2016

Expression of the Non-Structural Proteins NS3/4A of the Hepatitis C Virus Using a Genetically Modified Vesicular Stomatitis Virus Vector System

Angela Ye Seul Kim
Western University, ayk.angelakim@gmail.com

Expression of the non-structural proteins NS3/4A of the hepatitis C virus using a genetically modified vesicular stomatitis virus vector system

Abstract

Hepatitis C virus (HCV) infection is one of the leading causes of chronic liver diseases. Despite advancements in the development of antivirals and efforts to combat HCV infections, there is currently no vaccine for HCV. Adopting traditional approaches to HCV vaccine development has been impractical due to the lack of reproducible cell culture systems that can support HCV replication. In this study, the New Jersey serotype of recombinant vesicular stomatitis virus ($rVSV_{NJ}$) was used as the vector to express non-structural proteins NS3/4A of genotype 1a HCV for potential vaccine purposes. The $rVSV_{NJ}$-GMM vector was genetically modified by changing glycine to glutamic acid at position 22 and methionine to arginine at positions 48 and 51 of the matrix protein to reduce inhibition of host gene expression and cytopathic effects. This study examined replication efficiency, attenuation, and capacity of $rVSV_{NJ}$-GMM vector to express high protein levels. It was hypothesized that the genetically modified $rVSV_{NJ}$-GMM vector with the HCV NS3/4A insert would demonstrate reduced cytopathogenesis without compromising viral replication and efficiently express functional NS3/4A proteins. The $rVSV_{NJ}$-GMM-NS3/4A was recovered by reverse genetics and amplified to construct viral growth kinetics that generated a high viral titre. The degree of cytopathogenesis of $rVSV_{NJ}$-GMM without insert and $rVSV_{NJ}$-GMM-NS3/4A was compared and results showed that $rVSV_{NJ}$-GMM-NS3/4A achieved fewer structural changes in the infected cells compared to $rVSV_{NJ}$-GMM without insert. In addition, the proper expression and processing of HCV NS3/4A proteins were confirmed, and the function of NS3/4A protein complex as a serine protease was confirmed by its ability to cleave at the NS5A/NS5B polyprotein junction. Electron microscopic visualization showed normal morphology of rhabdovirus particles and clear projection of glycoproteins. The replication efficiency, safety, and capacity to achieve high expression level of

functional NS3/4A proteins indicate that the $rVSV_{NJ}$.GMM vector can be used for recombinant HCV vaccine development.

Key words: vesicular stomatitis virus/hepatitis c virus/HCV NS3/HCV NS4A/recombinant vector vaccine

Introduction

Hepatitis C virus (HCV) infection poses significant public health burden with over 150 million people affected worldwide (Mohd Hanafiah, Groeger, Flaxman, & Wiersma, 2013). The virus can cause both acute and chronic hepatitis infections. Majority of individuals acutely infected with HCV develop chronic hepatitis which potentially can lead to liver cirrhosis, end-stage liver disease, and liver cancer (Jacobson, Davis, El-Serag, Negro, & Trépo, 2010). Despite the recent advances in antiviral treatment of HCV infection, hepatitis C remains a major problem in public health. Current therapy has shown to be effective in some patients, but is still daunting for millions of people due to the high cost, complex regimen, and side effects (Liang, 2013). Although several vaccine candidates are in preclinical and clinical trials, no effective vaccine is currently available. Therefore, the development of a safe, affordable, and efficacious vaccine is urgently needed.

Hepatitis C virus is a single-stranded positive-sense RNA virus of the family *Flaviviridae* (Blondel, Harmison, & Schubert, 1990). It encodes a polyprotein that is cleaved by cellular and viral proteases into structural proteins (Core, E1, and E2), a small membrane polypeptide (p7), and non-structural proteins (NS2/NS3/NS4A/NS4B/NS5A/NS5B). The progress of HCV vaccine development has been circumscribed by the genetic heterogeneity of the virus and its ability to

evade host immune responses. HCV is extremely variable due to its high mutational rate caused by the lack of proofreading capacity of the viral polymerase (Simmonds et al., 2005). It exists in several major genotypes (1-7) and in quasispecies variants which are groups of related, but distinct viral populations that differ in sequences within the hypervariable regions of the viral genome (Farci et al., 2000). HCV persists in majority of infected individuals by inhibiting interferon induction and evading neutralizing antibodies by circulating complexed with host lipoproteins (Horner & Gale, 2013; Timpe et al., 2008). Previous studies have used the non-structural gene product NS3 in vaccine designs since it is a highly conserved region and a major target of T cell-mediated immunity (Ahlén, Holmström, Gibbs, Alheim, & Frelin, 2014; Arribillaga et al., 2002; Ratnoglik et al., 2014; Zhu et al., 2015). NS3 is a multifunctional protein that has serine protease, nucleoside triphosphatase (NTPase), and helicase activities which are involved in proteolytic processing of the polyprotein, deregulation of normal cellular functions, and viral RNA replication respectively (Shiryaev et al., 2012). The NS4A protein acts as a cofactor essential for the full function of NS3. The NS3/4A protein complex cleaves downstream junctions including NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B. It has been shown that T-cell immunity mediates HCV viral control in natural infections (Ratnoglik et al., 2014). Since NS3 carries numerous T cell epitopes and induces strong HCV-specific T cell responses associated with viral clearance and resolution of acute HCV infection, the NS3 and NS4A proteins have been identified as ideal antigens for a novel vaccine and are therefore the proteins of interest in this study.

