCAAGGACAC
CGACGACC

TCTGCCGCAGCAACATCATCGTCATCGACGAGTGCGGCGTCATCCTCCGACACATGCTGCACG
TCGTCGT

CTTCTTCTACTACTTCTACAACGCCCTCAACGACAGCGAGCTCTACCGCCAGCGCGCGGCGCC
CTGCATC

GTCTGCGTCGGGTCGCCCACGCAGTCCGAGGCCCTCGAGAGCCGCTACGACCACCGCACGCA
GAATCGGG

ACGTGCAGCGCGGCATGGACGTCCTCTCCGCGCTCATCAGCGACCCGGTCCTCTCCGAGTACT
GCGACGT

CGCCCACAACTGGGTCATGTTCATCAACAACAAG

Figure 20 MCMV CRISPR treatment targets design. Five targets were chosen for the chimeric CRISPR/Cas9 system. The detection region start from 151124 to 152035 in MCMV Smith Strain genome.

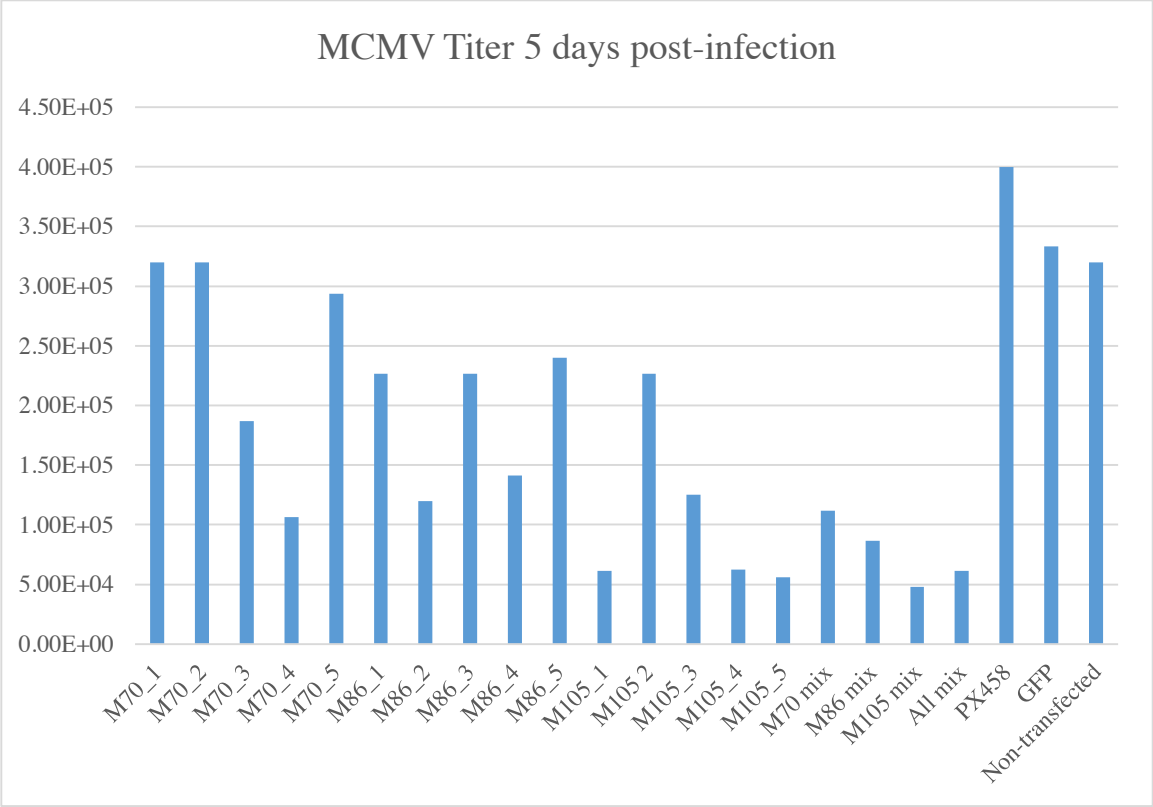

Figure 21 MCMV titer 5 days post-infection after chimeric CRISPR/Cas9 transfection. MCMV infected NIH3T3s were either mock treated or transfected with different CRISPR/Cas9 expression plasmid targeting different regions in viral genome. NIH3T3s were then incubated at 37 °C. MCMV virus was harvested at 5 days post-infection. Virus titer was determined by plaque forming assay.

