SIN NOMBRE VIRUS: REVERSE GENETICS AND HOST GENOMIC RESPONSE

AN ABSTRACT

SUBMITTED ON MARCH 24, 2017

TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF MEDICINE

OF TULANE UNIVERSITY

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

BY

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New World hantaviruses are emerging zoonotic viruses that cause disease with high mortality rates, ranging from 35 to 50% for Sin Nombre Virus (SNV). Hantaviruses are transmitted through aerosolization of rodent excreta and New World viruses cause a disease called hantavirus pulmonary syndrome (HPS), due to infection of endothelial cells and subsequent capillary leakage. In order to gain a greater understanding of hantaviruses, an attempt at a reverse genetics system was (RGS) preformed. In addition, the role the individual viral proteins play in pathogenesis of endothelial cells was evaluated. To date, no reliable RGS has been successfully constructed for the hantavirus genus. We hypothesize that this is due to incompatibility of a DNA-based RGS with hantavirus replication. The genome segments of SNV contain termini with unique chemical moieties that potentially play a very important role in replication. DNA-based systems create termini that end in nucleosides and nucleotides that differ from that of the virus. To circumvent these problems, we attempted an RNA-based RGS that will allow for exact formation of the unique chemical groups found on the termini of the naturally occurring vRNA and cRNA genome segments.

We determined the contribution viral protein production has on pathology and disease. We used a biologically relevant cell type, human primary lung microvascular endothelial cells (HMVEC-Ls). We expressed viral proteins through
nucleofection from the vectors we created from our RGS in HMVEC-Ls, allowing us to determine the impact these proteins in addition to whole virus replication have on the transcriptomic response of the cells. We discovered pathways of importance during SNV infection, including platelet activation and aggregation, endothelial cell-to-cell junction disorganization, and extracellular matrix disruption. The glycoproteins (Gc and Gn) appear to be responsible for the platelet activation and aggregation during infection. Prior to this work, the function of the non-structural protein (NSs) was unknown. We discovered that NSs functions as an interferon antagonist and potential antigen presentation antagonist for MHC I.
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ACKNOWLEDGEMENTS

First, I'm forever thankful to Nito, who accepted a graduate student orphan into his lab after my former PI left. You have provided a great environment for me to complete my dissertation research. Thank you for giving me guidance and supporting me through all the setbacks that inevitably come during a PhD. Thank you for helping to nurture my love of emerging viruses and high containment research.

To Vibha, Kaarthik, and especially Elizabeth for accepting me into the lab and ‘showing me the ropes’. Elizabeth, you have been the best work wife one could ask for and have been incredibly supportive and so helpful in lab work and in life. Nick has also been in my eyes, my secondary mentor. Thank you, Nick, for being a great committee member first of all, but also a great mentor and a great role model.

To Theresa Secrist, you have single handedly saved me from multiple headaches. Thank you for being my advocate and making sure my stipend and health insurance are secured. To Dr. Flemington and Dr. Melody Baddoo of the Cancer Crusader Next Generation Sequence Analysis Core, you have made my dissertation research infinitely easier, thank you.

To my friends here in New Orleans and afar, thank you for being supportive during my PhD time. I needed the laughs and the distractions during these stressful 5+ years. To my BMS buddies, thanks for going through this tough graduate school process with me and keeping me sane. Special thanks to my ‘first grad school friend’ turned best friend, Hope, for being the best. 6 Mardi Gras’ of friendship! Thanks to my office mates Nell and Marissa for being a great sounding board. Thanks to Alan and Amanda for making the journey with me to the primate center each day in a carpool for those years.

Thank you to my family for being there for all of my 28 years. My dad who gave me my love of science by our medical talks around the dinner table in my childhood. My mother for being there for phone calls while I was stressed and for visiting me countless time in New Orleans. To my brothers and sister-in-laws, and their broods of children, thanks for being awesome and sending me photos of all my beautiful nieces and nephews. I hope I can convince a few of them to become scientists!

To Brian, thanks so much for your support. You were forever optimistic for me when my research had me down. You were there to pick up the slack when I was stressed and frazzled and provided never-ending love and support. You’re the best and some other mushy stuff. I can’t forget Mona, who will never know I thanked her for being her. Thanks for sleeping on my feet or by my side while I wrote my
dissertation or practiced talks. Thanks for forcing to go outside and take walks and refresh my mind.

To my committee members who have provided support throughout my dissertation time, thank you for looking out for me as a researcher and providing great insight towards my dissertation work.
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CHAPTER 1: HANTAVIRUS BACKGROUND

OVERVIEW

Hantaviruses are members of the family *Bunyaviridae* and are found throughout the world. The hantaviruses are split into two groups, the New World and Old World hantaviruses and they differ in the disease they cause. Old World viruses (found in Europe, Asia, and Africa) cause hemorrhagic fever with renal syndrome (HFRS), which results in a more systemic infection of endothelial cells in the host and a more mild disease with lower mortality rates than New World hantaviruses. New World hantaviruses (found in North and South America) cause hantavirus cardiopulmonary syndrome (HCPS) which primarily affects the lungs and results in much higher mortality rates than HFRS, rates of 30-40%. The two New World strains most prevalent include Sin Nombre Virus (SNV), found predominantly in the American southwest and Andes Virus (ANDV), found in South America. Each strain of hantavirus has a specific asymptomatic rodent reservoir. SNV is carried by deer mice and ANDV is carried by the long tail rice rat. Transmission to human occurs when aerosolized rodent excreta, including feces, urine and saliva, enters the human respiratory tract. (Figure 1) Human to human transmission cases have been documented for ANDV and involve close contact between individuals either in a family or a hospital.
Figure 1. Transmission of New World hantaviruses. Excreta (feces, urine, or saliva) from the infected rodent reservoir is aerosolized. The infected excreta enters the upper respiratory tract of the human host through inhale and makes it way to the lungs, establishing infection.
The virus infects the lungs of the host. Early signs of disease are very similar to other viral diseases, causing flu-like symptoms. Later stages of disease include capillary leakage into the lungs, causing edema, due to an increase in vascular permeability. The viral infection is localized to the lungs but in later stages of disease can be found in endothelial cells of various organs of the body. Endothelial cells are the primary replication site. The virus is not cytolytic in these cells, so various mechanisms of pathology have been proposed to induce the capillary leakage seen during infection. Supportive care is the only treatment for disease as there are no vaccines or therapeutics currently. SNV and ANDV are both emerging viruses and there is much impetus to elucidate the virology and pathology in order to develop vaccines and therapeutics.

**DISCOVERY AND GEOGRAPHICAL LOCATION**

Old world hantaviruses were discovered in the 1950s during the Korean War. Soldiers came down with symptoms of hemorrhagic fever with renal syndrome, the disease associated with Old World hantaviruses. It was soon found that hantaviruses are endemic to Europe, Asia, United States, and Africa. The disease is more mild than that of New World hantaviruses with a mortality rate ranging from 0.1% to 10%. The different strains of virus are found in their specific rodent host, including voles, shrews, and rats, among others. Other geographical regions most likely also have endemic hantaviruses but as the disease symptoms are mild, can go undetected.
New world hantaviruses were discovered in 1993 after the Four Corners disease outbreak\textsuperscript{12}. There was immunological cross reactivity with Old World hantaviruses, leading to the discovery of the etiological agent, a hantavirus. The disease differs from the Old World hantaviruses and has a much higher mortality rate (30-40\%)\textsuperscript{10}. The virus was named Sin Nombre Virus. Outbreaks through the Americas have led to the discovery of many more New World hantaviruses, including the primary species of South America, Andes Virus. Andes Virus has been documented to spread from human to human through close contact\textsuperscript{5}. This is an ominous sign since, as Sin Nombre Virus and ANDV are closely related, mutation of SNV has the potential to adapt to person-person transmission.

**Hantavirus cardiopulmonary syndrome (HCPS) vs hemorrhagic fever with renal syndrome (HFRS)**

Both Old and New World hantaviruses infect human hosts in the same way. Aerosolized excreta from the rodent host enters the respiratory track. HFRS symptoms begin 1-2 weeks post exposure. The disease is split into five phases. The initial phase is called the febrile phase where flu-like symptoms occur along with gastro-intestinal problems. This leads to the hypotensive phase, which involves a blood platelet level drop, leading to tachycardia and hypoxemia. Next, the oliguric phase occurs, leading to renal failure and proteinuria. The diuretic phase follows, and leads to a massive production of urine. The final convalescent phase results in recovery if complications and death do not arise.\textsuperscript{13-16} Because the symptoms of HFRS are found in many diseases, the diagnosis is done using serological evidence,
screening blood for hantavirus antibodies\textsuperscript{17}. In order to treat HFRS, supportive care is used as there is no therapeutic. Supportive care includes renal dialysis to counteract renal failure. The mortality rate from HFRS strongly depends on the strain of hantavirus, ranging from 0.1\% for mild strains and 10\% for more severe strains\textsuperscript{1,2}.

New World hantaviruses enter the respiratory track and establish infection in the lungs of the host. Unlike Old World hantaviruses, the infection is generally contained in the lungs. Later in infection, virus is found in endothelial cells throughout the host but initially is found in the lungs, where the main pathology occurs. HCPS begins with flu-like symptoms and the disease can progress to acute respiratory failure. Initial symptoms include shortness-of-breath with subsequent pulmonary edema. As with HFRS, there are no therapeutics against the New World hantaviruses and late stage disease requires mechanical ventilation and administration of diuretics. Regardless of the supportive care the mortality rate of hantavirus-induced HCPS is much higher than for HFRS, ranging from 30-40\%.

**REPLICATION CYCLE**

Hantaviruses are single stranded negative sense RNA enveloped viruses. There are three genome segments termed small (S), medium (M), and large (L). The M segment codes from the two glycoproteins found on the lipid membrane, Gn and Gc. L encodes for the RNA-dependent-RNA-polymerase. The S segment encodes for the nucleopcapsid protein and in 80\% of viruses in the *Bunyaviridae* family encode a
non-structural protein NSs, which has been found to have various functions during infection\textsuperscript{18}. ANDV has been shown to transcriptionally express NSs by a leaky scanning mechanism\textsuperscript{19}. There is also a putative ORF site for NSs in SNV but it remains to be shown if it encodes a protein that is expressed during infection and, if so, what role the protein has in infection\textsuperscript{20}. (Figure 2) Once virus enters the upper respiratory track of the human host, it likely infects macrophages and epithelial cells prior to reaching its site of predominant replication, capillary endothelial cells. In-vitro work has shown that β\textsubscript{3}-integrin is a receptor for the virus and that the virus undergoes receptor-mediated endocytosis upon binding\textsuperscript{21}. The virus enters the endosome and upon maturation and acidification, the fusion loop of the Gc protein is exposed and binds to the endosomal membrane inducing fusion of the viral and cellular membranes\textsuperscript{22}. Viral transcription and replication occurs in the cytoplasm\textsuperscript{23}. Negative sense single stranded vRNA is found within the virion associated with a trimerized hantavirus nucleocapsid in a RNP. When the RNPs are released, the vRNA is available for transcription to mRNA by the virus associated RNA dependent RNA polymerase (RdRp). As discussed above, hantaviruses utilize a cap snatching mechanism using caps from the P bodies in the cytoplasm. This mRNA can then be translated by the host cell ribosomes. Synthesis of vRNA starts with synthesis of cRNA from the original template vRNA brought into the cell. This is de-novo synthesis, done without a primer, the RdRp uses a prime and align mechanism of transcription to synthesize cRNA. This is done using a triphosphorylated G attaching to the C at the 5’ end of the cRNA then the RdRp cleaves these residue, leaving a monophosphorylated 5’ terminus. A similar process takes place during the
Figure 2. **Virus and genome structure.** A. Hantavirus has a lipid envelope with two transmembrane glycoproteins, Gc and Gn. These glycoproteins combine in a tetrameric spike complex. The three genomes segments are vRNA that is associated with a trimerized form of the nucleocapsid protein. The vRNA forms a panhandle structure and inverted repeats on the termini allow for hydrogen bonding. The RNA-dependent-RNA-polymerase is thought to be associated with the termini of the genome segments. B. The genome segments are termed small, medium, and large. The S segment is 1.6 kb long and contains
The M segments is 3.6 kb long and contains the sequence for the glycoprotein precursor. This polypeptide is cleaved to mature Gn and Gc peptides by cell proteases. The L segment contains the sequence for the RNA-dependent-RNA-polymerase. All three segments contain a monophosphorylated uridine on the 5’ termini and a hydroxylated adensine on the 3’ termini.
synthesis of vRNA, leaving that same monophosphorylated 5’ terminus.\textsuperscript{24} Transcription is initiated by a cap-snatching mechanism by virus components in processing bodies (P-bodies)\textsuperscript{25}. Host cells mRNAs that are sent to P-bodies for degradation are utilized by the virus for caps and subsequently used for transcription of viral mRNA\textsuperscript{25}. Other members of the bunyaviruses replicate in the ER-Golgi intermediate compartment (ERGIC) and bud from the Golgi, with a Golgi-derived lipid membrane\textsuperscript{23}. Exocytosis results in progeny virions release. This general mechanism of virus release is likely used by hantaviruses as well. Many of the replication details of the virus are unknown and research has been hindered by the lack of a viable reverse genetics system for the New World hantaviruses.

**PATHOLOGY**

Infection with the New World hantaviruses results in HCPS. The initial symptoms are flu-like but later symptoms include capillary leakage in the lungs. Capillary leakage results in edema of the lungs and cardiogenic shock\textsuperscript{10}. Increased vascular permeability is the major cause of the pathology seen during infection\textsuperscript{8}. The basis of this increased vessel permeability is an important on-going research topic in the hantavirus field. There exist various proposed mechanisms and contradictory information. Hantavirus infects macrophages, dendritic cells, epithelial cells, and the main site of replication, endothelial cells. The capillary leakage seen during infection is caused by endothelial dysfunction. The virus is not cytolytic or even cytopathic in endothelial cells\textsuperscript{6} so proposed mechanisms include upregulation of vascular permeabilizing factors, including bradykinin\textsuperscript{26}; downregulation of cell-to-
cell junction proteins, including VE-cadherin\textsuperscript{27}; and immune system involvement, including the complement cascade\textsuperscript{28}. (Figure 3)

Proposed mechanisms for increased vascular permeability start with the process of virus-receptor binding. It has been shown \textit{in-vitro} that virions utilize $\alpha_v\beta_3$ as a receptor for viral entry into endothelial cells\textsuperscript{21}. This integrin is also present on platelets. Hantaviruses bound to endothelial cells are able to bind platelets as well, possibly contributing to the thrombocytopenia send during clinical infection in patients\textsuperscript{29}. It is proposed that this also allows for ‘masking’ of the virus on the endothelium surface\textsuperscript{30}. $\alpha_v\beta_3$ is an integrin that regulates vascular permeability in endothelial cells and if they act as a receptor \textit{in-vivo}, may play an important role in virus-induced capillary leakage. VE-cadherin is another cell-cell junction protein in which its internalization results in increased vascular permeability. There are many stimuli that can induce VE-cadherin loss or internalization, and current investigations with hantavirus is determining whether the virus may degrade cellular VE-cadherin. One mechanism of VE-cadherin reduction is through the upregulation of VEGF, a secreted factor that binds to VEGFR and results in a signaling pathway that eventually leads to the internalization and degradation of VE-cadherin\textsuperscript{8}. This process may be triggered by a specific viral protein during viral replication. Some studies have shown an increase in production of VEGF during hantavirus infection but others have observed no significant changes\textsuperscript{27,31-33}. 