The traditional approaches of vaccine development such as using live attenuated virus are impractical due to the lack of a reproducible cell culture system supporting HCV replication. Instead, the recombinant viral-vector based vaccine approach has been used to express foreign

genes and elicit host immune responses (Cobleigh, Wei, & Robek, 2013; Pietschmann et al., 2002). Among other gene expression vectors, the vesicular stomatitis virus (VSV) has shown to be a promising candidate for recombinant vaccine development due to its genetic malleability, rapid replication, high expression level of foreign gene inserts, wide host range, and mild pathogenicity in humans (An, Kim, Wu, & Kang, 2013; Ezelle, Markovic, & Barber, 2002). VSV is a non-segmented, negative-sense RNA virus of the *Rhabdoviridae* family. The viral genome encodes five viral proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large polymerase protein (L). The M protein has been shown to be critical in the cytopathogenesis of VSV and inhibition of host gene expression in addition to its regulatory and structural roles (Black & Lyles, 1992; Blondel et al., 1990). Furthermore, VSV is comprised of two major serotypes, Indiana (VSV_{Ind}) and New Jersey (VSV_{NJ}) (Gould et al., 1998). The New Jersey serotype has been less frequently used and studied as a gene expression vector.

In this study, the New Jersey serotype of vesicular stomatitis virus was engineered to express NS3/4A of genotype 1a HCV. The M protein of the $rVSV_{NJ}$-GMM vector was modified by changing glycine to glutamic acid at position 22, and methionine to arginine at positions 48 and 51 (Kim and Kang 2007). Manipulation of the M protein has been shown to reduce inhibition of host gene expression and cytopathic effects (Kim & Kang, 2007). The NS3/4A genes were inserted into the *G* and *L* gene junction of $rVSV_{NJ}$-GMM. This study generated high viral titres while achieving reduced cytopathogenesis using $rVSV_{NJ}$-GMM-NS3/4A, examined the capacity to achieve high levels of NS3/4A protein expression using the recombinant VSV_{NJ}-GMM vector, and confirmed the proper NS3 function as a protease.

Hypothesis

It was hypothesized that the genetically modified $rVSV_{NJ}$-GMM vector would efficiently express NS3/4A genes based on the property of VSV to accommodate large foreign gene inserts and demonstrate reduced cytopathogenesis without compromising viral replication (An et al., 2013; Kim, Wu, Hong, Awamleh, & Kang, 2015). Generation of high viral titre of $rVSV_{NJ}$-GMM-NS3/4A while achieving reduced cell death, detection of high NS3/4A protein expression level using the $rVSV_{NJ}$-GMM vector system, and confirmation of the proper function of NS3/4A as a serine protease would indicate that $rVSV_{NJ}$-GMM vector can be used for recombinant HCV vaccine development.

Materials and Methods

Cells. BHK_{21} (baby hamster kidney, ATCC) cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) containing 5% fetal bovine serum (FBS, Gibco BRL), 100 μg/ml of penicillin (Gibco BRL), 100 μg/ml of streptomycin and kanamycin (Gibco BRL), and 2 mM L-Glutamine (5% FBS c-DMEM). The BHK cells constitutively expressing bacteriophage T7 RNA polymerase (BHK-T7, Buchholz, Finke, & Conzelmann, 1999) were maintained in 5% FBS c-DMEM containing 500 μg/ml G418 (Invitrogen) for transfection. Approximately 20 hours before transfection, BHK-T7 cells were cultured in 5% FBS DMEM containing 2 mM of L-Glutamine without antibiotics. The Vero E6 (green monkey kidney) cells (ATCC) were used for plaque assay to purify the virus and were cultured in Minimum Essential Medium Eagle (MEM, Invitrogen) supplemented with 10% FBS, 100 U/ml of penicillin, 100 μg/ml of streptomycin and kanamycin, 2 mM of L-glutamine, and 5 ml of sodium pyruvate (10% FBS c-MEM). The cells were incubated at 37°C and 5% CO_2.

Cloning of HCV NS3/4A gene into prVSV$_{NJ}$-GMM (G22E+M48R+M51R). The recombinant prVSV$_{NJ}$-GMM vector and KS-NS3/4A plasmid were provided for this study by the Kang laboratory. The plasmid rVSV$_{NJ}$-GMM-NS3/4A was constructed by treating prVSV$_{NJ}$-GMM vector and KS-NS3/4A plasmid with the restriction enzymes *Pme* I (BioLabs) and *Mlu* I (BioLabs). The NS3/4A and prVSV$_{NJ}$-GMM DNA fragments were ligated by ligase (BioLabs), and XL-10 gold cells (Agilent Technologies) were transformed using the ligated sample. The prVSV$_{NJ}$-GMM-NS3/4A isolated from the transformants were digested by restriction enzyme *Nco* I (BioLabs) and the digested DNA fragments were confirmed using 1.0% agarose gel electrophoresis.

Transfection. Recombinant VSV$_{NJ}$-GMM-NS3/4A was recovered by reverse genetics. Using Lipofectamine 2000 (Invitrogen), BHK-T7 cells were transfected with 15 μg cDNA clones of prVSV$_{NJ}$-GMM-NS3/4A and three plasmids expressing pBKS-IRES/N$_{NJ}$ (nucleocapsid protein), pBKS-IRES/P$_{NJ}$ (phosphoprotein), and pBKS-IRES/L$_{NJ}$ (large polymerase protein) in concentrations of 10 μg, 10 μg, and 5 μg respectively. The control group was cultured containing Lipofectamine 2000 without any plasmids. The culture medium was harvested when 80% of cells showed cytopathic effects (CPE) after 6-8 days of incubation at 37°C after transfection.

Purification, amplification, and determination of viral titre. The recovered virus was purified three times by plaque picking using a monolayer culture of Vero E6 cells incubated at 37°C and the virus was then amplified from one plaque using BHK$_{21}$ cells at 37°C and harvested 18 hours postinfection. The viral titre of rVSV$_{NJ}$-GMM-NS3/4A was determined by plaque assay using Vero E6 cells. Using the viral titre, BHK$_{21}$ cells were infected at multiplicity of infection (MOI) of 0.1 to propagate the recovered virus and attain a viral stock.