Introduction of Indel mutation in CMV genome by CRISPR/Cas9

Since CRISPR/Cas9 treatments targeting M105 showed the best antiviral effect among all the of the specific treatments, we chose M105 to investigate the indel mutation that might have been caused by CRISPR/Cas9 treatment. To determine if the CRISPR/Cas9 system cleaves the virus genome at the targeted site, we performed Surveyor assay to assess the cleavage efficiency caused by CRISPR treatment. Surveyor nuclease is an endonuclease that can recognize the mismatch site in DNA then cleave the mismatch site resulting in DNA fragments. The principle of Surveyor assay is shown in the Figure 22

For the surveyor assay, we amplified the DNA sample for 944 bp spanning the cleavage site(Figure 23). The amplified viral genome fragment contained both mutated and wild-type fragments. To create heterogeneous DNA duplex that can be recognized and cleaved by SURVEYOR nucleases, the DNA mixture was then reannealed to form heteroduplex. Surveyor recognizes the heteroduplex complex at the mismatch site and cleaves at the mismatch site. SURVEYOR reaction mixtures were analyzed via gel electrophoresis. As shown in the image (Figure 23), cleavages were created after SURVEYOR assay. In CRISPR/Cas9 targeting M105 at position 1, 2, 3, and 4, we can observe mutation at the predicted site. However, we could not observe any cleavage in M105_5. The reason might be the target site fell into the inaccessible region in MCMV genome, preventing Cas9 from creating double stranded break. As expected, there was no specific cleave in viral genome when cells were transfected with empty CRISPR/Cas9 treatment, indicating the specificity of the targeting activity.

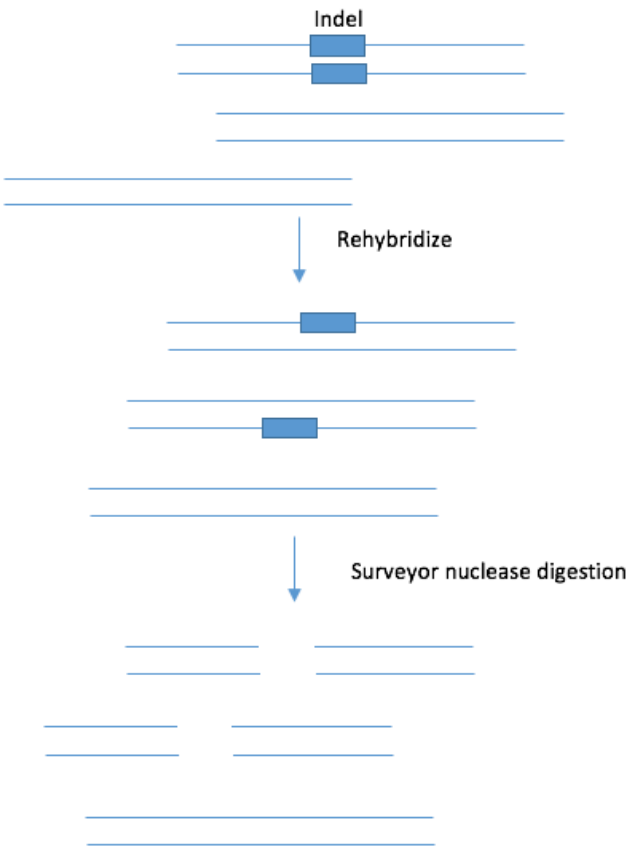

Figure 22 The principle of SURVEYOR nuclease assay.