Figure 3. Hantavirus pathology in endothelial cells. Figure is taken from Vaheri et al. Various pathways are thought to contribute to hantavirus pathology. These pathways include coagulation from blood platelets as well as thrombocytopenia. Increase production of VEGFA and bradykinin is linked in increased permeability. Certain proteins in the complement cascade also increase permeability, indicating that immunopathology may be contributing to hantaviral disease.
The Kallikrein-Kinin system has also been implicated in the increased vascular permeability seen during hantavirus infection of endothelial cells\textsuperscript{26}. This system results in the expression of bradykinin, a potent vasodilator. It has not been previously determined if any hantavirus proteins have a direct effect on this system but previous research shows that nucleic acids and misfolded proteins activate the Kallikrein-Kinin system among other stimulus\textsuperscript{34}. In addition to the Kallikrein-Kinin system, ANDV N was found to upregulate the Rheb and RhoA GTPases through interaction with TSC (tuberous sclerosis complex), inhibiting it’s repression of the Rheb-mTOR-pS6K pathway, resulting in enlargement and increased permeability of endothelial cells\textsuperscript{35}. With our study we hope to determine if the viral proteins have any transcriptional effect on these systems as it relates to SNV infection.

Immunopathology mechanisms include production of interferon during infection, resulting in expression of a constellation of factors that mediate innate immunity through multiple mechanisms, activation of CD4+ T cells, B-cells, and CTLs, and the innate complement cascade. Tumor necrosis factor $\alpha$ (TNF-$\alpha$) is an important immune factor involved in the antiviral response and directly increased vascular permeability in endothelial cells\textsuperscript{36}. Complement cascade components, mainly SC5b-9, may be involved during infection. SC5b-9 can also bind $\beta$3 integrins and results in release of bradykinin and platelet activation factor, contributing to increased permeability\textsuperscript{28}. Thus, the activation of innate complement cascade may be a mechanism for the pathology seen during hantavirus infection.
NSs

Some members of the Bunyaviridae family contain a non-structural protein (NSs) that is either expressed through ambisense transcription, an alternate open reading frame, or antisense transcription. A majority of the NSs proteins are interferon antagonists, primarily working on the IFN-β response and to a lesser extent the IFN-α response. Some NSs proteins inhibit host mRNA synthesis, resulting in decreased host protein synthesis. In addition, RNAi has been implicated as a target of NSs. The orthobunyaviruses code a NSs protein through an alternative reading frame. Bunyamwera, Schmallenberg, and Lacross virus NSs inhibits host interferon signaling. Bunyamwera virus targets the RNA-polymerase II, inhibiting mRNA elongation and 3’ end processing as well as activation of pathogen recognition receptors (PRRs). La Crosse virus inhibited RNAi in mammalian cells in addition to inhibiting the interferon response.

REVERSE GENETICS

Reverse genetics is a system allowing the de novo creation of RNA viruses from cloned cDNA. Reverse genetics has greatly aided the field of virology, with many successful systems in various genera of RNA viruses. Influenza virus has one of the better known reverse genetics systems (RGS) and the first for a negative sense RNA virus. The most recent RGS contains 8 plasmids that have bidirectional transcription that results in synthesis of mRNA and vRNA. The early days of reverse genetics utilized helper viruses for creation of new viruses, then minireplicons were utilized. Minireplicons consisted of only transcribing and
translating the proteins necessary for viral replication and including all vRNA segments. While DNA based systems have worked for various viruses, there are incompatibility issues with hantavirus. DNA based systems contain promoters, either T7 or RNA pol I that initiate transcription and these results in a uniform start with a 5’ purine (G or A) tri-phosphate nucleoside. Transcription termination results in a pyrimidine (C or U). Hantaviruses contain a 5’ mono-phosphorylated uridine and end with an A on the 3’ end with a hydroxyl group. The termini of the hantavirus genome segments are important for initiation of transcription by the viral derived RdRp. There are inverted repeats found on the 5’ and 3’ ends of the genome segments that are complementary, and that result in hydrogen bonding to form a panhandle structure of the ends in the viral RNA. This panhandle structure and termini serve as a promoter for transcription for the RdRp. In addition, having these unique chemical moieties on the genome segments may assist in immune evasion. Pathogen recognition receptors in the host cell recognize foreign RNAs and hantaviruses can avoid recognition through their unique termini. Some DNA-based reverse genetic systems have utilized a ribozyme to obtain a specific genome segment end in transcribed RNA, but ribozymes leave a hydroxyl group on the 5’ end and a 2’3’ cyclic phosphate at the 3’ end, which does not mimic hantavirus genome segment ends. Our system for generating correct chemical moieties includes the in use of a hammerhead ribozyme, restriction enzyme site, and T4 polynucleotide kinase. Treatment of DNA with restriction enzyme and then T7 transcription will give run-off transcription, leaving a hydroxylated adenosine. The hammerhead ribozyme allows cleavage of the synthesized RNA at the uridine
residue on the 5’ end of the RNA, leaving a hydroxyl group. Subsequence
treatment with a T4 polynucleotide kinase will phosphorylate this residue, giving a
monophosphorylated uridine. This will allow for in-vitro transcription of full-length
hantavirus genome segments with correct chemical moieties.

Production of the nucleocapsid, two glycoproteins, and RNA-dependent-RNA-
polymerase will allow for the proteins to interact with the genome segments,
undergoing transcription, translation, and replication. The hope is that this will
produce infectious virus. Creation of DNA vectors for production of viral proteins
will also allow their use for studies related to gene expression relating to expression
of individual or combined proteins in primary site of replication, human pulmonary
lung endothelial cells. The expression can then be compared to whole virus
infection, giving the scientific community better understanding of how the proteins
contribute to the pathology of the virus.

**RNA-SEQ**

Recent times have seen a great increase of the use of RNA-Seq technology to
determine transcriptomics of organisms. The increasing popularity and decreasing
cost have contributed to a great understanding of genetics. The process for RNA-
seq includes isolating and purifying total RNA from virus-infected or plasmid-
nucleofected cells. For analysis of gene expression, polyadenylation enrichment of
mRNA is used. In other cases, ribosomal RNA deletion is used, if the experimental
design is to sequence all RNAs, not just mRNAs. The RNA sample is digested to give
short RNA segments, in a process termed fragmentation. The small fragments are then converted to cDNA through reverse transcription. Adapters are added to the ends of the cDNA fragments in order to label the cDNAs from individual samples. This allows multiplexing, or running multiple samples in the same lane for sequencing. As RNA-seq technology has become more common, sequencing depth has improved, allowing multiple samples to be run in the same lane while still retaining extensive coverage of the genome, while being cost effective. All the cDNA segments are then sequenced. The sequencing data is then aligned to a reference genome and the frequency of sequenced cDNA fragments is ranked and the expression of mRNA at the point in time that the RNA was isolated can be determined.  

RNA-seq is replacing microarrays gradually for analyzing gene expression for biological samples. It has various advantages over microarray analysis. RNA-seq provides better reproducibility, allows for detection of novel transcripts, better detection and quantification of rare transcripts, and detection and quantification of splice variants.
**Figure 4. RNA-sequencing overview.** Figure taken from Wang et al.

RNA-sequencing begins with processing whole RNA from the cell. RNA is then fragmented in order to be compatible for deep sequencing. The whole RNA isolated via the Directzol kit by Zymo. RNA can be analyzed for quality via the Aligent Bioanalyzer. A quality score above 8 indicated high quality RNA. The whole RNA isolated from the cell must thus be fragmented into 200-500 bp fragments in order for deep sequencing.
In our protocol, RNA fragmentation is done by RNase III treatment. After fragmentation, the RNA can be purified for polyA tails but this is not necessary and not all transcripts have a polyA tail. Also rRNA depletion kits can be used to reduce the amount of ribosomal RNA in the sample. After fragmentation, the Bioanalyzer can then be used to assess the quality of the RNA fragments. After fragmentation, hybridization and ligation of the RNA with adapters is done in order for it to be sequenced. The cDNA is then sequenced. These small reads are then processed to trim the ends of the ligation sequences and these small reads are aligned to a reference genome (in this case the human genome). Data is then processed in a BAM file and give back to us for use in data analysis.
CHAPTER 2: DEVELOPMENT OF A SIN NOMBRE VIRUS REVERSE GENETICS SYSTEM

INTRODUCTION
New World hantaviruses are emerging zoonotic viruses that cause disease with high mortality rates, ranging from 35 to 50% for both Sin Nombre Virus (SNV) and Andes Virus (ANDV), the prototype North and South American hantaviruses respectively. Hantaviruses are transmitted through aerosolization of rodent excreta. Additionally, in South America, cases of human-to-human transmission have been recorded with ANDV. SNV and ANDV cause hantavirus pulmonary syndrome (HPS), due to infection of endothelial cells and subsequent capillary leakage. The ease of aerosolization leads to potential for use as a bioterrorism agent or bioweapon, combined with the high virus-associated mortality rate and lack of viable therapeutics, provide impetus to develop countermeasures against hantaviruses. The NIH has designated the hantaviruses as Category A agents.

In order to gain a greater understanding of hantaviruses, we attempted to develop a novel reverse genetics system (RGS). Hantaviruses comprise a genus in the *Bunyaviridae* family, and have a negative sense tripartite RNA genome. A RGS has been successfully constructed for several other negative sense segmented viruses in the bunya-, arena-, and orthomyxovirus families, among others. This has greatly facilitated our understanding of replication, protein expression and
mechanisms of pathogenesis of these viruses. A prime example of how a viable RGS has facilitated virus analysis is that of influenza virus, a member of the *Orthomyxovirus* family. Development of a DNA-based RGS for influenza has made it possible, through creation of mutant and reporter viruses, to define the function of individual viral proteins and the regulatory regions of the RNA\(^{44,56}\). Availability of an RGS has facilitated a greater understanding of viral factors contributing to the lethality of 1918 Spanish flu (H1N1)\(^{57,58}\). In another example, a successful DNA-based RGS has been developed for the orthobunyavirus, Schmallenberg virus, another member of the bunyavirus family\(^{53}\). To date, no reliable RGS has been successfully constructed for the hantavirus genus. We hypothesized that this might be due to incompatibility of a DNA-based RGS with hantavirus replication. The genome segments of SNV and ANDV contain termini with unique chemical moieties that potentially play a important roles in replication (Figure 5). These specific chemical moieties likely also contribute to immune evasion of the virus during host cell infection. Each of the three hantavirus genome segments contain a 5’ monophosphorylated uridine and a 3’ adenosine with a hydroxyl group. DNA-based systems create termini that end in nucleosides and nucleotides that differ from that of the virus (Figure 6). In addition, in DNA-based systems various strategies that have been used to create the correct terminal nucleotide or nucleosides
**Fig. 5. Hantavirus genome segments.** All hantaviruses have 3 genome segments in minus-sense orientation. The S segment encodes the nucleocapsid (N), and some viruses encode an additional nonstructural S peptide (NSs). The M segment encodes the envelope glycoproteins Gn/Gc, and the L segment encodes the RNA-dependent RNA polymerase (RdRp). Genes are shown in antisense orientation since the virus is minus stranded.
Figure 6. Incompatibilities with a DNA based RGS for hantavirus. Likely incompatibilities with hantavirus replication derived from DNA-based RGSs are shown in red. Transcription initiation by Pol I or T7 RNA polymerase, used in RGSs, uniformly starts with a 5’ purine (G or A) tri-phosphate nucleoside whereas the 5’ nucleotide of hantaviral RNA is a mono-phosphorylated U. Transcription termination at a Pol I terminator ends with a pyrimidine (C or U) whereas the terminal nucleoside of viral RNA is A. Ancillary intracellular RGS strategies that trim RNA using ribozymes generate a 5’ hydroxyl whereas hantavirus RNA contains a 5’ monophosphate. Ribozymes also leave an unusual 2’3’ cyclic phosphate at the 3’ end whereas authentic viral RNA has a 3’ hydroxyl.

RGS that allows exact formation of the unique chemical groups found on the termini of the naturally occurring vRNA and cRNA genome segments. This approach i
expressed RNA introduce chemical moieties that differ from that of the virus (Figure 5). To circumvent these problems, we attempted to develop an RNA-based system involved the in vitro synthesis of vRNA, modification to produce correct chemical moieties at the genome termini, and direct transfection of the vRNA and cRNA into cells. In addition to transfection of vRNA and cRNA (Figure 7), for translational expression of viral protein we synthesized viral mRNA from expression plasmids. Subsequently, we created DNA vectors for direct transfection for protein production. By co-transfecting with mRNA or DNA vectors in addition to the RNA segments containing correct terminal moieties, we hoped to create a tractable reverse genetic system for the production of infectious virions. Unfortunately, this system was unsuccessful in creating robust viral expression. We describe our attempts and hypothesize where these were unsuccessful.

MATERIALS AND METHODS

Plasmids used for in vitro synthesis of viral RNA

Plasmids expressing the entire viral M and L complementary segment RNA were generated by DNA synthesis (GenScript). The S segment was cloned from cDNA that was transcribed from vRNA isolated from SNV strain 77734 infected VeroE6 cells. Each of these plasmids expressed the viral c- or vRNA
Fig. 7. Synthesis of chemically correct hantavirus vRNA. The figure outlines the steps for generation of vRNA for transfection into cells and encapsidation into progeny virus particles. T7 indicates T7 promoter; HH indicates hammerhead ribozyme; circled P indicates a 5’ mono-phosphate. BsmBI is an example of a type I restriction enzyme that would cleave the DNA template at the nucleotide corresponding to the 3’ end of the vRNA.
from a T7 promoter sequence. The expressed synthetic RNA contained a hammerhead ribozyme sequence, full Sin Nombre virus strain 77734 segment sequence (S, M, or L), and a BsmB1 DNA restriction enzyme sequence. All DNA sequences encoding these pertinent RNA regions were cloned into the Eco RV site of pUC57. In some experiments, to generate additional template for in vitro synthesis using T7 RNA polymerase, primers containing terminal and flanking sequences used for PCR amplification of the region (See Table 1). In some of these experiments we amplified the M and L cRNA expression plasmids using primers that introduced T7 promoter, and hammerhead ribozyme sequences in the minus sense orientation to facilitate synthesis of vRNA rather than cRNA.