Growth kinetics of $rVSV_{NJ}$-GMM-NS3/4A. The growth kinetics of $rVSV_{NJ}$-GMM-NS3/4A was determined by infecting BHK_{21} cells with $rVSV_{NJ}$-GMM-NS3/4A at MOI of 0.1. The culture medium was harvested every 2 hours from 18 hours postinfection. The viral titres of samples were determined by plaque assay in Vero E6 cells.

Expression of NS3/4A protein in $rVSV_{NJ}$-GMM-NS3/4A. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM without insert and $rVSV_{NJ}$-GMM-NS34/A at MOI of 6, incubated at 37°C, harvested at 6 hours postinfection, and treated with Lysis Buffer prepared from 10 mM Tris-Cl adjusted to pH of 7.4, 1% Nonidet P40, 0.4% sodium deoxycholate, and 10 mM EDTA. The cell lysates were electrophoresed by 10% SDS-PAGE and the expression of NS3/4A proteins were analyzed by Western blot using rabbit monospecific polyclonal antibody against NS3, provided by the Kang laboratory, diluted to 1:3000, and polyclonal antibody against VSV_{NJ} (Choi, 1997) diluted to 1:5000. The samples were subsequently treated with goat anti-rabbit IgG secondary antibody (Sigma-Aldrich) diluted to 1:5000. The VSV and NS3/4A proteins were detected using ECL prime chemiluminescence Western blotting detection reagents (GE Life Sciences).

Comparison of cytopathogenesis. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM and $rVSV_{NJ}$-GMM-NS3/4A at MOI of 0.1 each. The control group was incubated in culture medium. Images were taken at 16 hours postinfection at 10X magnification. Standard deviation was used for the results to be expressed as mean ± SD.

Cleavage of HCV NS polyprotein junctions by NS3 protease with NS4A protein. BHK_{21} cells were individually infected with $rVSV_{NJ}$-GMM without insert, $rVSV_{NJ}$-GMM-NS3/4A, $rVSV_{NJ}$-GMM-NS5A/5B, and co-infected with $rVSV_{NJ}$-GMM-NS3/4A and $rVSV_{NJ}$-GMM-NS5A/5B at MOI of 6 each. The infected cells were incubated at 37°C, harvested, and lysed at 6 hours postinfection. The cell lysates from the infected cells were analyzed by 10%

SDS-PAGE followed by Western blot. The VSV and HCV proteins were detected by using rabbit monospecific polyclonal antibody against NS3 diluted to 1:3000, monoclonal antibody against NS5A (Abcam) diluted to 1:5000, and polyclonal antibody against VSV_{NJ} diluted to 1:5000. Following treatment with goat anti-rabbit IgG secondary antibody diluted to 1:5000, the protein bands were detected using ECL prime chemiluminescence Western blotting detection reagents.

Electron microscopy of $rVSV_{NJ}$-GMM-NS3/4A. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM-NS3/4A using MOI of 1 and incubated at 37°C. The supernatant of the infected BHK_{21} cells was harvested at 6 hours postinfection. The virus was concentrated by ultra-centrifugation, purified by 20% sucrose cushion in TNE, and negatively stained with 2% PTA adjusted to pH 6.8. The samples were viewed on Phillips CM10 transmission electron microscope (TEM) at 92000X magnification.

Results

Confirmation of DNA fragment using restriction enzyme *Nco* I. The plasmid $rVSV_{NJ}$-GMM-NS3/4A was constructed by using the restriction enzyme *Pme* I and *Mlu* I to clone *NS3/4A* genes into the *G* and *L* gene junction of the $rVSV_{NJ}$-GMM vector (Fig. 1). The plasmid was detected at a size greater than 10 kb (Fig. 2, lane 1). Following treatment with *Nco* I and separation by gel electrophoresis, the DNA fragments were detected at proper sizes (Fig. 2, lane 2).

Determination of viral titre. The $rVSV_{NJ}$-GMM-NS3/4A virus was recovered using reverse genetics, purified by three consecutive plaque assays, and amplified in BHK_{21} cells. The

viral titre was determined to be 2.75×10^8 plaque forming units (PFU)/ml after infecting BHK_{21} cells using MOI of 0.1 and harvesting the stock virus at 18 hours postinfection.

Growth kinetics of $rVSV_{NJ}$-GMM-NS3/4A. In order to assess viral replication of $rVSV_{NJ}$-GMM-NS3/4A, the kinetics of infectious virus particle production were examined. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM-NS3/4A at MOI of 0.1 and incubated at 37°C. The culture medium was harvested every 2 hours from 18 hours to 26 hours postinfection. The viral titre was determined by plaque assay using Vero E6 cells. The viral titres of $rVSV_{NJ}$-GMM-NS3/4A at 18, 20, 22, 24, and 26 hours postinfection were 1.2×10^8 PFU/ml, 2.1×10^8 PFU/ml, 2.1×10^8 PFU/ml, 2.5×10^8 PFU/ml, and 3.2×10^8 PFU/ml respectively (Fig. 3).

Protein expression level of HCV NS3/4A from $rVSV_{NJ}$-GMM. The expression of NS3 protein from BHK_{21} cells infected with $rVSV_{NJ}$-GMM-NS3/4A was assessed by Western blot using antibodies against NS3 (Fig. 4). The expression of $rVSV_{NJ}$-GMM proteins was confirmed by the proper sizes of G protein at 56 kDa, N protein at 46 kDa, and P protein at 30 kDa. The data also showed detection of NS3 using antibody against NS3 in the proper size of 69 kDa, but only in the cell lysates infected by $rVSV_{NJ}$-GMM-NS3/4A (Fig. 4, lane 5).

Comparison of cytopathogenesis. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM and $rVSV_{NJ}$-GMM-NS3/4A at MOI of 0.1 each. The structural changes in the infected cells were examined at 16 hours postinfection at 10X magnification. BHK_{21} cells infected with $rVSV_{NJ}$-GMM-NS3/4A showed lower level of cytopathic effects compared to those infected with $rVSV_{NJ}$-GMM (Fig. 5).