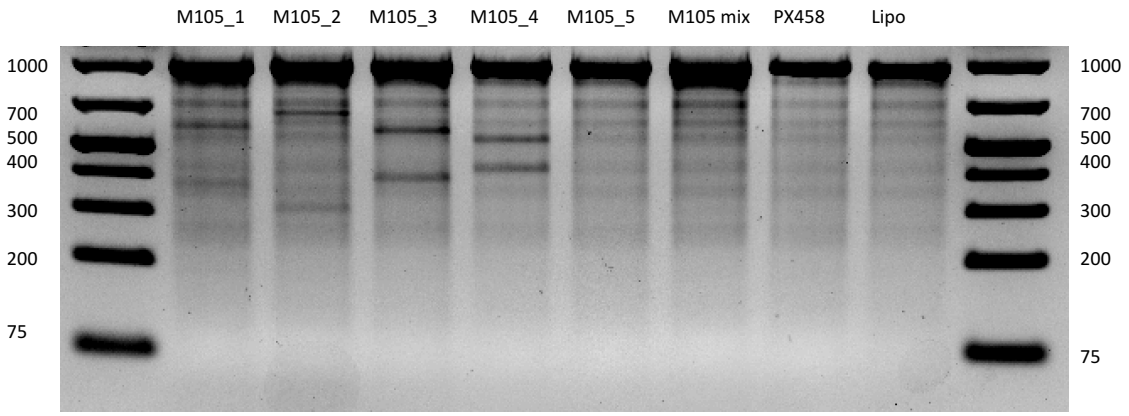

Figure 23 CRISPR/Cas9 treatment targeting M105 create mutation in the targeted region. MCMV infected NIH3T3s were either mock treated or transfected with specific CRISPR/Cas9 expression plasmid targeting viral genome. NIH3T3s were then incubated at 37 C. Total DNAs were harvested at 2 days post-infection. Collected DNA were subject to Surveyor mutation detection assay. The total length of the DNA fragment is 944bp. gRNA M105_1 causes cleavage at the predicted location, leaving 2 bands: one at 407bp, the other at 537bp. gRNA M105_2 causes cleavage at the predicted location, leaving 2 bands: one at 312bp, the other at 632bp. gRNA M105_3 causes cleavage at the predicted location, leaving 2 bands: one at 416bp, the other at 528bp. gRNA M105_5 did not show any cleavage from Surveyor assay.

4.4 Discussion

HCMV infection causes mild or subclinical diseases in immunocompetent adults but leads to severe, life-threatening complications in people who are immunocompromised, including AIDS patients and transplant recipients, as well as newborn infants. As the state of art treatment is using nucleotide analogue to block viral polymerase replication, another difficulty in CMV or any other latently infected virus treatment is failing to control the reactivation. Treatment is only effective when virus genome is actively replicating, hence the remaining silenced viral genome can be reactivated at any time. A gene therapy that can edit and eliminate the CMV virus is the only solution for curing CMV latent infection.

In our study, we explored the potential of using CRISPR/Cas 9 in treat HCMV and MCMV infection. We designed our treatment to target CMV genome replication, gene expression, or virus production. The ORFs chosen are critical members of genome replication and virion production. In current models of herpesvirus DNA synthesis, the viral DNA helicase unwinds the template at the replication fork, then primase primes lagging-strand synthesis. HCMV-UL70 encodes the primase for CMV DNA synthesis, HCMV-U105 encodes the helicase during viral DNA replication event, are targeted to inhibit viral DNA replication. In MCMV, M70 and M105 encode the primase, helicase, respectively. They were chosen to be the treatment targets in MCMV infection. Another proposed target for treating CMV infection is the structural protein, major capsid protein. HCMV-UL86 encodes the major capsid protein were chosen as the structural protein target that could prevent viral capsid assembly. We constructed all six gRNAs expression vectors. Our data show that the CMV virus titer is reduced by at least 90% after CRISPR/Cas9 treatment. To promote the efficacy of CRISPR/Cas09 system, we added 5 additional targets in each ORF. Furthermore, we applied chimeric CRISPR/cCas9 expression system to improve the delivery and expression of sgRNA and Cas9. Surprisingly, sgRNA targeting M105, a helicase complex in MCMV, had the best anti-viral effect. Interestingly, the anti-viral effect of a mixture of gRNA was not significantly higher than individual gRNA/Cas9. Increasing the number of targets in the viral genome does not relate to the strength of the antiviral effect. Moreover, we examined the indel mutation introduced in the MCMV genome by Cas9. In the SURVEYOR assay, we were able to detect the mutation at the expected location,

suggesting that CRISPR/Cas9 could introduce indel mutations that lead to a suppression of viral replication. To conclude, CRISPR/Cas9 system could disrupt HCMV and MCMV replication in the cell model. CRISPR/Cas9 can be safely delivered into mice and has the potential to be studied as an animal model for CRISPR/Cas9 treatment in vivo.

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