**Production of polynucleotide kinase treated vRNA and cRNA**

Vectors and purified PCR products were digested with BsmB1 for 3 hours at 55C. DNA was purified with the DNA clean and concentrator from Zymo Corporation. 1 ug of DNA was then used in the RiboMax T7 RNA synthesis kit from Promega and RNA was transcribed for 4 hours at 37C for the S and M vRNA and cRNA constructs and 30C for the L vRNA and cRNA constructs. After synthesis of full length RNA, RNA was ethanol precipitated following the addition of LiCl using a standard protocol. RNA was then measured via nanodrop and 5’ ends monophosphorylated with polynucleotide kinase T4 (3’ phosphatase minus) for 30 minutes. RNA was
### Primers for synthesis of vRNA DNA templates

<table>
<thead>
<tr>
<th>vRNA</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tr>
<td>SNV M vRNA</td>
<td>GCCGGGAATTTAATACGACTCCTATAGGAGCCGCTACTACTGATGAGTC CGTGCGCGGACGAAACGAGGTGACTCGTCTAGTAGTATGCTCCGACGAACAAA AAGCCTCGTAATAAGCAA-3’</td>
<td>SNV M vRNA</td>
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<tr>
<td>SNV L vRNA</td>
<td>GCCGGGAATTTAATACGACTCCTATAGGAGCCGCTACTACTGATGAGTC CGTGCGCGGACGAAACGAGGTGACTCGTCTAGTAGTATGCTCCGACGAACAAA AAGCCTCGTAATAAGCAA-3’</td>
<td>SNV L vRNA</td>
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| SNV L vRNA | REV 5’-CTACGTCTCATAGTAGTAGACTCCGAGATAGAGAAATTCAGGAAAAATGG-3’ |

**Table 1.** Primers for synthesis of vRNA from cRNA synthesis plasmids.
then ethanol precipitated again in the presence of LiCl. To verify that the RNA was full length, a portion of the RNA was analyzed on a formeldahyde denaturing agarose gel per standard protocol. In addition RNA was run on Bioanalyzer for further confirmation of size and concentration.

**Transfection**

RNA was transfected into VeroE6 cells using MirusBio TransIt-mRNA transfection reagent. DNA was transfected using MirusBio LT-1 transfection reagent. In experiments involving both RNA and DNA transfection, DNA was first transfected into cells, 24 hours later media was changed, and RNA was transfected into the cells. Following transfection cells were moved into an incubator at 37°C 5%CO2 in BSL-3 containment.

**Protein expression and detection**

VeroE6 cells in 12 well plates were transfected with the DNA expression constructs for synthesis of the nucleocapsid protein and the glycoprotein precursor. Cells were then incubated at 37°C and 5% CO2 for 48 hours. After 48 hours, media was removed, cells were washed with PBS and 200 uL cell lysis buffer supplemented with protease inhibitor were added to each well. After a 10 minute incubation on ice, a cell scaper was used to recover the cell lysis solution. Samples were suspended in 4x Lammeli buffer with BME and 20 ul of each was loaded on a 12% Tris-Glycine gel. Gels were run for 1 hour at 120 volts. Western blots were then generated by transferring protein to a PVDF membrane and stained with either anti-N (Sigma) antibody or
anti-Gc (Sigma) at 1:1000 in TBS-T with 5% BSA or anti-G2 (Sigma) antibody at 1:1000 in TBST with 5% BSA for 1 hour. Membranes were then washed 3X for 15 minutes. Secondary HRP conjugated antibody (goat anti-rabbit, Biorad) was then diluted 1:5000 in TBST with 5% BSA and incubated for 1 hour at room temperature. After 3X for 15 minute washes with TBST, blots were imaged using Protein Simple device with chemiluminescense reagent (Biorad).

**Quantitative PCR**

Media was removed from VeroE6 cells and cells were washed with sterile PBS. 200 uL of Trizol was added to each well of the 12 well plates. After 5 minutes, lysates were collected. After removal from BSL-3, RNA was isolated using the Direct-zol RNA isolation kit from Zymo using their suggested protocol. 8 uL of RNA was then used for cDNA synthesis. Reactions were incubated at 48C for 45 minutes and 5 uL of each cDNA was then utilized with Power SYBR green mix (Thermo Scientific) in a 20 uL reaction with primers corresponding to the S, M, and L vRNA segments of Sin Nombre Virus. Relative expression differences were calculated by determining ΔΔCt. Statistical verification of the results were done with standard student’s t-test.

**RESULTS**

As discussed in the Introduction, the termini of SNV RNA contain nucleotides and chemical modifications difficult to recapitulate using DNA transfection. Consequently, we attempted to generate an SNV reverse genetic system that starting with creating synthetic viral RNA in-vitro rather than transfecting DNA to produce
RNA intracellularly. This allows modification of the RNA termini prior to transfection. We attempted a system using various combinations of vRNA, cRNA, mRNA, and DNA vectors for protein production. As the ORFs for the viral proteins are found on the cRNA, proteins could potentially be translated from merely transfecting cRNA. In addition, we also created mRNA and DNA vectors for viral protein production. Potentially, transfection with either vRNA or complementary (cRNA) could be used to initiate a reverse genetic system. cRNA may be preferable as the vRNA has the potential to bind to the mRNA used for protein production, creating a double stranded structure, possibly eliciting an immune response. As described below, we constructed plasmids containing synthetic SNV sequences that could be used for direct expression of cRNA, and also used these plasmids as template for PCR amplification to generate DNAs amenable for expression of vRNA.

Plasmids for cRNA expression contained a promoter for T7 transcription, a hammerhead ribozyme sequence, and restriction enzyme site for BsmB1 to enable cleavage so that transcription would terminate with 3’ nucleotides found in the viral genome (Figure 2). These synthetic sequences were confirmed by DNA sequencing, the plasmid was digested with BsmB1, and transcribed with T7 polymerase. This resulted in production of a hammerhead ribozyme at the 5’ end of the cRNA, with higher order structure that should form correctly under conditions of transcription 59. Hammerhead ribozyme cleavage would occur at nucleotide C17 relative to Stem II of the RNA secondard structure (Figure 8). This nucleotide is a uridine residue.
**Figure 8. Hammerhead ribozyme structure and cleavage site.** RNA sequence of hammerhead ribozyme and 5’ end of SNV L cRNA shown. Secondary structure forms under transcription conditions and cleaves the SNV L cRNA at nucleotide shown. Hammerhead ribozyme sequence then falls off SNV L cRNA, leaving a hydroxylate uridine residue on the 5’ end of the cRNA.
Transcription runoff would be expected to result in a hydroxylated adenosine on the 3’ end of the RNA. After transcription, the cRNA segments were treated with a T4 polynucleotide kinase to monophosphorylate the terminal 5’ uridine residue. RNAs treated in this way would now contain both the correct chemical moieties on the termini and the correct viral genome sequence of Sin Nombre Virus. The resulting cRNA was visualized on a formaldehyde-containing agarose gel. (Figure 9).

To create vRNA, primers were designed to amplify the DNA complementary strand with the addition of the hammerhead ribozyme, T7 promoter and BsmB1 restriction enzyme site (Table 1). The resulting PCR product would contain the same features as the cRNA expression construct flanking vRNA. Transcription of the resultant PCR products and subsequent treatment with T4 polynucleotide kinase allows for production of vRNA that mimics wild-type virus vRNA. vRNA was visualized on a foldmaldehyde-containing agarose gel to determine correct size (Figure 9).

Although synthetic cRNA contains the viral genes in sense orientation, it seemed likely that viral protein expression from these RNAs would be inefficient owing to lack of a 5’ cap and 3’ regulatory sequences including a polyA tail. Thus, we also constructed plasmids that could be used for mRNA and protein expression. In order to create mRNA, the open reading frames for the nucleocapsid, glycoprotein polypeptide precursor and the RNA-dependent-RNA-polymerase were cloned into an expression construct provided from the lab of Dr. Drew Weissman at University of Pennsylvania. Transcription with a T7 polymerase and cap analog (NEB kit)
Figure 9. SNV mRNAs, cRNA, and vRNAs. A. ~2 ug mRNA was transcribed from plasmids containing the genes for protein synthesis in addition to a T7 promoter, polyA sequence, and Kozak sequence. SNV N and SNV GPC plasmids were digested with EcoRV and SNV RdRp with ScaI to give linearized plasmids. Transcripts were additionally poly-adenylated and run on a 1% formaldehyde denaturing gel. 2 log ladder represents DNA migration rates and will run slower than ssRNA segments. B. ~2 ug cRNA synthesized from DNA plasmids cut with
cRNA was run on a 1% denaturing formaldehyde-agarose gel. 2 log ladder represents DNA migration rates and will run slower than ssRNA segments. C. ~2 ug vRNA was synthesized from PCR products digested with BsmB1 and transcribed using T7 polymerase. vRNA was run on 1% denaturing formaldehyde-agarose gel. 2 log ladder represents DNA migration rates and will run slower than ssRNA segments.
created full sized capped and poly-adenylated transcripts. mRNA transcripts were visualized on a formeldahyde containing agarose gel (Figure 5). The genes for the SNV proteins were also cloned into pTriEx1.1 vector that contains a CAG mammalian promoter and terminator, which allows for transcription and translation of the proteins by mammalian cells. We had both mRNA and DNA plasmids for protein production to test for the reverse genetics system. Production of proteins was tested by transfecting DNA vectors into VeroE6 cells and probing with anti-N and anti-Gn, and anti-Gc antibodies via Western blot (Figure 10). There is no antibody for the SNV polymerase, so expression was not tested outside of the test for viral production. Due to other groups creating bunyavirus reverse genetics system having issues with full length polymerase production, we created a human codon optimized sequence for production of the RdRp60. This codon optimized gene was then cloned into pTriEx1.1. The following attempts at creating a reverse genetics system were completed.

**cRNA only transfection**

We hypothesized that cRNA transfection of the S, M, and L SNV segments may result in transcription of mRNA and translation by the host cell as the cRNA segments contain the gene sequences in sense orientation. These proteins would subsequently interact with the transfected cRNA, resulting in viral replication. VeroE6 cells were transfected with the S, M, and L cRNAs either together (1 ug total RNA transfected, 333.3 ng each transcript), S supplemented with control RNA
Figure 10. Western blot of N expression in VeroE6 cells. An empty vector, his-tagged N vector, GFP fused N vector, N vector, and N-NSS- vector were transfected in VeroE6 cells. 20 uL of each well were run on a Tris-HCL Page gel. Proteins were transferred to a PVDF membrane and blocked with 5% BSA. N protein was probed for with 1:1000 dilution using Sigma anti-N. B-actin was probed for at a 1:400 dilution using Applie biosciences anti-B-actin.
(333.3 ng S and 666.7 ng control RNA), and control RNA (1 ug). After 48 hours, cells were washed with PBS and harvested with Trizol and RNA was purified using a spin column. RT-PCR with S specific primers allowed for quantification of S segments. Initially, transfection with a three PNK treated cRNAs resulted in a significant increase of S segments in the well that was transfected with S,M,and L genome segments as compared to the well only transfected with S cRNA and control RNA. Subsequence passages with supernatant resulted in a drastically reduced copy number of S segments, leading to the conclusion that there is no SNV production, rather dilution of the original RNA transfected into the VeroE6 cells. (Figure 11)

**cRNA with mRNA transfection**

In order to ensure protein production, we cloned the three genes for the viral proteins (N, GPC, and RdRp) into an mRNA synthesis vector that contains a T7 promoter, kozak, and polyA sequence. RNA synthesis yielded capped, polyadenylated mRNA transcripts that were visualized on a formaldehyde containing agarose gel (Figure 8). Transfection of combinations of 3 mRNAs and 3 cRNAs was preformed using VeroE6 cells in the following way: 1. SNV S cRNA and control RNA and control mRNA (166.7 ng S RNA, 833.3 ng control RNA) 2. SNV S, M, and L cRNA and N, GPC, and RdPr mRNAs (166.7 ng of each RNA) 3. Control RNA and control mRNA (500 ng of each) 24 hours post transfection, media was changed in order to remove the possibility of RNA carry over during subsequent passages. 48 hours post transfection, 25 uL of supernatant was transferred to VeroE6 cells
Figure 11. cRNA transfection. cRNAs transcribed with a T7 polymerase were transfected into a 6 well plate. SNV S segments were transfected alone and SNV S, M, and L segments were transfected together to give ‘All’ category. After 48 hours, 3 mL of media from the All transfected well was added to a confluent flask of VeroE6 cells (Passage 1). After 48 hours, 3 mL of media from the flask was passaged to another confluent VeroE6 flask (Passage 2). Cells were then harvested, RNA isolated, and qPCR analysis done. Shown here are fold difference SNV S segments compared to no treatment wells and flasks.
seeded 24 hours before. Cells were harvested with Trizol and RNA purified using a spin column. RT-PCR was preformed using the primers specific to the S genome segment. Transfection with all three PNK treated cRNAs and mRNAs resulted in the same significant difference in S genome copy numbers in the initial transfection well as compared to the S cRNA and control RNA transfected well. Subsequent passage of supernatant showed no significant differences between these well. No viral replication is presumed to have occurred. (Figure 12)

cRNAs and vRNAs with mRNAs and longer passage times

The protocol for plaque assays from many research groups\textsuperscript{26,27} for Sin Nombre Virus includes leaving the virus on the cells for 7 days prior to staining for plaques. Growing virus also yields the highest titer at 7 day post-infection. Our reasoning with this experiment was to give the cells more time with potentially infectious virus from our reverse genetics system to replicate. This would give us the best chance to see if the reverse genetics system indeed produced infectious virus. We transfected the combinations of the mRNAs, cRNA, and vRNAs into VeroE6 cells. The following combinations were used: 1. SNV S cRNA with control RNA and control mRNA (166.7 ng S, 416.7 ng control RNA, and 416.7 ng control mRNA) 2. SNV S cRNA with control RNA and control mRNA 3. SNV S, M, and L cRNA with N, GPC, and RdRp mRNA (166.7 ng each RNA) 5. SNV S, M, and L vRNA with N, GPC, and RdRp mRNAs (166.7 ng each RNA). 4 days post transfection, media was changed and 7 days post transfection, 25 uL of supernatant was transferred to VeroE6 cells seeded 24 hours prior. Cells were then washed and harvested with
Figure 12. cRNA and mRNA transfection. mRNAs coding for the four viral proteins and then PNK-treated cRNAs and were transfected together to give ‘All’ category. Control included SNV S only cRNA. Cells were transfected. 24 hours later, media removed, cells washed with PBS and media replaced. 48 hours post transfection, 25 uL of media was passaged to a new confluent VeroE6 12 well plate. Same protocol as before for passage 2. Cell were harvested with Trizol and RNA isolated. qPCR data analysis results in fold difference of SNV S genome segments as compared to no treatment control.
Figure 13. cRNA and vRNA and mRNA transfection with increased time between passages. mRNAs coding for the four viral proteins and then PNK-treated cRNAs and vRNAs were transfected together to give ‘All’ category.