The cleavage of HCV polyprotein NS5A/5B by NS3/4A protein. The protease activity of the NS3 protein to cleave the junctions of NS5A and NS5B was evaluated by infecting BHK_{21} cells with $rVSV_{NJ}$-GMM without insert, $rVSV_{NJ}$-GMM-NS3/4A, $rVSV_{NJ}$-GMM-NS5A/5B, and

co-infecting BHK_{21} cells with $rVSV_{NJ}$-GMM-NS3/4A and $rVSV_{NJ}$-GMM-NS5A/5B at MOI of 6 each. The cell lysates were electrophoresed by 10% SDS-PAGE followed by Western blot using antibodies against NS3 and NS5A. The polyprotein NS5A/5B was detected at 126 kDa in cell lysates infected with $rVSV_{NJ}$-GMM-NS5A/5B (Fig. 6, lane 6) using an antibody against NS5A. The proper size of the NS5A cleaved from the polyprotein NS5A/5B was confirmed to be 58 kDa, but only when BHK_{21} cells were co-infected with $rVSV_{NJ}$-GMM-NS3/4A and $rVSV_{NJ}$-GMM-NS5A/5B (Fig. 6, lane 7). Therefore, the function of NS3/4A protein complex as a serine protease was confirmed by examining its activity to cleave the NS5A/5B junction.

Electron microscopy of $rVSV_{NJ}$-GMM-NS3/4A. The production and morphology of virus particles were examined by infecting BHK_{21} cells with $rVSV_{NJ}$-GMM-NS3/4A at MOI of 1, harvesting the culture medium at 16 hours postinfection. The virus particles were concentrated, negatively stained, and viewed on Phillips CM10 TEM at 92000X magnification. The image showed bullet-shaped virions characteristic of normal rhabdovirus morphology with the presence of glycoproteins and electron-dense nucleocapsid bound by an envelope (Fig. 7).

Discussion

Despite the advances in the treatment of infectious diseases, there is currently no efficacious vaccine against HCV. The development of HCV vaccine using traditional approaches has been challenging since HCV does not replicate efficiently in cell cultures (Pietschmann et al., 2002). The attenuated and replication-competent recombinant VSV is an attractive vaccine platform for use in humans due to its immunogenicity, high gene expression, and capacity to accommodate large foreign inserts (An et al., 2013; Cobleigh et al., 2013; Ezelle et al., 2002). VSV has mild pathogenicity in humans and is safe since it does not integrate its genome into the

host cell DNA or recombine with the wild-type virus *in vivo* (Lawson, Stillman, Whitt, & Rose, 1995). In this study, the M protein of VSV was modified with three mutations (G22E+M48R+M51R) to reduce the inhibition of host gene expression and cytopathic effects since previous studies have shown that genetic manipulation of VSV can improve its safety and immunogenicity while maintaining its efficiency in introducing the insert (Flanagan, Zamparo, Ball, Rodriguez, & Wertz, 2001; Kim & Kang, 2007).

Previous studies have demonstrated that mutations in VSV vectors could lead to slower growth rates and lower peak titers (Cooper et al., 2008; Roberts, Buonocore, Price, Forman, & Rose, 1999). The reductions in growth rates and peak infectious particle production can be attributed to mutations that directly affect replication efficiency and virion formation. In this study, $rVSV_{NJ}$-GMM-NS3/4A achieved a viral titre of 2.75×10^8 PFU/ml after infecting BHK_{21} cells using MOI of 0.1 and harvesting the stock virus at 18 hours postinfection. The peak titre was measured to be 3.2×10^8 PFU/ml at 26 hours postinfection using MOI of 0.1 in BHK_{21} cells. Similarly using the former vector $rVSV_{NJ}$-M (M48R+M51R), the viral titre of $rVSV_{NJ}$-M-NS3/4A stock virus was shown to be 5.6×10^8 PFU/ml using MOI of 0.1 in BHK_{21} cells at approximately 16 to 18 hours postinfection (An et al., 2013). From infecting BHK_{21} cells at MOI of 10, the viral titre of the wild-type VSV_{NJ} was shown to be approximately 10^9 PFU/ml 8 hours postinfection (Kretzschmar, Peluso, Schnell, Whitt, & Rose, 1996). The $rVSV_{NJ}$-GMM without insert previously demonstrated replication efficiency of approximately10^9 PFU/ml from infecting BHK_{21} cells using MOI of 3, and harvesting the culture medium 8 hours postinfection (Kim et al., 2015). The altered $rVSV_{NJ}$-GMM-NS3/4A showed slower growth, but achieved a high viral titre despite attenuation by genetic modifications. The attenuation of the altered virus was confirmed by examining cytopathogenic effects in BHK_{21} cells as characterized by the rounding of infected

cells. Previously, infection of BHK_{21} cells using wild-type VSV_{NJ} showed extensive cell rounding and detachment; however, $rVSV_{NJ}$-GMM infected cells showed reduced structural changes (Kim et al., 2015). The reduction in cytopathogenesis was similarly demonstrated in this study with decreased CPE in BHK_{21} cells infected with either $rVSV_{NJ}$-GMM without insert or $rVSV_{NJ}$-GMM-NS3/4A. The cells infected with the latter construct showed greater attenuation.

The genetically modified $rVSV_{NJ}$-GMM vector encoding NS3/4A was capable of expressing functional viral proteins. The high expression level of viral proteins was demonstrated by clear Western blot bands and the capacity to express functional protein was shown by confirming the serine protease function of the NS3 protein in the NS3/4A complex to cleave the NS5A/5B polyprotein junction. Similar results were reported using the $rVSV_{NJ}$-M vector to express NS3/4A proteins and confirm the function of NS3 as a protease (An et al., 2013). The M gene of VSV_{NJ} was modified by changing methionine to arginine at positions 48 and 51. Introducing mutations by changing one or two nucleotides in the amino acid codon of the $rVSV_{NJ}$-M vector raised concerns regarding potential reversion of VSV to the wild-type phenotype with multiple passages. However, $rVSV_{NJ}$-GMM used in this study was modified from the former vector with additional mutations in the M protein gene with changes in all three nucleotides to generate a much more stable vector. A recent study has demonstrated that additional nucleotide changes in a codon increased genetic stability of mutations *in vitro* (Kim et al., 2015). Despite multiple consecutive passages, the mutations in the M protein gene of $rVSV_{NJ}$-GMM vector did not convert back to the wild-type amino acid codon. Although both $rVSV_{NJ}$-M and $rVSV_{NJ}$-GMM vector systems were able to achieve high protein expression levels, the $rVSV_{NJ}$-GMM vector showed greater stability and attenuation with triple mutations, mitigating safety concerns associated with using replication-competent vectors. Furthermore,

VSV allows for potential prime-boost immunization strategies since both serotypes, Indiana and New Jersey, have the capacity to stably express the HCV inserts and achieve high protein expression levels without exhibiting cross-neutralization (Kim et al., 2015).

The electron microscopic examination showed bullet-shaped virus particles of $rVSV_{NJ}$-GMM-NS3/4A with glycoproteins projecting from viral envelopes. The virion structures were consistent with normal rhabbdovirus morphology indicating that insertion of the foreign gene did not alter particle formation.

Some of the challenges involved in HCV vaccine development have been associated with the tendency of HCV to mutate as it replicates. It has been demonstrated that high genetic variation of the virus allows it to elude pre-existing immunity, and infections persist by escaping host immune responses (Forns, Bukh, & Purcell, 2002; Martell et al., 1992). The NS3 and NS4A proteins have been identified as ideal antigens to address the barriers to HCV vaccine development. The NS3 protein is highly conserved among strains which may help protect against escape mutants that persist during chronic infections. It also carries multiple CD4+ and CD8+ T cell epitopes and is the major target of T cell-mediated immunity which has been shown to mediate HCV viral control in natural infection (Ratnoglik et al., 2014). The NS4 protein is a required cofactor for the protease function of NS3 and the inclusion of NS4A in NS3-based genetic vaccines have shown to enhance the immunogenicity of NS3 (Zhu et al., 2015).

The stability and high levels of the NS3/4A protein expression indicate the potential use of the $rVSV_{NJ}$-GMM vector for vaccine purposes. The development of a safe, affordable, and effective HCV vaccine can replace expensive antiviral therapies that are limited in developing countries and potentially reduce the overall disease burden of HCV infections.

Acknowledgements

I thank my supervisors Dr. Chil-Yong Kang and Dr. Hwa-Yong An for their guidance and encouragement. This work performed in the Kang laboratory at Siebens-Drake Research Institute was supported by Canadian Institutes of Health Research, NHRDP of Health Canada, IRAP of the National Research Council Canada, Sumagen Co. Ltd., and Western University.

Appendix

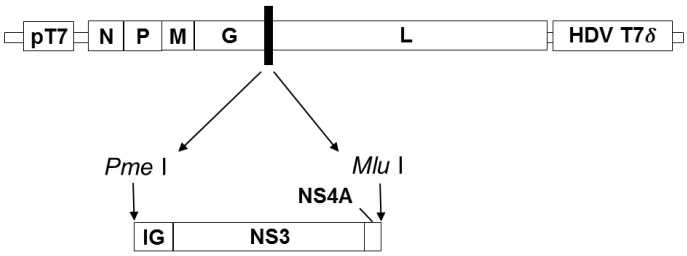

Fig.1. Construction of prVSV$_{NJ}$-GMM-NS3/4A. The plasmid prVSV$_{NJ}$-GMM-NS3/4A was constructed by using the restriction enzymes *Pme* I and *Mlu* I to clone *NS3/4A* genes into the *G* and *L* gene junction of the rVSV$_{NJ}$-GMM vector.

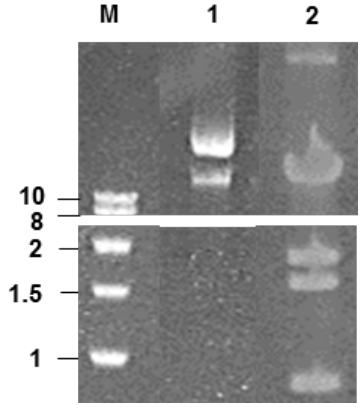

Fig. 2. Confirmation of DNA fragment using restriction enzyme *Nco* I. The plasmid was detected (lane 1) and treated with *Nco* I, to confirm its proper size by gel electrophoresis (lane 2).

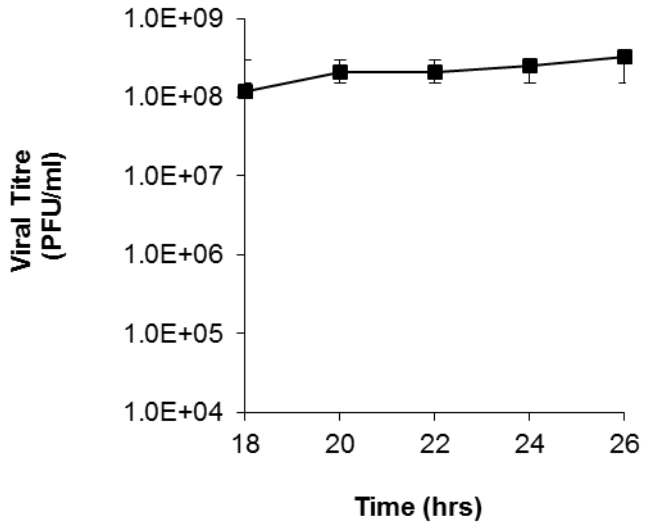

Fig. 3. Growth kinetics of $rVSV_{NJ}$-GMM-NS3/4A. In order to assess viral replication of $rVSV_{NJ}$-GMM-NS3/4A, the kinetics of infectious virus particle production were examined. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM-NS3/4A at MOI of 0.1 and incubated at 37°C. The culture medium was harvested every 2 hours from 18 hours to 26 hours postinfection. The viral titre was determined by three consecutive plaque assays using Vero E6 cells. Standard deviation was used for the results to be expressed as mean ± SD.