Control included SNV S only cRNA. Cells were transfected. 24 hours later, media removed, cells washed with PBS and media replaced. 48 hours post transfection, 25 uL of media was passaged to a new confluent VeroE6 12 well plate. Same protocol as before for passage 2. Cell were harvested with Trizol and RNA isolated. qPCR data analysis results in fold difference of SNV S genome segments as compared to no treatment control.
Trizol. RNA was purified using a spin column and RT-PCR was preformed using primers specific to the S segment. The results indicated that in the wells with all 6 segments transfected, there was a significant increase in number of S genome segments as compared to the S cRNA/vRNA and control RNA transfected well. Subsequent passaging resulted in exponentially decreased copy numbers of S, even with significant differences between control well and all transfected well. Because of the drastic reduction in overall segments numbers for all wells, we conclude that no viral replication took place. (Figure 13)

cRNA and vRNA with DNA vectors
Due to potentially poor protein production from the use of mRNAs, we created DNA vectors that will be transcribed and translated by mammalian cells. In order to account for replicating virus intracellularly, rather than virus in the supernatant, we devised a new protocol that includes seeding new cells with transfected and passaged cells. In addition, we used a codon-optimized RdRp vector in order to insure full length translation of the viral RNA-dependent-RNA-polymerase. A reverse genetics system established for Crimean Congo Hemorrhagic Fever Virus was successful due in part through the use of a codon-optimized RdRp vector\textsuperscript{60}. VeroE6 cells in a 12 well plate were transfected with DNA vectors. 24 hours post transfection, cells were washed and transfected with cRNA and incubated for 7 days with media change 4 days post RNA transfection. Combination include: 1. GPC, N, RdRp-co, S cRNA, M cRNA, L cRNA 2. GPC, N, S cRNA, M cRNA, L cRNA (333.3 ng each DNA vector, followed with 333.3 ng each RNA) 3. Control vector, S cRNA, M cRNA, L
cRNA (333.3 ng each RNA) 4. Control vector, S cRNA, control RNA. (333.3 ng and 666.7 ng control RNA) 5. Control vector, control RNA. (1 ug each) After 7 days incubation, cells were trypsinized and passaged using half transfected cell and half new VeroE6 cells. Non passaged cells were harvested with Trizol and purified using a spin column. RT-PCR was preformed using primers specific to the S genome segment. This protocol was repeated for passage 2. Transfection resulted in no significant differences between control wells and wells with all 3 DNA vectors and all 3 cRNA genome segments. Passaging resulted in a decrease in genome copy numbers and no significant differences between control wells and all 6 DNA/RNA transfected well. In addition, we passaged supenatant to VeroE6 seeded chamber slides and probed with anti-N antibodies to determine nucleocapsid production. We found no nucleocapsid production (data not shown). We conclude that no viral replication occurred but the overall number of S genome segments was higher than other attempts, leading us to conclude that this protocol of passaging cells rather than supernatant is the better route of creating a tractable reverse genetics system. (Figure 14)

**DISCUSSION**

In the series of attempts at creating a reverse genetics system for Sin Nombre Virus, the most promising was a sequential DNA transfection followed by an RNA transfection 24 hours later. This allows for expression of the viral proteins first and then addition of the cRNA for viral replication. Expression of the RNA-dependent-
Figure 14. **Codon optimized RdRp vector transfection.** Combined 1 ug of DNA vectors (pT-N, pT-GPC, pT-RdRp-co, and pTriEx) were transfected into 12 well plate of confluent VeroE6 cells in listed combinations. 24 hours post transfection, media was changed and 1 ug total of RNA was transfected (SNV S cRNA, SNV M cRNA, SNV L cRNA and TriEx RNA) in various combinations. Cells were then placed in BSL-3.
4 days post RNA transfection, media was changed. 8 days post RNA transfection, cells were trypsinized and passaged using half transfected cells and half VeroE6 cells. This passaging was repeated 8 days later to yield P2. RNA was harvested and probed with SNV S specific primers to yield relative expression via qPCR.
RNA-polymerase is vital for viral replication, leading to the impetus to create a codon optimized DNA vector. This allows for the full-length expression of the mRNA transcripts and full translation. We utilized a different protocol for the final experiment in order to account for any viral presence in the cells rather than just the supernatant. We found higher levels of the genome segments in these passage plates as compared to the plates where only the supernatant was passage. This may be due to the RNA being stably held in the cells for prolonged periods of time or potentially poor viral replication. Future directions include optimizing the ratios between viral genome segments and plasmids to insure that enough nucleocapsid is present to allow for transcription by the RNA-dependent-RNA-polymerase. The amount of RdRp plasmid may need to be reduced as well due to potential cytoxicity to the cells after transfection, resulting in poor conditions for viral replication.
CHAPTER 3: RNA-SEQ OF SIN NOMBRE VIRUS INFECTED HMVEC-Ls

INTRODUCTION

The pathology associated with hantavirus infection in endothelial cells is not well understood. Increased permeability of the endothelium induces capillary leakage, leading to edema of the lungs. Research has indicated certain pathways that contribute to the increased vascular permeability during infection and these include VEGFA signaling, bradykinin production through the kallikrein-kinin pathway, complement cascade, thrombocytopenia and platelet coagulation, matrix metalloproteinease production, and immunopathology. In order to determine a complete picture of what is happening transcriptionally during infection, we preformed RNA-seq on human pulmonary microvascular endothelial cells (HMVEC-Ls) infected with Sin Nombre Virus (SNV). Differentially expressed genes indicate various pathways of importance that relate to vascular permeability. In addition to permeability factors, we discovered immune response genes that are important for the antiviral response to SNV. Lastly, various long non-coding RNAs are greatly impacted during infection and we hypothesize a role these may play in viral replication and infection.

BACKGROUND
New World hantaviruses are the causative agent of hantavirus pulmonary syndrome, an acute illness that has a high mortality rate (40-80%)\textsuperscript{10}. Hantavirus pulmonary syndrome is caused by an increase in capillary permeability in the lungs, resulting in edema of the lungs\textsuperscript{6,61}. The exact mechanism for the increased permeability seen in the endothelial cells of the lung capillaries hasn’t been well elucidated. Various mechanisms have been proposed and we will review some of them and how our data support or differ from these findings. Endothelial cells are the primary site of hantavirus replication but infection of these cells is not cytolytic. Endothelial cell junctions include various proteins that form adherens junctions and tight junctions (Figure 15). The adherens junctions include VE-cadherin and PECAM1 but strong cell-to-cell contact is predominantly mediated by VE-cadherin. Scaffold proteins connect the cytoplasmic component of the proteins to the cytoskeleton of the endothelial cell. Tight junctions include the proteins JAM, claudins and occludins, and connexins. These are also connected to the cytoskeleton of the endothelial cell via scaffold proteins. These junction proteins contribute to homeostasis of the endothelium and allow for leukocyte transmigration in event of damage to endothelium layer. Changes in these proteins greatly influence the permeability of the endothelial cell layer.\textsuperscript{62} Viral replication in endothelial cells is not cytolytic so the increased permeability could result from pathway initiation in the cells and/or immunopathology\textsuperscript{10}. Some research groups have found an upregulation of VEGFA during infection with hantavirus, which results in a signaling cascade that internalizes and degrades VE-cadherin in the endothelial cell junctions
Figure 15. Normal endothelial cell-cell junction. Adherens junctions include the cell-to-cell adhesion molecules VE-cadherin and PECAM1. Tight junctions include claudins and occludins, JAMS, and connexins. These junctions maintain homeostasis of the endothelium and control cellular permeability. The extracellular matrix of the endothelium consists of collagen, proteoglycans, fibronectin, casein, and laminin and help to maintain homeostasis.
and subsequently induces increased vascular permeability\textsuperscript{26,27,32,33}. However, other research groups have attempted to replicate these data and found no significant difference in VEGFA and VE-cadherin levels during infection\textsuperscript{26,31}. This has led to the search for additional mechanisms for the increased permeability seen during infection. Activation of the kallikrein-kinin pathway has been implicated during infection. This pathway increases permeability by releasing bradykinin, a vasodilator\textsuperscript{26}. In addition, Andes virus, a New World hantavirus, has been shown to interact with platelets, leading to dysregulated platelet aggregation and coagulation, another path leading to increased vascular permeability\textsuperscript{63}. We hypothesize that hantavirus-induced increased permeability is a complex process involving many pathways. Our data here indicate that in our infection model, the most likely candidates for increased vascular permeability include upregulated L1CAM and SPARC expression, increased production of matrix metalloproteinases (MMPs), dysregulated signaling for platelet activation and aggregation, and increased TNF-α and leukocyte adhesion molecule expression.

**MATERIAL AND METHODS**

**Viral titer**

VeroE6 cells were seeded in 12 well plates at 80% confluency and incubated for 24 hours at 37°C and 5%CO₂. Cells were infected with 20 uL resuspended lyophilized Sin nombre virus (SNV) 77734 (courtesy of UTMB The World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). After 7 days of viral replication, supernatants were centrifuged to remove cellular debris. Amicon Ultra-15
centrifugal filter units (MilliporeSigma) were then used to concentrate viral supernatant. 20 ul of concentrated viral supernatant was harvested using Trizol and RNA extracted using Zymo Directzol-RNA MiniPrep Plus (Zymo corp). cDNA was created using GeneAmp reverse transcriptase reagents and SYBR green RT-PCR using PowerSYBR Green MasterMix (Thermo Scientific) was preformed to quantify cDNA. DNA standard curves were created for all three genome segments to allow for exact quantification. We utilized the measurement of M segments in the supernatant as representative of physical virus titer. As qPCR gives a higher physical titer than does biological titer due to quantification of non-infectious virus, we did an immunofluorescence test to determine if our MOI dilutions were accurate. Cells were infected with MOIs of 1 and 0.1 and cells were fixed 24 hours later and permeabilized with 0.1% Triton-X, then treated with an anti-SNV N antibody (MilliporeSigma) and counterstained with AlexaFluor 488 conjugated anti-rabbit (ThermoScientific). N-positive cells were quantified using a confocal microscope and approximate biological MOIs were calculated.

**HMVEC-L infection**

Passage 5 HMVEC-Ls were seeded for 80% confluence in 6 well plates in EGM-2MV media (Lonza) and incubated at 5% CO2 and 37C for 24 hours. Cells were infected with MOI 0.5 SNV 77734 and MOI 0.05 SNV 77734. Negative control cells were mock-infected with concentrated control MEM media. Each infection was done in replicates of three. 48 hours post infection, media was removed, cells were washed with sterile PBS and RNA extracted from cells with Trizol. RNA was isolated using
Directzol RNA kit (Zymo Corp.) and eluted in 50 μL ultra-pure water. RNA Integrity Number (RIN) number was then determined via Bioanalyzer.

**RNA-Sequencing**

Novogene prepared libraries from RNA samples using NEBNext Ultra Directional RNA Library Prep Kit for Illumina. RNA was sequenced using HiSeq X. PE150 55M Raw reads sequencing was preformed and data was screened to remove incomplete sequences and adapter sequences. Files were returned in FastQ format.

**RNA-Seq Data Analysis**

FastQ files were assessed for quality using FASTQC. Reads were aligned to Homo_sapiens.GRCh38.78 chromosomes 1-22, X,Y,M plus SNV genome using STAR aligner (version 2.5.2). Expression values for cellular and viral genes were calculated using RSEM (version 1.2.31). EBSeq was performed to determine differential expression of host genes in SNV-infected cells. RSEM (RNA-Seq by Expectation-Maximization) results in quantification of transcript quantification through normalization of expected read counts. RSEM uses an Expectation-Maximization (EM) algorithm to estimate the maximum likelihood expression levels using predictive distributions. These transcript quantifications are then used in EBSeq to determine differential gene expression. EBSeq is a parametric distribution model that evaluates posterior probabilities associated with differential expression (DE) and equal expression (EE). Prior to evaluating differential gene expression, a predicted distribution of data is generated and then after, after data acquisition, a
real distribution is determined. These values then are used to evaluate posterior probability. Based on this analysis, a cutoff value of 5% false discovery rate (FDR) was used for inclusion in data composed of significant equal and differential gene expression (i.e. values with 0 to 0.05 posterior probability of equal expression (PPEE) were included and values with 0.95 to 1 posterior probability of differential expression were included (PPDE). FDR is a statistical method of having greater power but increased incidence of Type I errors (incorrect rejection of a true null hypothesis).126

RESULTS

Based on the results of our RNA-seq analysis, we decided to focus our pathway analysis on data from the MOI 0.05 infections as these data resulted in better differential gene expression. The MOI 0.5 infection condition may have been too high a viral burden as many cell death protein transcripts were upregulated. Figures 16 and 17 show all the genes differentially expressed in alphabetical order in the MOI 0.05 infection condition. We used a cutoff of equal to or larger than 1.5 fold increased and equal to or larger than -1.5 fold decrease. In certain cases, genes not within that cutoff range were included in our analysis and discussion if they shared great relevant biological significance with genes within the cutoff. Here we discuss the various pathways we think are important in infection of HMVEC-Ls with Sin Nombre Virus.

VEGFA and VE-cadherin
Figure 16. First 300 differentially regulated genes of MOI 0.05 infected

**HMVEC-Ls.** Monolayers of HMVEC-Ls were infected with MOI 0.05 of Sin Nombre Virus. RNA was isolated and sequenced using RNA-seq. Statistics were preformed to discover differentially expressed genes as compared to control (uninfected). Genes >1.5 fold and <−1.5 fold changed were included in heatmap representation. Scales are shown in log₂ fold change. Subsequent RNA-seq data figures will have a log₂ fold change scale.
**Figure 17. Remainder differentially expressed genes MOI 0.05 infected**

**HMVEC-Ls.** Monolayers of HMVEC-Ls were infected with MOI 0.05 of Sin Nombre Virus. RNA was isolated and sequenced using RNA-seq. Statistics were preformed to discover differentially expressed genes as compared to control (uninfected). (a) Genes >1.5 fold and <-1.5 fold changed were included in heatmap representation. (b) Genes beyond >48 and <-48 were included in separate heatmap in order to not skew heatmaps.
Previous studies have shown that hantavirus infection results in increased expression of VEGFA and through binding to VEGFR2, causes internalization and degradation of VE-cadherin, an important cell-to-cell junction protein\textsuperscript{26,27,32,33}. Loss of VE-cadherin would cause increased vascular permeability in the capillaries as the junctions between endothelial cells are loosened\textsuperscript{64}. However, as noted previously, other research groups have found no change in the expression of VEGFA and VE-cadherin in hantavirus infected endothelial cells\textsuperscript{26,31}. Our RNA-seq study similarly showed no significant change in transcriptional expression of VEGFA and VE-cadherin in either of the infection conditions relative to the mock-infected control HMVEC-Ls. Our data does indicate an upregulation of cell adhesion molecules that bind to platelets, monocytes, and T-cells, other sources of VEGFA production\textsuperscript{65-68}. Therefore, we cannot conclude that VEFA is not a source of vascular permeability during SNV infection, but rather that infected endothelial cells are not the source of increased VEGFA production. (Figure 18)