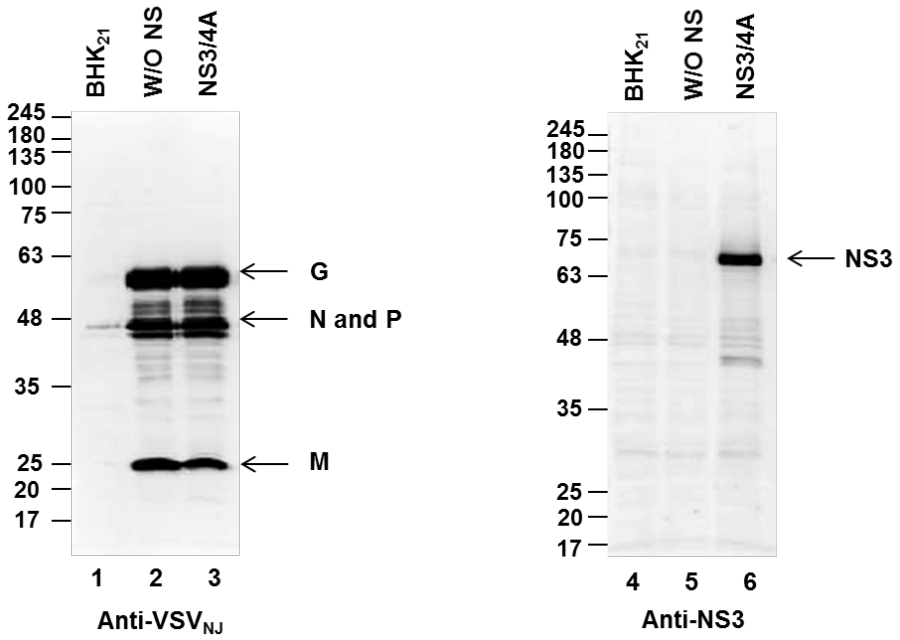

Fig. 4. Protein expression level of VSV_{NJ} and HCV NS3. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM and $rVSV_{NJ}$-GMM-NS3/4A at MOI of 6. Cell lysates were harvested at 6 hours post-infection, electrophoresed using 10% SDS-PAGE, and analyzed for VSV_{NJ} proteins in lanes 1 to 3 or for NS3 proteins in lanes 4 to 6.

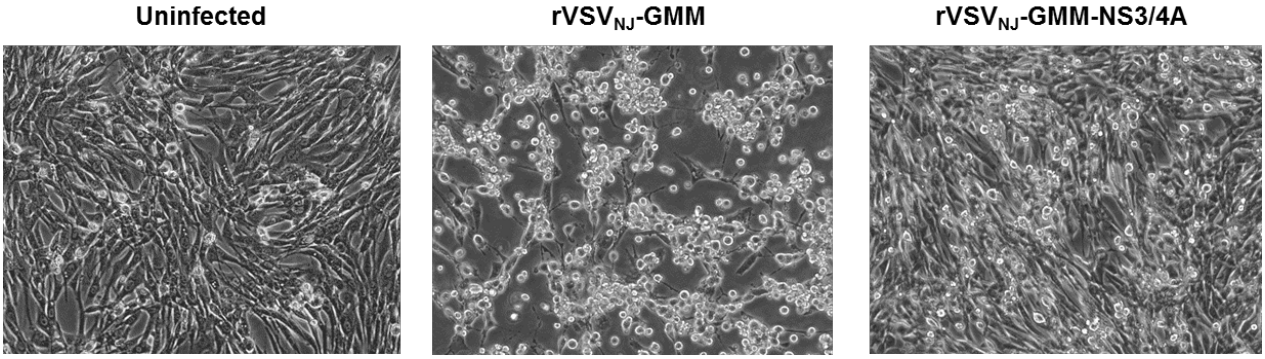

Fig. 5. Comparison of cytopathic effects. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM and $rVSV_{NJ}$-GMM-NS3/4A at MOI of 0.1 each. The structural changes in the infected cells were examined at 16 hours postinfection at 10X magnification.

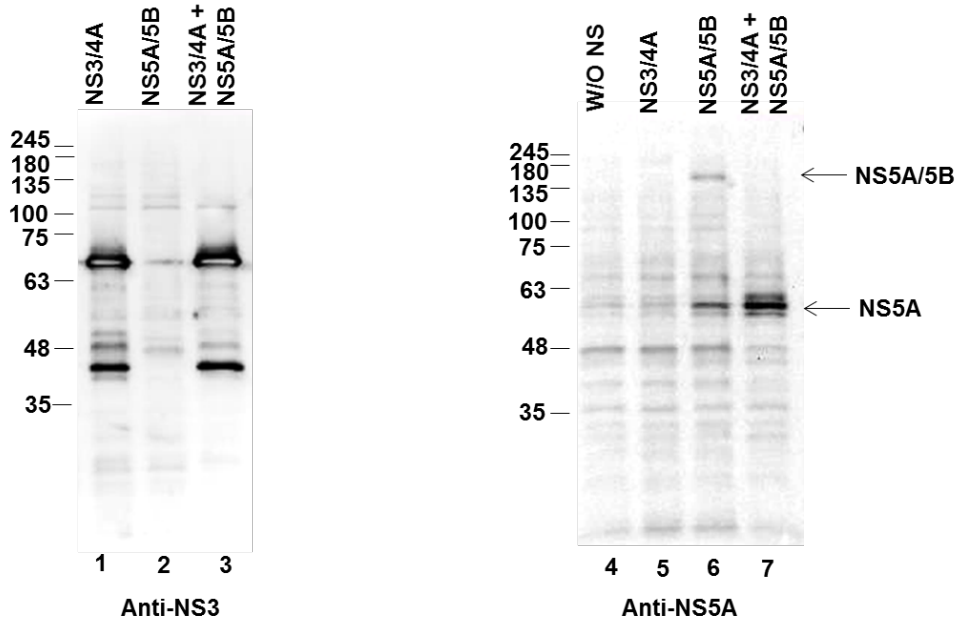

Fig. 6. Cleavage of HCV polyprotein NS5A/5B by NS3/4A protein. BHK_{21} cells were infected with $rVSV_{NJ}$-GMM-NS3/4A or $rVSV_{NJ}$-GMM-NS5A/5B or co-infected with both using MOI of 6. The infected cells were harvested at 6 hours post-infection and the cell lysates were separated by 10% SDS-PAGE and analyzed by Western blot to detect NS3 in lanes 1 to 3 or NS5A proteins in lanes 4 to 7.

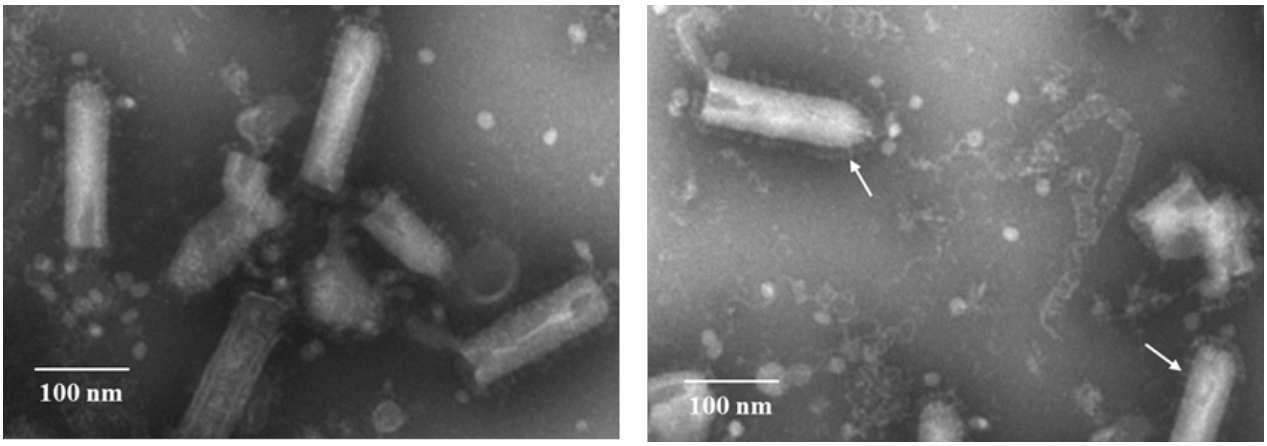

Fig. 7. Electron microscopic examination of rVSV$_{NJ}$-GMM-NS3/4A. BHK$_{21}$ cells were infected with rVSV$_{NJ}$-GMM-NS3/4A at MOI of 1. The culture medium was harvested 16 hours postinfection. The sample was purified by 20% sucrose in TNE, resuspended in TNE, and negatively stained with 2% phosphotungstic acid. The image was viewed on Phillips CM10 transmission electron microscope at 92000X magnification. The glycoproteins present on the virions are indicated by the white arrows.

References

Ahlén, G., Holmström, F., Gibbs, A., Alheim, M., & Frelin, L. (2014). Long-term functional duration of immune responses to HCV NS3/4A induced by DNA vaccination. *Gene Therapy*, *21*(8), 739–750. http://doi.org/10.1038/gt.2014.48

An, H.-Y., Kim, G. N., Wu, K., & Kang, C. Y. (2013). Genetically modified VSVNJ vector is capable of accommodating a large foreign gene insert and allows high level gene expression. *Virus Research*, *171*(1), 168–177. http://doi.org/10.1016/j.virusres.2012.11.007

Arribillaga, L., de Cerio, A. L. D., Sarobe, P., Casares, N., Gorraiz, M., Vales, A., … Lasarte, J. J. (2002). Vaccination with an adenoviral vector encoding hepatitis C virus (HCV) NS3 protein protects against infection with HCV-recombinant vaccinia virus. *Vaccine*, *21*(3-4), 202–10. http://doi.org/10.1016/S0264-410X(02)00456-5

Black, B. L., & Lyles, D. S. (1992). Vesicular stomatitis virus matrix protein inhibits host cell-directed transcription of target genes in vivo. *Journal of Virology*, *66*(7), 4058–4064.

Blondel, D., Harmison, G. G., & Schubert, M. (1990). Role of matrix protein in cytopathogenesis of vesicular stomatitis virus. *Journal of Virology*, *64*(4), 1716–1725. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2157054

Buchholz, U. J., Finke, S., & Conzelmann, K. K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *Journal of Virology*, *73*(1), 251–259.

Choi, W. Y. (1997). Molecular biological characterization of defective interfering particles of vesicular stomatitis virus. University of Ottawa, Ottawa. Canada.

Cobleigh, M. A., Wei, X., & Robek, M. D. (2013). A vesicular stomatitis virus-based therapeutic vaccine generates a functional CD8 T cell response to hepatitis B virus in transgenic mice. *Journal of Virology*, *87*(5), 2969–73. http://doi.org/10.1128/JVI.02111-12

Cooper, D., Wright, K. J., Calderon, P. C., Guo, M., Nasar, F., Johnson, J. E., … Clarke, D. K. (2008). Attenuation of recombinant vesicular stomatitis virus-human immunodeficiency virus type 1 vaccine vectors by gene translocations and g gene truncation reduces neurovirulence and enhances immunogenicity in mice. *Journal of Virology*, *82*(1), 207–19. http://doi.org/10.1128/JVI.01515-07

Ezelle, H. J., Markovic, D., & Barber, G. N. (2002). Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector. *Journal of Virology*, *76*(23), 12325–12334. http://doi.org/10.1128/JVI.76.23.12325