**Kallikrein-kinin pathway genes**

Taylor et. al. reported activation of the kallikrein-kinin pathway during infection with Andes Virus in which they used a 3D model of HMVEC-Ls\textsuperscript{26}. Factor XII is activated to initiate the signaling cascade resulting in bradykinin being released from high molecular weight kinongen(HK)\textsuperscript{34}. Our data indicate no expression of HK transcriptionally (KNG1). In addition, there were no significant differences seen between control and infection for the bradykinin receptors (BDKRB1 and BDKRB2) and the only tissue kallikrein present in our cells (KLK6). We found a significant
Figure 18. VEGFA and VE-cadherin transcript levels during Sin Nombre Virus infection. Transcripts per million, a quantification normalized for gene length, was determined for each MOI condition (0.05 and 0.5). (a) Counts were normalized for VEGFA expression in two infection conditions and control. (b) Counts were normalized for VE-cadherin in two infection conditions and control. There was no significant difference between control and infected conditions for both VEGFA and VE-cadherin.
(c) Counts were normalized for Bradykinin-1 receptor expression in two infection conditions and control. (d) Counts were normalized for Bradykinin-2 receptor expression in two infection conditions and control. (e) Counts were normalized for bradykinin expression in two infection conditions and control.
Figure 19. Kallikrein-kinin system for production of bradykinin. No significant differences for the main players in the kallirein-kinin synthesis. (a) Counts were normalized for Factor XII expression in two infection conditions and control. Factor XII was significantly downregulated in MOI 0.05 condition. (b) Counts were normalized for Kallikrein 6 expression in two infection conditions and control.
downregulation between control and MOI 0.05 for the Factor XII transcript, which would result in decreased Factor XII in infected cells. Due to this fact and lack of bradykinin seen in our system, we conclude that this pathway likely is unlikely to be important for increased permeability seen during infection with SNV using HMVEC-Ls. (Figure 19)

SPARC and VCAM1

SPARC (secreted protein acidic and rich in cysteine) is an extracellular matrix protein that regulates interactions between cells and their surrounding extracellular matrix\textsuperscript{69}. Over expression of SPARC in endothelial cells causes rounding of the cells and increased permeability\textsuperscript{70}. SPARC causes increased vascular permeability through its effect on VCAM1. SPARC binds VCAM1, resulting in a signaling cascade mediated by p38 and MAPK. The effects of this signaling cascade include actin skeleton rearrangement of the endothelial cells and loosening of cell-to-cell junctions.\textsuperscript{71} Infection by SNV by HMVEC-Ls resulted in a upregulation of both SPARC and VCAM1 (Figure 20). Therefore, we conclude that SPARC and VCAM1 potentially have a role in the increased permeability of endothelial cells during SNV infection. In addition to its direct effect on vascular permeability, SPARC also modifies the expression of matrix metalloproteinases, which also have a role in regulating vascular permeability\textsuperscript{72}. This is further described in the following section.

Matrix Metalloproteinases
Figure 20. Permeability genes affected during MOI 0.05 SNV infection. MOI 0.05 SNV infection group had genes related to endothelial cell permeability up or downregulated.
Andes virus and dengue infected dendritic cells secrete matrix metalloproteinases (MMPs) and these proteins are implicated in the increased vascular permeability of infection\textsuperscript{73,74}. Metalloproteinases are responsible for degradation of extracellular matrix components of the endothelium, leading to increased permeability. In our data, two MMP genes are upregulated (MMP10 and MMP28) during infection and one (MMP1) is downregulated (Figure 21). MMP10 is responsible for the degradation of proteoglycans and fibronectin. MMP28 is responsible for the degradation of casein.\textsuperscript{75} Thus, we think that upregulation of MMP10 and MMP28 contribute directly to increased vascular permeability. MMP1 is responsible for degradation of collagen. While downregulation of MMP1 is superficially consistent with unchanged or decreased permeability, we hypothesize that the downregulation of MMP1 correlates with increased platelet activating and pro aggregation genes found upregulated in our data set since sub-endothelial collagen functions as an important initiator of platelet coagulation\textsuperscript{76} (discussed further below). This idea is further consistent with the finding that endothelial cells infected with the Old World hantavirus, Pumuula virus, did not exhibit impaired platelet adhesion to collagen during thrombocytopenia\textsuperscript{77}. In sum, these data indicate that infected endothelial cells, as well as dendritic cells, are a source of secreted MMPs. These likely contribute to the degradation of junction components, increasing the vascular permeability of the endothelium during infection with SNV.

L1CAM and ADAM12 and DLL4
Figure 21. Matrix metalloproteinases significantly changed during infection. In the MOI 0.05 infection group, MMP1 was downregulated, MMP10 upregulated, and MMP28 upregulated. In the MOI 0.5 group, MMP10 was upregulated.
L1CAM functions in binding to the integrins of platelets and promoting coagulation\(^7^8\), L1CAM is also directly involved in increasing endothelial cell permeability. L1CAM is a cell adhesion molecule in the immunoglobulin superfamily. The protein has various interactions with other proteins including integrins and other Ig-CAMs. It contains an intracellular and extracellular domain. L1CAM can undergo cleavage mediated by the ADAM family of metalloproteinases and is further processed by γ-secretase, leaving an intracellular domain that translocates to the nucleus and acts as a transcription factor. (Figure 22) L1CAM is thus able to regulate the expression of certain genes. Some of the genes whose transcriptional expression can be modified by L1CAM have been found to be altered in our data (DLL4 and CD55, among others).\(^7^9\)-\(^8^1\) DLL4, involved in Notch signaling, was down regulated during infection and this could result in increased permeability as well as downregulation of Dll4 increases eNOS activation, resulting in increased production of NO, resulting in increased endothelial permeability\(^8^2,8^3\). Our data show a significant upregulation of both L1CAM and ADAM12 transcripts (Figure 6). In addition to the ability to cleave L1CAM, ADAM12 also cleaves the ectodomains of VE-cadherin, VCAM1, VEGFR-2, and TEK (angiopoietin-1 receptor)\(^8^4\). Loss of the ectodomains of these endothelial cell surface proteins directly induce increases in vascular permeability\(^6^2\).

In addition to the transcriptional regulation of the intracellular domain of L1CAM, L1CAM has additional significant potent effects on vascular permeability. L1CAM is expressed at low level in healthy endothelium but in various pathological
Figure 22. L1CAM functions as a transcription factor. The ADAM family of proteinases cleave the ectodomain of L1CAM and the intracellular domain is further processed by γ-secretase. The intracellular domain then translocates to the nucleus where it functions as a transcription factor for various genes.
conditions, including cancer, it is upregulated\textsuperscript{85,86}. Studies on over expression of L1CAM in healthy endothelial cells shows that L1CAM negatively regulates the recruitment of pericytes to the vascular wall, disrupts endothelial cell polarity, and promotes dysregulation of cell junctions\textsuperscript{87}. The cell junctions were found to be disorganized but with total level of PECAM-1 and VE-cadherin unchanged, consistent with our expression data. It was therefore concluded that L1CAM over expression in healthy endothelium induces increased vascular permeability by altering three different endothelial cellular processes. These effects were found to occur through the IL-6/JAK/STAT3 signaling pathway.\textsuperscript{87} L1CAM has a pleiotropic role in our infection model. It's ability to bind platelets, act as a transcription factor, and induce increased endothelial cell-to-cell junctions disorganization all contribute to increased permeability.

**Platelet activation, adhesion, and aggregation**

Multiple genes that are involved in platelet activation, attachment, and aggregation were upregulated or downregulated in our data set (Figure 23). This supports data from clinical settings where patients with hantavirus pulmonary syndrome have thrombocytopenia and increased platelet activation\textsuperscript{14,88}. In addition, other hemorrhagic fever viruses, including dengue fever and Crimean-Congo hemorrhagic fever virus, have a dysregulated platelet response to infection of the endothelium\textsuperscript{89-91}. The process of platelet activation and aggregation for clot formation on the endothelium surface involves disruption of the endothelial layer and exposure of the subendothelial matrix. Initial steps in the process include endothelial production of
Figure 23. Platelet activation, adhesion, and aggregation genes during infection. (a) vWF and ADAMTS13 had increased amount of normalized transcript reads per million during MOI 0.5 and MOI 0.05 respectively but these changes were not significant. (b) Significantly altered genes are shown in a heatmap with those downregulated on top and those upregulated on the bottom.
vWF, which binds directly to GP IB-IX-V complex on the platelet surface. vWF does not appear to be significantly increased in SNV-infected cells (Figure 9). ADAMTS13 releases vWF from endothelial cells and while we found an increase in ADAMTS13 transcripts in MOI 0.05, it was not significant. After capture of platelets by vww, platelets then interact with various types of collagen in the subendothelial matrix. MMP1, responsible for cleavage of collagen, was downregulated during infection (Figure 6), supporting the role of collagen in infected cells matrix in platelet aggregation. Research has shown the ability of hantavirus to bind platelets via the β3 integrins on the platelet surface and the endothelial surface. We saw no changes in expression of β3 integrins. However, ITGA8 and ITGA4 were elevated during infection, and both of these integrins are able to bind fibronectin, an important component of platelet clot formation and closely associated with collagen. Hantavirus infection leads to increase thrombin and fibrinolysis. We identified two plasminogen activator factors, PLAU and PLAT, upregulated in our data set (Figure 23). The following genes have an inhibitory role in platelet activation and aggregation: A2M, VASP, and MGLL. All of these genes were found to be downregulated during infection with SNV (Figure 23). Binding of platelets to the endothelium leads to the degranulation of platelets, releasing proteins that have various effects on the endothelium in relation to growth, permeability, and activation. We found dysregulation of many of the genes involved in recruitment and activation of platelets. Disruption of the endothelium by dysregulated aggregation of platelets has been shown to increase vascular permeability. Platelet aggregation and fibrinolysis could be a
contributing factor to increased vascular permeability of endothelial cells during SNV infection.

**Immune response**

Unsurprisingly, many interferon-induced genes are upregulated during infection with SNV (Figure 10). The most upregulated pathogen recognition receptors (PRRs) include IFI44L, IFIT1, MX1, MX2, RNASE6, and OASL. These PRRs are further upregulated with increased viral burden, as seen by the increase in expression in the MOI 0.5 data set, including additional PRRs (RSAD2, TLR3) (Figure 24).

Upregulation of these PRRs is consistent with that occurring following infection with other members of the bunyavirus family and previous hantavirus microarray data. OASL, OAS1, and OAS2 are 2’-5’-oligoadenylate synthetases. They are sensors for dsRNA and result in activation of RNASEL, causing degradation of viral RNA. MX1 and MX2 are GTP-binding proteins that antagonize the replication process of many viruses. IFIT1 binds RNA with a triphosphate 5’ group. IFI44 and IFI44L have been found to inhibit viral promoter activity of HIV and may function in a similar way following infection by other viruses. SOCS3 is a STAT-induced STAT inhibitor and negatively regulates cytokine signaling. RSAD2 is an ER-associated interferon induced protein that has been implicated in the antiviral response of various viruses. Various TNF-α induced genes are upregulated in our MOI 0.05 infection data set (Figure 25). TNF-α signaling directly causes changes in vascular endothelial permeability and our endothelial data set shows a
Figure 24. Immune response to infection. (a) Various genes related to the immune response were found to upregulated and one to be downregulated. (b) These fold changes were greatly increased with increasing viral burden. Interferon stimulated genes that were present in both MOI 0.05 and MOI 0.5 groups are displayed, showing increase with increased viral burden.
Figure 25. TNF-α related genes and leukocyte adhesion genes. (a) Various TNF-α induced genes were found to be significantly upregulated in our MOI 0.05 infection group. (b) 2 leukocyte adhesion genes were significantly upregulated in our MOI 0.05 group and 2 were downregulated.
sensitization of the cells to TNF-α. (Figure 25) This may be an additional mechanism of increased permeability by the cytokine TNF-α. Leukocyte adhesion molecules were found to upregulated in infected HMVECs, including VCAM1 and TNFSF18 (Figure 25). Transendothelial migration is a source of increased vascular permeability as binding of leukocytes to their adhesion molecules results in a signaling cascade mediated by RHO GTPases that in turn results in cytoskeletal rearrangement of endothelial cell junctions, allowing migration of leukocytes. In addition to rearrangement of the cytoskeleton, this signaling cascade also upregulates the expression of MMPs, discussed above, facilitating transendothelial migration. Surprisingly, E-selectin (SELE), a leukocyte adhesion molecule, was found to downregulated, contrary to other bunyaviruses (Crimean Congo Hemorrhagic fever virus and Andes virus) where patients were found to have an increased amount of SELE during infection. This may be a specific immune evasion mechanism of SNV.

**lncRNA, antisense transcripts, and run through transcription**

lncRNAs are long non-coding transcripts longer than 200 nucleotides. Their name notwithstanding, it has recently been discovered that lncRNAs do indeed code for proteins. lncRNAs have can modulate the immune response to viral infection. NEAT1, a well characterized lncRNA, has been associated with an antiviral effect to Hantaan virus, an Old World Hantavirus. Our data indicates various lncRNAs that are significantly either upregulated or downregulated in response to SNV infection, one as much as 32 fold downregulated (RP11-641D5.1) (Table 2). 

The
Table 2. Expression of IncRNAs, antisense RNAs, and run through transcripts during MOI 0.05 SNV infection. Various unknown function long non-coding RNAs were altered during infection. Antisense and run through transcripts of unknown function were also altered during MOI 0.05 SNV infection of HMVECs.
IncRNAs found in our data set that are differentially expressed currently have no known function but are intriguing as they might have a role in the antiviral response to Sin Nombre Virus. In addition to IncRNAs, we also noted changes in the expression of various antisense and run through transcripts of unknown function. CORO7-PAM16 is downregulated 120 fold during infection. (Table 2) The gene CORO7 is involved in Golgi apparatus morphology and the PAM16 gene is involved in mitochondrial matrix protein transport. The fusion protein encoded by this gene shares similarities with both individual proteins and the function of the peptide is believed to be Golgi maintenance and Golgi protein export. As research progresses and these IncRNAs, antisense transcripts, and run through transcripts have better identified functions, we can look further into the role they play in Sin Nombre Virus infection.

DISCUSSION

Through the technology of RNA-seq, we were able to obtain a snapshot of what is happening in HMVEC-Ls during SNV infection transcriptionally. This aids in the understanding of how infection impacts endothelial cells. By analyzing differentially expressed genes, we were able to discover multiple pathways that likely contribute to increased permeability, and this information adds significantly to current mechanisms proposed in the hantavirus field. We also defined changes in the expression of immune response genes that recognize replicating virus, as well as changes in
expression RNA of unknown function, including lncRNA, antisense transcripts, and run-through transcripts. We propose a model for the increase in permeability of the capillary endothelium during SNV infection in which upregulation of the transmembrane protein L1CAM plays a key direct role causing disorganization of the cell-to-cell junctions. In addition to this direct role, the cytoplasmic domain of L1CAM is released through cleavage by ADAM12, resulting in altered transcription of other genes related to permeability. ADAM12 is upregulated during SNV infection, and cleaves the ectodomain of VE-cadherin in addition to L1CAM, loosening the cell-to-cell junctions. Upregulation of SPARC results in increased expression of MMPs contributing significantly to further disorganization of the endothelial cell gap junctions. Increased MMP expression of MMP10 and MMP28 increases the degradation of the extracellular matrix. (Figure 26) Decreased MMP1 expression would facilitate exposure of collagen for subsequent platelet adhesion and aggregation. Platelet adhesion genes were found to be upregulated and platelet cascade inhibitory genes were found to be downregulated, initiating coagulation on the endothelium surface and causing a decrease in vascular barrier function, increasing permeability. (Figure 27) Prior to this work, SPARC and L1CAM had previously never been implicated in Sin Nombre Virus infection. Both of these proteins are implicated in cancer and are currently targets for developing therapeutics. These therapeutics could potentially be used to treat hantavirus pulmonary syndrome. Future directions include performing phenotypic experiments involving L1CAM, SPARC, and MMPs to confirm these proteins are being expressed at a higher levels and influencing Sin Nombre Virus infection of
endothelial cells. Functional assays regarding the lncRNAs influenced in our infection model would also greatly facilitate understanding how they contribute to infection.
**Figure 26. Model of endothelial cell junction during Sin Nombre Virus Infection.**

Upregulation of the transmembrane protein L1CAM induces disorganization of the endothelial gap junction proteins (VE-cadherin and PECAM1). In addition, the cytoplasmic domain is released through cleavage from ADAM12, resulting in transcription of genes. ADAM12 was found to be upregulated, it also cleaves the ectodomain of VE-cadherin. Upregulation of SPARC results in expression of MMPs and disorganization of the endothelial cell gap junctions. MMP28 degrades casein in the extracellular matrix and MMP10 degrades proteoglycans and fibronectin in the extracellular matrix, both of these MMPs are upregulated during infection. MMP1 was found to be downregulated, it is responsible for cleavage of collagen.
Figure 27. Schematic of platelet recruitment and coagulation at site of Sin Nombre Virus infection in endothelium. Based on our data regarding up or downregulated genes pertaining to platelet activation, adhesion, and aggregation, this is a schematic representing what we hypothesize in happening in-vivo. Infection endothelial cells upregulates platelet adhesion factors and exposed collagen of the subendothelium are binding partner for circulating platelets, leading to aggregation and coagulation.
CHAPTER 4: RNA-SEQ OF HMVEC-Ls EXPRESSING SIN NOMBRE VIRUS PROTEIN

INTRODUCTION

Sin Nombre Virus (SNV) is a New World hantavirus that causes a disease called hantavirus pulmonary syndrome. The virus primarily infects endothelial cells and causes increased permeability in the capillaries of the lung inducing vascular leakage and edema of the lungs. Virus replication is not cytolytic in endothelial cells, thus capillary endothelial cell death is not the cause of increased permeability. As discussed in the previous chapter, RNA-sequencing of SNV infected endothelial cells resulted in altered expression of genes related to platelet activation, focal adhesion, disorganization of endothelial cell junctions, and immune response. In order to determine the contribution the individual viral proteins towards the pathology seen during infection with Sin Nombre Virus, we preformed RNA-sequencing on human lung primary microvascular endothelial cells (HMVEC-Ls) nucleofected with DNAs expressing individual SNV proteins.

BACKGROUND

Prior to this project involving expression of viral proteins, we preformed RNA-seq on HMVEC-Ls infected with Sin Nombre Virus (SNV) at two MOIs. An MOI of 0.05 yielded better differential gene expression and antiviral response whereas infection at an MOI of 0.5 produced higher level expression of genes associated with cell
death, likely indicating too high a viral burden. In the MOI 0.05 infection condition, we discovered pathways that are important to understanding the host response to virus infection. In terms of vascular permeability, we found two main genes (SPARC and L1CAM) that were upregulated and that have a significant effect on endothelial cell junctions, that could account for increased vessel permeability. In addition, many genes involved in platelet activation and the coagulation cascade were altered, indicating that coagulation on the endothelium surface may be contributing to increased permeability and thrombocytopenia seen during infection\textsuperscript{14,92}. The HMVEC-Ls were also found to have upregulated genes related to leukocyte recruitment and adhesion, matrix metalloproteinases (MMPs), and TNF-\(\alpha\) signaling. Leukocyte adhesion facilitates loosening of the cellular gaps for cellular transmigration\textsuperscript{62}. Infected cells also expressed genes indicative of sensitization to TNF-\(\alpha\), which directly increases vascular permeability\textsuperscript{36}. MMPs degrade the extracellular matrix, contributing to increased vascular permeability\textsuperscript{75}. In addition to the discovery of modified expression of genes related to vascular permeability, we observed upregulation of pathogen recognition receptors during SNV infection. Finally, we found that a large number of lncRNAs, antisense RNA, and run-through RNA transcripts were significantly altered in their expression during infection.

The results of this RNA-seq experiment lead us to look into the role of individual viral proteins and their contributions to these changes during infection. We used mammalian expression vectors containing some of the individual viral genes and nucelofected them into HMVEC-Ls. These included vectors for the viral
nucleocapsid (N), with a mutant non-structural protein (NSs), and viral glycoprotein precursor (GPC). Following translation, GPC is further cleaved into Gc and Gn\textsuperscript{22}. In this chapter, I show how these viral proteins are likely to contribute to the pathology seen during virus infection. The nucleocapsid (N) appears to be fairly innocuous expressed on its own. Interestingly, the SNV non-structural protein (NSs) appears to be a potent interferon antagonist and likely plays a role in inhibiting antigen presentation through the MHC I pathway. Glycoproteins Gc and Gn seem to be the viral proteins that influence the platelet activation and coagulation cascade.

In addition to their influence on platelets, Gc and Gn appear to be contributing factors in cytoskeleton rearrangement at the endothelial cell junctions through their influence on the myosin light chain kinase pathway. Finally, I will discuss the influence the viral proteins have on MMP expression and IncRNA, antisense RNA, and run-through RNA transcripts.

**MATERIALS AND METHODS**

**HMVEC-L nucleofection**

Passage 5 HMVEC-Ls (Lonza) were harvested from monolayers. 650,000 cells were mixed with 100 ul nucleofection reagent (Lonza) and 3 ug of plasmid (SNV N-NSs+, SNV N-NSs-, SNV GPC, SNV N/GPC, and control plasmid). See reverse genetics chapter for methods of plasmid creation and preparation. Cells were nucleofected using program FP-100 on a 4D Nucelofector (Lonza). Cells were then transferred to a 6 well plate and incubated at 37C at 5% CO2 in EGM-2 MV media (Lonza) for 48
hours. 70% of cells produced protein based on GFP conjugated SNV N reporter protein expression (data not shown). Media was removed, cells were washed with sterile PBS and RNA harvested using Trizol. RNA was purified using Directzol RNA Mini-prep Plus (Zymo Corp) and eluted in 100 uL of nuclease free H2O. RIN was determined using Bionalayzer.

**RNA-Seq**

Novogene prepared libraries from RNA samples using NEBNext Utra Directional RNA Library Prep Kit for Illumina. RNA was sequenced using HiSeq X. PE150 55M Raw reads sequencing was preformed and data was screened to remove incomplete sequences and adapter sequences. Files were returned in FastQ format.

**RNA-Seq Analysis**

FastQ were assessed for quality using FASTQC. Reads were aligned to Homo_sapiens.GRCh38.78 chromosomes 1-22, X,Y,M plus Sin Nombre Virus genome using STAR aligner (version 2.5.2). Expression values for cellular and viral genes were calculated using RSEM (version 1.2.31). EBSeq was preformed to determine differential expression. EBSeq uses an empirical Bayesian method, Negative Binomial model for differential gene expression. Genes/transcripts are called differentially expressed by EBSeq at a false-discovery-rate (FDR) of 5% or less.

**RESULTS**

**N-NSs+ expression**
**Interferon**

In addition to encoding the N protein, the SNV S segment potentially encodes a second protein (NSs). This putative NSs gene begins at start codon following the N start codon and in an alternative reading frame relative to N. In order to determine the function of this NSs protein, we created a mutant vector in which we introduced a stop codon near the beginning of the NSs sequence that retained the amino acid sequence of N through an alternative codon. (Figure 28) This results in nucleocapsid expression without NSs.

RNA-seq analysis of N expression in the absence of NSs from this N-NSs- construct, indicated no change in interferon gene expression as compared to control nucleofections with empty vector. The one pathway of interest for viral infection upregulated for N expression was phagosome processing, leading to antigen presentation (Figure 29a and 30). While these genes were below a 1.5 fold change, they are probably significant due to low PPEE (posterior probability that gene is equally expressed) and high PPDE (posterior probability that gene is differentially expressed)\textsuperscript{126}. Major histocompatibility complexes (MHC) function to display peptides derived from pathogens and display them to T-cells, and in the case of HLA-E, natural killer (NK) cells. MHC I molecules acquire their peptides in the endoplasmic reticulum of the host cell, with assistance from TAP proteins. MHC II molecules acquire their peptides from degradation of pathogen proteins in
Figure 28. Sin Nombre Virus N protein with NSs ORF. The sequence for the SNV N protein includes an alternative reading frame for a putative NSs protein. We created a stop codon at the beginning of protein sequence that does not affect the amino acid of the nucleocapsid protein in order to have a NSs KO expression vector.
endosomes. The genes that code for the MHC molecules are called human leukocyte antigens (HLA). MHC class I genes include HLA-A, -B, and -C. MHC class Ib genes include HLA-E. MHC class II genes include HLA-D. MHC class I is primarily associated with viral infection. HLA-A, B, C, D, E, and F, CANX, and TAPBP are (associated with TAP in antigen presentation) were upregulated with N-NSs-expression, indicating that N may be an important source for antigen presentation by infected endothelial cells of SNV. (Figure 29 and 30)

The effect of NSs expression

Interferon

Prior to our RNA-sequencing experiment, the role of the Sin Nombre Virus NSs protein was not known. Other members of the bunyavirus family code an NSs protein and this protein functions in different roles depending on virus. These roles include interferon antagonism, host cell protein synthesis shut down, and RNAi interference. The bunyaviruses Bunyawera and La Crosse have NSs proteins that are interferon antagonists. The NSs of La Crosse virus was also found to interfere with host cell RNAi. The NSs of Rift Valley Fever Virus is involved in inhibiting host mRNA transport from the nucleus. 39,40,43,128,129.

Our observations are consistent with an experimental model in HMVEC-Ls, in which the function of NSs is as an interferon antagonist. N expression (N in the absence of NSs) produced no significant change in interferon stimulated gene expression but in the presence of NSs (N-NSs +) many interferon stimulated genes were found to be
Figure 29. NSs interaction with antigen presentation pathways. (a) Expression of N protein without NSs resulted in upregulation of various HLA genes and TAP associated protein. CANX and HSPA8 are downregulated. (b) Expression of N protein with NSs resulted in downregulation of HLA-E, TAP 1 and 2, and IFI30, a protein associated with the MHC II response.
Figure 30. Antigen presentation pathway with highlighted genes altered due to N. Pathway adapted from KEGG pathways. Schematic shows process of antigen presentation to T-cells and NK cells via MHC I and II pathways. N with NSs expression in HMVEC-Ls is represented. Genes found to be downregulated are highlighted in blue and genes found to be upregulated are highlighted in red.
Interferon and immune response genes

![Heatmap displaying down or up regulation of genes related to interferon and immune signaling. Genes found downregulated are shown in blue and genes found upregulated are shown in red.]

**Figure 31. NSs augmentation of interferon and immune signaling.** Heatmap displaying down or up regulation of genes related to interferon and immune signaling. Genes found downregulated are shown in blue and genes found upregulated are shown in red.
downregulated (Figure 31). Many of these interferon-stimulated genes are part of the main antiviral response of cells. Important members include DDX58 or RIG-I, four members of the 2’5’-oligoandeylate synthase family of genes (OAS1, OAS2, OAS3, and OASL), and the influenza antiviral protein MX1\textsuperscript{130}. In addition, cytokine genes, antigen processing genes, and one of the main pathogen recognition receptors (TLR3), were found to be downregulated as well, further supporting the idea that NSs functions as an immunomodulator. (Figure 31)

In addition to downregulation of interferon-stimulated genes, NSs appeared to also significantly downregulate five genes closely related in MHC I antigen processing and one gene associated with MHC II antigen processing. (Figure 29b and 32) These five NSs-downregulated genes associated with MHC I are TAP 1 and 2, HLA-E, and NLRC5. NLRC5 is a positive transcription factor regulator for the MHC class I genes, HLA-A, HLA-B, HLA-C, TAP1, which are closely associated on chromosome 6\textsuperscript{131}. That NLRC5 is downregulated may indicate that NLRC5 interacts more proximally with NSs to cause downregulation of the other antigen processing genes associated with the MHC class I process. Other viruses, including Herpes Simplex Virus and Cytomegalovirus have mechanisms by which viral proteins interfere with antigen processing\textsuperscript{132,133}. Sin Nombre Virus may also participate in inhibiting antigen presentation through the viral protein NSs.

The effect of N-NSs+ and GPC coexpression
Figure 32. Antigen processing pathway with altered genes due to N and NSs.

Pathway adapted from KEGG pathways. Schematic shows process of antigen presentation to T-cells and NK cells via MHC I and II pathways. N without NSs expression in HMVEC-Ls in represented. Genes found to be downregulated are highlighted in blue and genes found to be upregulated are highlighted in red.
We expressed N-NSs+ and GPC together in order to determine the synergistic effect these proteins may have in the endothelial cell response. It was found that for the interferon antagonism of Andes Virus with the TBK1-dependent IFN responses, expression of both Andes glycoproteins and nucleocapsid were necessary\textsuperscript{134}

**eNOS activation pathway**

Expression of GPC resulted in HBA1 and HBA2, genes which code for hemoglobin, to be significantly downregulated in cells expressing N-NSs+ and GPC. (Figure 33 and 34) Hemoglobin produced by endothelial cells has been found to inactivate nitrous oxide (NO) produced by endothelial cells and that is present in the myoendothelial junctions\textsuperscript{135,136}. In the eNOS signaling pathway, various genes were altered by N-NSs+ and GPC expression. (Figure 5) NOS3 was found to be slightly downregulated but AKT3, HSP90 (HSP90AB1 and HSP90AB2P), and PI3K were upregulated, which would potentially increase the phosphorylation of eNOS and concomitantly increase production of NO\textsuperscript{137}. The NO released into the myoendothelial junction would then able to act upon the smooth muscle cells without becoming sequestered by endothelial produced hemoglobin\textsuperscript{135}. NO signaling between endothelial cells and smooth muscle cells results in vasodilation, which in turn increases vascular permeability\textsuperscript{138}. This hyperpermeability is amplified during an inflammatory response in the endothelial cells\textsuperscript{139}. The exact mechanism by which NO increases the permeability of endothelial cell junctions is not well understood but the effect on permeability is well documented\textsuperscript{137-139}. Some research implicates RHO GTPases and the actin cytoskeleton at endothelial junctions and other research is consistent with
**Figure 33. eNOS production in endothelial cells altered by N and GPC presence.** Pathway adapted from Ingenuity Pathway Analysis. Diagram shows endothelial cell and smooth muscle cell with extracellular space inbetween. Genes shown to be altered in our N and GPC expression data set are highlighted in pink. Genes colored red are found to be upregulated, genes colored in green and found to be downregulated. The higher the opacity, the larger the down or upregulation.
Figure 34. Hemoglobin expression in HMVECs with GPC and N expression.