Farci, P., Shimoda, A, Coiana, A, Diaz, G., Peddis, G., Melpolder, J. C., … Alter, H. J. (2000). The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. *Science*, *288*(5464), 339–344. http://doi.org/10.1126/science.288.5464.339

Flanagan, E. B., Zamparo, J. M., Ball, L. A., Rodriguez, L. L., & Wertz, G. W. (2001). Rearrangement of the genes of vesicular stomatitis virus eliminates clinical disease in the natural host: new strategy for vaccine development. *Journal of Virology*, *75*(13), 6107–14. http://doi.org/10.1128/JVI.75.13.6107-6114.2001

Forns, X., Bukh, J., & Purcell, R. H. (2002). The challenge of developing a vaccine against hepatitis C virus. *Journal of Hepatology*, *37*, 684–695. http://doi.org/10.1016/S0168-8278(02)00308-2

Gould, A. R., Hyatt, A. D., Lunt, R., Kattenbelt, J. A., Hengstberger, S., & Blacksell, S. D. (1998). Characterisation of a novel lyssavirus isolated from Pteropid bats in Australia. *Virus*

Research, *54*(2), 165–187. http://doi.org/10.1016/S0168-1702(98)00025-2

Horner, S. M., & Gale, M. (2013). Regulation of hepatic innate immunity by hepatitis C virus. *Nature Medicine*, *19*(7), 879–88. http://doi.org/10.1038/nm.3253

Jacobson, I. M., Davis, G. L., El-Serag, H., Negro, F., & Trépo, C. (2010). Prevalence and challenges of liver diseases in patients with chronic hepatitis C virus infection. *Clinical Gastroenterology and Hepatology, 8*(11), 924–33; quiz e117. http://doi.org/10.1016/j.cgh.2010.06.032

Kim, G. N., & Kang, C. Y. (2007). Matrix protein of VSV New Jersey serotype containing methionine to arginine substitutions at positions 48 and 51 allows near-normal host cell gene expression. *Virology*, *357*(1), 41–53. http://doi.org/10.1016/j.virol.2006.07.022

Kim, G. N., Wu, K., Hong, J. P., Awamleh, Z., & Kang, C. Y. (2015). Creation of matrix protein gene variants of two serotypes of vesicular stomatitis virus as prime-boost vaccine vectors. *Journal of Virology*, *89*(12), 6338–6351. http://doi.org/10.1128/JVI.00222-15\rJVI.00222-15 [pii]

Kretzschmar, E., Peluso, R., Schnell, M. J., Whitt, M. A, & Rose, J. K. (1996). Normal replication of vesicular stomatitis virus without C proteins. *Virology*, *216*(2), 309–316. http://doi.org/10.1006/viro.1996.0066

Lawson, N. D., Stillman, E. A, Whitt, M. A, & Rose, J. K. (1995). Recombinant vesicular stomatitis viruses from DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *92*, 4477–4481. http://doi.org/10.1073/pnas.92.19.9009c

Liang, T. J. (2013). Current progress in development of hepatitis C virus vaccines. *Nature Medicine*, *19*(7), 869–878. http://doi.org/10.1038/nm.3183

Martell, M., Esteban, J. I., Quer, J., Genescà, J., Weiner, A., Esteban, R., … Gómez, J. (1992).

Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. *Journal of Virology*, *66*(5), 3225–9. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=241092&tool=pmcentrez&rendertype=abstract

Mohd Hanafiah, K., Groeger, J., Flaxman, A. D., & Wiersma, S. T. (2013). Global epidemiology of hepatitis C virus infection: New estimates of age-specific antibody to HCV seroprevalence. *Hepatology*, *57*(4), 1333–1342. http://doi.org/10.1002/hep.26141

Pietschmann, T., Lohmann, V., Kaul, A., Krieger, N., Rinck, G., Rutter, G., … Bartenschlager, R. (2002). Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *Journal of Virology*, *76*(8), 4008–21. http://doi.org/10.1128/JVI.76.8.4008-4021.2002

Ratnoglik, S. L., Jiang, D., Aoki, C., Sudarmono, P., Shoji, I., Deng, L., & Hotta, H. (2014). Induction of cell-mediated immune responses in mice by DNA vaccines that express hepatitis C virus NS3 mutants lacking serine protease and NTPase / RNA helicase activities, *PLoS One, 9*(6), 1–9. http://doi.org/10.1371/journal.pone.0098877

Roberts, A., Buonocore, L., Price, R., Forman, J., & Rose, J. K. (1999). Attenuated vesicular stomatitis viruses as vaccine vectors. *Journal of Virology*, *73*(5), 3723–3732.

Shiryaev, S. A, Thomsen, E. R., Cieplak, P., Chudin, E., Cheltsov, A. V, Chee, M. S., … Strongin, A. Y. (2012). New details of HCV NS3/4A proteinase functionality revealed by a high-throughput cleavage assay. *PLoS One*, *7*(4), e35759. http://doi.org/10.1371/journal.pone.0035759

Simmonds, P., Bukh, J., Combet, C., Deléage, G., Enomoto, N., Feinstone, S., … Widell, A.

(2005). Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology*. http://doi.org/10.1002/hep.20819

Timpe, J. M., Stamataki, Z., Jennings, A., Hu, K., Farquhar, M. J., Harris, H. J., … McKeating, J. A. (2008). Hepatitis C virus cell-cell transmission in hepatoma cells in the presence of neutralizing antibodies. *Hepatology*, *47*(1), 17–24. http://doi.org/10.1002/hep.21959

Zhu, F., Chen, T., Zhang, Y., Sun, H., Cao, H., Lu, J., & Zhao, L. (2015). A novel adeno-associated virus – based genetic vaccine encoding the hepatitis C virus NS3 / 4 protein exhibits immunogenic properties in mice superior to those of an NS3-protein-based vaccine, *PLoS One*, *10*(11), 1–15. http://doi.org/10.1371/journal.pone.0142349