Transcript levels of both HBA1 and HBA2 were found to be drastically downregulated during N and GPC expression.
increased VEGF sensitivity of the endothelial cell and subsequent changes in VE-cadherin\textsuperscript{140,141}. Increased NO synthesis during infection related to N-NSs+ and GPC protein expression may be associated with increased permeability seen during infection of endothelial cells with Sin Nombre Virus.

**Myosin light chain kinase and endothelial cell permeability**

Many of the processes that could account for increased permeability during hantavirus infection involve reorganization of actin cytoskeleton at the endothelial cell junctions. Pathways implicated from our experiments with of virus infection using RNA-seq include those controlled by L1CAM and SPARC expression. These proteins elicit a signaling cascade that induces reorganization of the cytoskeleton, causing disorganization of cell endothelial junction proteins, resulting in increased permeability\textsuperscript{69-72,81,87,125,142}. This is pathway is mediated by a series of kinases and GTPases that alter the actin cytoskeleton. Phosphorylation of myosin light chain (MLC) by myosin light chain kinase (MLCK) induces contraction, resulting in permeability increases at the cell-cell junctions. The main players in this signaling cascade include myosin light chain phosphatase (MLCP), myosin light chain kinase (MLCK), Rho A GTPase, and Rho kinase (ROCK) (Figure 35).\textsuperscript{143-146} Many of these genes were found to be altered in cells expressing N/GPC (Figure 6). This includes a 4-fold increase in myosin light chain kinase 4 (MYLK4), which would result in an increase in phosphorylation of MLCs in the endothelial cells. These changes in the myosin light chain signaling cascade were not found for the cells where only N was expressed. Consequently, we favor the idea that Gc and Gn are solely
Figure 35. Myosin light chain kinase pathway with genes altered by N and Gn and Gc highlighted. Figure adapted from KEGG pathways. Extracellular matrix components were found altered in our data set, they interact with integrin A and B on the endothelial cell surface. This interaction results in a signaling cascade mediated by Src, RhoGAP, RhoA, ROCK, MLCP, MLCK, and MLC. Of these mediators, we found upregulation in ECM, ITGA, ITGB, ROCK, MLCP, and MLCK (red). MLC was found downregulated (blue). Pathways related to myosolining light chain contraction were found to have upregulated genes as well, including Paxillin and Parvin (red).
Figure 36. Myosin light chain kinase signaling altered during N and GPC protein expression in HMVEC-Ls. Genes involved in the myosin light chain kinase signaling pathway that leads to actin cytoskeleton contraction are shown.
responsible for these changes in expression, but cannot rule out the possibility
that a combination of Gc and Gn with N-NSs+ is necessary for these changes in
expression. Nonetheless, it is significant that the modification of the myosin light
chain kinase cascade and remodeling of the actin cytoskeleton, resulting in
reorganization of endothelial cell-to-cell junctions and increased permeability, is
induced by viral protein expression.

**Platelet recruitment and activation**

The process of platelet activation and aggregation for clot formation on the
endothelium surface involves initial disruption of the endothelial layer and
exposure of the subendothelial matrix. The first steps in the signaling pathway
include endothelial production of vWF, which binds directly to GP IB-IX-V complex
on the platelet surface\(^{147}\). As discussed previously in our RNA-seq experiments with
SNV-infected cells, we found increases in vWF and ADAMTS13, but they were not
statistically significant. However, in cells expressing N/GPC following nucleofection,
we found a significant increase in both vWF and ADAMTS13 (Figure 37). vWF was
increased 4-fold, indicating likely strong involvement of the HMVEC-Ls in
recruitment of platelets. In addition, a recently identified platelet activation factor,
SCUBE1 was found to be 12 fold upregulated in response to N/GPC expression\(^{148}\).
SCUBE1 is found in platelets and endothelial cells and forms homophilic
interactions, activating platelets\(^{149,150}\). The upregulation of SCUBE1 further
Figure 37. Platelet activation and aggregation genes in HMVEC-Ls with N and GPC expression. vWF and the enzyme that releases the active form ADAMTS13, are found to be upregulated with N and GPC expression. SCUBE1, a platelet activation factor is found to be upregulated with N and GPC expression.
Figure 38. Schematic of platelet activation and aggregation during infection.

Our data demonstrate that in addition to factors found during our RNA-seq study with infection with Sin Nombre Virus, other factors found with expression of N and GPC in HMVEC-Ls may be important for the platelet response during infection. These factors include vWF which is released from endothelial cells, and acctaches to glycoproteins found on the platelet surface, activating them. The platelets then bind the exposed collagen on the subendothelialium, initiating aggregation. SCUBE1 is also upregulated with N and GPC expression and binds to SCUBE1 on the platelet surface, activating the platelet.
reinforces the idea that N and GPC play an important role in activating platelets and initiating aggregation and coagulation. A diagram depicting the platelet response in the endothelium shows the contribution that vWF and SCUBE1 appear to make to the platelet aggregation response during infection (Figure 38).

**Leukocyte adhesion**

VCAM1, a leukocyte adhesion molecule was upregulated 6-fold in cells expressing N/GPC. VCAM1 mediates adhesion of endothelial cells to monocytes, lymphocytes, eosinophils, and basophils. Binding of VCAM1 to leukocytes leads to a signaling cascade that results in loosening of the cell-cell junctions of endothelial cells to facilitate transmigration of immune cells. Other leukocyte adhesion molecules and cytokines were also upregulated (Figure 39). While we saw an upregulation of VCAM1, CD34, and ITGB1, we observed a downregulation of leukocyte recruitment and adhesion molecules CXCL1, CXCL3, CXCL8, and ICAM1. NSs expression resulted in downregulation of CXCL1, CXCL3, and CXCL8 so this affect may be due to the expression of the NSs protein. These results indicate that the glycoproteins may be responsible for the increase in VCAM1 and CD34 expression during whole virus infection.

**MMP expression**

Similar to SNV infection, matrix metalloproteinase expression was altered when N-NSs+ and GPC were expressed following nucleofection (Figure 40). In the N-NSs+ group, MMP10 was found to be downregulated. N and GPC expressing cells also
Figure 39. Leukocyte adhesion genes altered with N and GPC expression.

Cytokines for recruitment of leukocytes to endothelial cells were either up or downregulate during N and GPC expression. ICAM1, ITGB1, and VCAM1 are leukocyte adhesion factors and they were found to be either up or downregulated during N and GPC expression.
downregulated MMP10 along with an additional metalloproteinase (MMP1), consistent with our observations with infection. MMP7 and MMP19 were upregulated but the exact functions of these MMPs is unknown so we can’t conclude that these MMPs are contributing to increased permeability seen during infection. The gene expression data support the role of collagen in platelet aggregation seen during whole virus infection with the similar downregulation in MMP1. MMP7 and MMP19 are upregulated and may increase permeability by degrading the extracellular matrix similar to the MMPs found upregulated during whole virus infection, but this cannot be confidently concluded.

**IncRNA, antisense transcripts, and run-through transcription**

Similar to our infection data set, all three protein expression conditions had various IncRNAs, antisense transcripts, and run-through transcripts altered. (Table 3 and 4) The IncRNA NEAT1 has been shown to promote the interferon response to hantaviruses and this gene was upregulated in the N-NSs+ and GPC co-expression group. We did not find any changes in the N-NSs+ group, so NEAT1 may be transcribed in response to expression of the glycoproteins. As research progresses and these unidentified transcript functions are identified, it will be interesting to see how they fit in with SNV infection.

**DISCUSSION**

RNA-Seq of SNV infected HMVEC-Ls resulted in discovery of pathways of importance. These pathways include platelet activation and aggregation, expression
Figure 40. Matrix metalloproteinase expression in N/GPC and N+NSs expression groups. MMP1 and MMP10 were found to be downregulated in the N and GPC expression group. MM7 and MMP19 were found to be upregulated in the N and GPC expression group. MMP10 was the only differentially expressed MMP in the N+NSs group and it was downregulated.
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Table 3. IncRNAs, anti-sense transcripts, and run-through transcripts of N-NSs- and N-NSs+ expression in HMVEC-Ls. (a) IncRNAs, anti-sense transcripts, and run-through transcripts found differentially expressed in the N-NSs+ group. (b) IncRNA and run-through transcripts found differentially expressed in the N-NSs- group.
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**Table 4.** lncRNAs, anti-sense transcripts, and run-through transcripts of N-NSs+ and GPC coexpression group. (a) Antisense transcripts differentially expressed listed. (b) lncRNAs differentially expressed listed. (c) Run-through transcripts differentially expressed listed.
of genes that lead to reorganization of endothelial cell junction proteins, and expression of genes that alter the extracellular matrix. Our protein expression study revealed contributions of the viral proteins to these overall infection effects. N without the NSs protein appears to be an important antigen as it upregulated many genes involved in MHC I antigen presentation. N-NSs+ revealed expression data that indicates the NSs protein is an interferon antagonist and may inhibit MHC I antigen presentation. Expression of N-NSs+ with GPC indicates that the glycoproteins may be important for the platelet activation and aggregation cascade. This could also be a synergistic effect of the glycoproteins combined with N and NSs. L1CAM and SPARC were upregulated during SNV infection and overexpression of these proteins in endothelial cells induces cytoskeletal rearrangement at endothelial cell junctions, increasing vascular permeability. These rearrangements are induced by phosphorylation of myosin light chains by myosin light chain kinase. In cells expressing N and GPC, we found an upregulation of many of the genes involved in the signaling cascade, that would be expected to result in increased phosphorylation of myosin light chain. The combination of these changes with the upregulation of L1CAM and SPARC, indicate a large change in the cytoskeleton at the endothelial cell junctions, resulting in the increased endothelial permeability. L1CAM and SPARC were not found to be altered in any of the protein expression groups so we hypothesize that viral replication needs to occur for these changes in these genes in the endothelial cells. The changes during infection of leukocyte adhesion molecule and MMP expression does not appear to be induced by merely expressing viral
Figure 41. Endothelial cell gap junction connected to actin cytoskeleton via scaffold proteins. Endothelial gap junctions include adherens junctions and tight junctions composed of VE-cadherin, PECAM1, claudins and occluding, JAMS, and connexins, among others. These gap junctions proteins are connected to the actin cytoskeleton via series of scaffold proteins. Various signaling processes in the endothelial cell can cause rearrangement of the actin cytoskeleton, altering the organization of the gap junction.
Figure 42. Myosin light chain kinases changes to actin cytoskeleton at endothelial cell gap junctions results in increased permeability. Endothelial gap junctions include adherens junctions and tight junctions composed of VE-cadherin, PECAM1, claudins and occluding, JAMS, and connexins, among others. These gap junctions proteins are connected to the actin cytoskeleton via series of scaffold proteins. Various signaling processes in the endothelial cell can cause rearrangement of the actin cytoskeleton, altering the organization of the gap junction. On of these signaling cascades is through the phosphorylation of myosin light chain, inducing contractions of the actin cytoskeleton.
These contractions result in reorganization of the gap junction proteins, disruption binding to neighboring endothelial cell gap junction proteins and inducing increased permeability in the paracellular space.
proteins, and may require virus replication as well. A schematic showing how the endothelial cell junction proteins are connected to the actin cytoskeleton via scaffold proteins and how changes in these actin filaments can induce dissociation of the junction proteins, inducing vascular permeability (Figures 41 and 42). Future directions include phenotypic studies looking at the phosphorylation state of myosin light chains to confirm this is the correct mechanism for changes in endothelial cell junction organization. In addition, looking further into the antigen presentation pathway to determine exactly how NSs may be interfering is intriguing. We hypothesize that NLRC5 is important and we can test for this via siRNA studies in combination with expression of NSs. In addition we would like to do further studies to determine the interferon antagonism potential of NSs and what the cellular target may be in endothelial cells.

**Acknowledgements**

RNA-seq data differential gene expression analysis for chapters 3 and 4 was done by the Cancer Crusader Next Generation Sequence Analysis Core. The Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P20GM103518. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
CHAPTER 5: PROTEIN INTERACTIONS OF INFLUENZA HA AND M1

BACKGROUND

Influenza virus causes a disease that results in annual epidemics that infect millions of people each year. Pandemics can arise, resulting in millions of deaths, like the pandemic of 1918\textsuperscript{152}. It has a large impact on both health and the economy. The disease manifests itself in a stuffy nose and sore throat, body aches and fatigue, and headache. The virus is very contagious and those that are infected are encouraged to remain home from work and school for the duration of the disease. This creates an economic burden. Infection can result in hospitalization, especially in the elderly and young. 80 to 90\% of those that die due to influenza infection are over 65 years old. Children under 4 years old also have a higher mortality rate than the general population.\textsuperscript{153} If two influenza viruses infect the same cell, an occurrence called reassortment, or gene mixing, can occur where a new virus will develop that contains components from each of the original two viruses. This is thought to be the cause of many of the human pandemics seen from influenza virus.\textsuperscript{152} There is much impetus to develop therapeutics for influenza to lessen the mortality and morbidity associated with infection in addition to avoid catastrophic pandemics like that seen in 1918. The virus is a segmented negative sense ssRNA virus from the family \textit{Orthomyxovirus}. The 8 segments code for 11 to 12 viral proteins. There are two glycoproteins found on the lipid envelope of the virus, neuraminidase and
hemagglutinin. In addition, there are two membrane proteins, M1 and M2. Within the virion, the genome is enclosed in a nucleoprotein and the RNA-dependent polymerase is associated with the genome segments and together, it is termed the ribonucleoprotein. These proteins are involved in the viral replication cycle within cells of the host in addition to non-structural proteins coded by the viral genome. Influenza virus primarily infects epithelial cells within the upper respiratory track. One of the glycoproteins on the envelope, hemagglutinin, binds to sialic acid on the host cell surface, inducing receptor-mediated endocytosis. The virus travels through the various stages of maturation of endosome and the acidic environment of the late endosome triggers a conformational change in hemagglutinin and the fusion peptide is exposed, fusing the viral membrane with the endosomal membrane. The M2 protein on the viral envelope allows an influx of protons into the virus, causing acidification, allowing for release of the core proteins and viral RNA. The viral RNA and core proteins are transported across the nuclear membrane by the assistance of a viral protein, NRF2. Within the nucleus, viral replication takes place, positive strand RNA is synthesized, which are then used as templates for the synthesis of negative strand RNA for progeny virions. Viral proteins assemble at the cell membrane of the host cell and new virions bud with cell derived lipid envelopes. Hemagglutinin is a glycoprotein that has 18 antigen types, H1 through H18. The hemagglutinin is responsible for receptor binding in addition to membrane fusion during viral genome escape. In addition to these roles, it has a known role during viral budding. HA is known to alter the membrane curvature of the host cell membrane. Sole expression of HA results in budding of
viral-like particles (VLPs) from the cell membrane but co-expression with other viral proteins results in more efficient production of VLPs\textsuperscript{158}. Another viral protein thought to assist with viral budding is M1 matrix protein. The matrix protein is responsible for recruitment of the ribonucleotide proteins (RNPs) to the membrane for viral budding\textsuperscript{154}. We also hypothesize that it interacts with HA, likely the cytoplasmic tail, to facilitate viral budding and exit.

**REVERSE GENETICS**

Reverse genetics is a system that allows for the creation of virus using plasmids that express both the viral RNA and the mRNA for the synthesis of viral proteins. Because of the flexibility of the use of plasmids, reverse genetics allows for the synthesis of mutant viruses, reassorted viruses, among other uses.\textsuperscript{159} Reverse genetics allowed for the synthesis of the 1918 pandemic flu virus, allowing for researchers to investigate what exactly made that flu virus especially lethal\textsuperscript{160}. In addition to resurrecting past viruses, researchers are able to mutate current strains, including H5N1, or avian flu, to determine what mutations would cause the transmission rate to increase, in order to set a ‘watch’ for mutations that could lead to a deadly pandemic\textsuperscript{161}. The plasmid system we use is an 8 plasmid system. They plasmids are bidirectional, with a RNA polymerase I promoter that allows for transcription of vRNA in one direction and a RNA polymerase II promoter that allows for transcription of mRNA that once transcribed, allows synthesis of viral proteins in the transfected cell. Once the vRNA is transcribed from the plasmid, the viral proteins are able to use the genome segments to transcribe to cRNA and mRNA
and replicate to vRNA. (Figure 43) All these components combined allow for synthesis of new virions. Influenza has various strains and they are named based on their heamgglutinin and neuraminidase antigen (for influenza A viruses), category (either A, B, or C), geographical origin, the strain number, and the host of origin (but for human origin viruses, no origin destination is given)\textsuperscript{154}. For our studies, we used a strain of influenza called A/California/07/04 (H3N2). The 8 plasmid system was created based on the protocol of Brown et al.

DEVELOPMENT OF THE REVERSE GENETICS SYSTEM

Introduction

The establishment of a reverse genetics system for influenza virus has greater increased the understanding of this pathogen. By creating a system for a virus isolated from the 2009 swine flu pandemic, we can understand what made this virus especially virulent in addition to understanding the basic virology of influenza replication.

Materials and Methods

Production of influenza reverse genetics vectors

Production of reverse genetics vectors was done by Dr. Naveen Sommana, a research scientist in the Voss lab. Influenza viruses A/Cal/07/2004(A/Cal Egg P2 12/29/07), A/Cal/04/09/Egg P2 062909 HA-4 and A/HongKong/H1N1 Tamiflur-esistant Egg P1 6/10/10 propagated in 10-day old embryonated eggs. Viral RNA was extracted from 250 μl volumes of allantoic fluid using the Invitrogen total RNA isolations mini kit (USA).
Figure 43. **8 plasmid Influenza reverse genetics system.** Figure taken from Hoffman et al. 8 plasmids with bidirectional transcription are transfected into mammalian cells. RNA pol I transcribes vRNA while RNA pol II transcribes mRNA. mRNA is translated into viral proteins and interacts with vRNA to produce progeny virions.
Isolated RNA quality and quantity was checked in nanodrop spectrophotometer. Influenza cDNA were subsequently prepared using a universal plus sense primer mixture U12 5’GGGGAGCAAAAGCAGG plus 5’GGGGAGCGAAAGCAGG, and reverse transcription was performed in a reaction mixture containing 300ng of viral RNA, 1µM primer, 2µl of annealing buffer, 20µl of first strand reaction mix, 4µl of SuperScript III/RNaseOUT Enzyme Mix in a total volume of 40µl, then incubate at 50°C for 1hr. Each influenza virus segment was amplified from the cDNA mixture for each of the 3 virus subtypes by PCR using the appropriate segment specific primers. Universal forward (G): UF/G = GGGGAGCGAAGCAGG, Universal forward (A): UF/A = GGGGAGCAAAAGCAGG, Universal reverse: UR = GGTTATTAGTAGAAACAAGG, PB2/H1N1 UF/G-TCAATTATATTC UR-TCGTTTTTAAAAC, PB1/H1N1 UF/G-CAAACCATTTGA UR-CATTTTTTTC, PA/H1N1 UF/G-TACTGATCC UR-TACCTTTTTTG, HA/H1N1 UF/A-GGAAAAT UR-GTGTGTTTTTC, NP/H1N1 UF/A-GTAGATAATC UR-GTATTTTTTTC, NA/H1N1 UF/A-AGTTTTAAAATG UR-AAGTTTCTTG, M1,2/H1N1 UF/A-TAGATATTG UR-TAGTTTTTTTAC NS 1,2/H1N1 UF/A-GTGACAAA UR-GTGTGTTTTTATC. The PCR reactions were carried out in a final volume of 50µl that contained 5µl cDNA, 0.3 µM dNTP, 5 µl 10x PCR buffer, 0.4 unit platinum taq DNA polymerase (Invitrogen) and 0.3 µM of each oligonucleotide primer. For PCR the samples were heated for 5min at 94°C, followed by 40 cycles of 94°C for 15sec, annealing at 55°C for 30sec and elongation at 68°C for 1min/Kb, followed by single 5 min incubation at 68°C to extend incomplete templates. PCR products were purified by agarose gel electrophoresis and eluted into water using the QIAgent Gel Elution kit (Qiagen).
Immunohistochemistry of influenza viral protein expression

200 ng of the vector that codes for HA and for M1 were transfected into MDCK cells that were seeded for ~85% confluency in a NUNC 8 well chamber slide using Lipo-LTX Plus (Thermo Fisher Scientific). Cells were fixed and permeabilized with ice cold acetone for 10 minute on ice. Cells were stained with anti-HA antibody (Santa Cruz Biotechnology) and anti-M1 (Santa Cruz Biotechnology) at a concentration of 1:500 in Tris buffered solution with 1% Tween-20 for 1 hour. Cells were then incubated with anti-mouse antibody conjugated to fluorophore 488. Cells were stained with SyTox green (Thermo Fisher Scientific) to stain the nucleus of the cells and mounted using ProLong Gold anti-fade mountant (Thermo Fisher Scientific). Cells were then visualized on Zeiss Confocal (look up exact microscope).

Western blot of influenza viral protein expression

MDCK cells were plated for 85% confluency in a 6 well plate. 2500 ng of each Influenza protein construct was transfected using Lipo-LTX Plus (Thermo Fisher Scientific). 48 hours post transfection, cells were harvested. Immunoprecipitation was performed on cell lysates using anti-HA, anti-M1, and anti-M2 antibodies (Santa Cruz Biotechnology) using Protein G sepharose magnetic beads (Thermo Fisher Scientific). 20 ul of each sample was then run on a 12% SDS-Page gel (Biorad). Gel was transferred using an iBlot (Thermo Fisher Scientific) onto a nitrocellulose membrane. Proteins were probed for using anti-HA, anti-M1, and anti-M2 antibodies at a concentration of 1:1000. Secondary antibodies conjugated to HRP
and developed using the Chemiluminescence Detection Kit (Thermo Fisher Scientific).

**Co-capping of hemagglutinin and matrix protein 1**

MDCK cells were seeded for 60% confluency in a NUNC 8 well chamber slide (NUNC). After incubation at 37°C at 5% CO2 for 24 hours, 300 ng of the M1 and HA construct were transfected into the cells using Lipo-LTX Plus (Thermo Fisher Scientific). Empty vector was transfected into control wells. After 48 hours incubation at 37°C and 5% CO2, media was removed and serum free media plus primary anti-HA antibody (1:500 dilution) were added and slides were allowed to incubate at room temperature for 1 hour. Cells were washed and secondary antibody to anti-HA was added in serum free media and cells were allowed to incubate at room temperature for 1 hour. Media was removed and growth media plus 5% FBS was added and cells were incubated for 1 hour at 37°C and 5% CO2. Following incubation, cells were fixed and permeabilized with ice cold acetone. Then cells were stained for M1 expression. Slides were visualized with Zeiss confocal (look up model).

**Ferret infection with influenza and treatment with FF-3**

Donor ferrets were treated with FF-3 12 hours prior to infection with influenza A virus (A/New Cal/20/99) by intranasal administration under anesthesia. 24 hours post influenza challenge, donor ferrer was co-housed with two naïve contact ferrets. Ferrets were monitored for clinical signs of disease, including temperature,
symptoms (coughing, sneezing, etc) and viral loads were calculated from nasal aspirates for 10 days post infection. Viral titer was determined using TCID50 using MDCK cells using nasal aspirates collected under anesthesia.

**Results**

Virus was propagated in embryonated eggs in order to create a working stock of viral RNA for production of cDNA to be used to clone into vectors for synthesis of vRNA and mRNA. This allows for creation of the vectors that exactly mimic the sequence of infectious virus isolate. The 8 genome segments were cloned via PCR (Figure 44) Sequencing was done on the vectors to verify the correct viral sequences. Vectors viral protein expression was verified by transfecting into MDCK (Madin-Darby Canine Kidney Epithelial Cells) and utilizing immunofluorescence and detection via confocal microscope. MDCK cells were chosen because epithelial cells are a target during influenza infections and we are able to visualize the migration of viral proteins to either apical or basal membranes, important placement during viral packaging and budding. M, NP, and HA were chosen as viral proteins to detect expression via lipofectamine transfection into cells. HA localized to the host cell membrane and M1 was found within the cytosol and membrane. NP was found in the perinuclear region. (Figure 45) In order to determine the interaction between the two viral proteins, HA and M1, I performed a methodology termed co-capping or antibody-induced redistribution. MDCK cells were transfected with both the M1 and HA expressing plasmids. Live cells were then stained with an anti-HA antibody and allowed to incubated to allow for the antibody-induced redistribution. Cells were
Figure 44. Full-length RT-PCR amplicons from genes of the strain A/Cal/07/2004 H3N2.

Lanes: M DNA size marker (0.5 ug), Lanes 1-8 PCR from cDNA transcribed from viral RNA (+)

(each 5 µl from 50 µl total reaction volume).
Figure 45. MDCK cells transfected with reverse genetic plasmids efficiently. Chamber slides seeded with MDCK cells were transfected with 200 ng of reverse genetic plasmids derived from A/Cal/04/07 using Lipo-LTX Plus. MDCK cells were fixed and permeabilized and stained. Cells were visualized by a confocal microscope. A. Hemagglutinin type 3 is expressed in permeabilized MDCK cells, its location being cell surface and cytoplasmic and visualized by anti-HA antibody. B. Anti-M1 antibody is used to visualize matrix protein located in the cytoplasm and at a higher concentration below the cell membrane. C. Anti-NP antibody is used to visualize nuclear protein and the NP is located in the nucleus.
then fixed and stained for M1. The resulting effect was the co-localization of the HA and M1 proteins on one end of the cell, or a 'capping' effect. (Figure 46) This demonstrates a possible direct interaction between HA and M1, something that has not been shown before. There may be a possible important role for this interaction during influenza budding at the cell membrane and egress of progeny virions.

**FLUFIRVITIDE-3**

Flufirvitide-3 (FF-3) is a 16 amino acid peptide that was created from a conserved region of the stalk of the influenza A hemagglutinins. It is from a region termed the fusion initiation region of a helical domain of HA2, thought to contribute to the triggering and fusion of the viral membrane with that of the host cell. Researchers at Tulane University and Autoimmune Technologies of New Orleans discovered and synthesized FF-3 and discovered a broad-spectrum antiviral effect. Ferrets serve as an animal model for influenza infection in humans and the effect of FF-3 was determined for the morbidity of disease as well as transmission. (Figure 47) (Figure 48) We hypothesize that FF-3 localizes to the cell membrane, interfering with the interaction between HA and M1, causes the virus to be incapable of budding and thus inhibits cell-to-cell viral spread. In order to determine the mechanism of action of FF-3, we first need to determine if an interaction between HA and M1 occurs, then determine the cellular localization of FF-3, and finally if FF-3 interacts with either proteins during viral egress.
**Figure 46. Co-capping of HA and M1.** MDCK cells were transfected with plasmids for Influenza protein HA and M1. Cells were permeabilized and stained using co-capping procedure. HA and M1 were found to be colocalized on the cell surface.
Figure 47. FF-3 abrogates influenza virus transmission from infected ferrets to naïve cage mate by contact. Twenty-four (24) hours prior to contact, donor ferret was treated with FF-3 and infected with Influenza A or B virus (12’ post FF-3 treatment) by intranasal administration under anesthesia. At 24’ post challenge, donor ferret was co-housed with two (2) contact ferrets. Animals were observed for clinical signs twice daily with body temperature collected. Nasal aspirate samples were collected (under anesthesia) for 10 days following virus challenge.
Figure 48. Ferret challenge results.

C. Mean clinical scores (1 - 4 scale) where 1 = normal, 2 = mild, 3 = severe; 4 = moribund

D. Body temperature (°F) were collected twice daily (AM/PM) and mean body temperature calculated for each animal

F. Nasal aspirates were collected daily for 10 days and virus titer determined by TCID$_{50}$ (Log$_{10}$/ml) in MDCK cells

Results are for challenge with A/New Cal/20/99 (H1N1)
DISCUSSION

FF-3 reduces the transmission rate when infected ferrets are treated with the peptide and housed with non-infected ferrets. The ferrets who received FF-3 and were infected with influenza A showed signs of infection, including fever and viremia. The lack of transmission to naïve ferrets indicated a potential inhibitory effect of viral spread during viral budding and egress. Hemaglutinin and M1 play important roles in assembly at the cell membrane and viral budding. A direct interaction between these two proteins has not been witnessed. We created a reverse genetics system of 8 plasmids and utilized the expression plasmids for M1/M2 and HA in order to look at the interaction between HA and M1. Through a methodology determined ‘co-capping,’ we saw what indicates an interaction between the two proteins on the cell membrane. Future directions include treatment of cells with FF-3 and transfection with HA and M1 in order to determine if a disruption of this protein interaction occurs, through ‘co-capping’ or co-immunoprecipitation. Determining the method of action of FF-3 is critical to progressing the treatment down the pipeline for eventual use in influenza infected humans.
BIBLIOGRAPHY


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Catherine “Katie” Arnold was born on January 19, 1989 to Dr. Kevin Arnold and Linda Arnold in Hartland, Wisconsin. She has two older brothers, Dan and Greg. She attended Arrowhead Union High School where she first developed her love for science. She then went on to attend the University of Wisconsin-Madison where she graduated with a dual major in Medical Microbiology and Immunology and Spanish Language and Culture. While studying at the university, she worked as an undergraduate research assistant in the Conversion group within the Great Lakes Bioenergy Research Center. It was there she realized how much she loved benchwork and molecular biology. She went on to attend Tulane University School of Medicine for her PhD starting Fall 2011. After graduation, she will begin a postdoctoral position at the U.S Army Medical Research Institute of Infectious Diseases within the Center for Genome Sciences in Frederick, Maryland.