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Enhancement of Vesiculovirus Oncolysis by Expression of Exotic Envelope Proteins

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ENHANCEMENT OF VESICULOVIRUS ONCOLYSIS BY EXPRESSION OF EXOTIC ENVELOPE PROTEINS.

By

Christopher W. Brown

A Thesis Submitted to the Faculty of Graduate Studies in Partial Fulfillment of the Requirements of the Degree of

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Abstract

Viral treatment of cancer has been described since the beginning of the twentieth century. With the increasing need for effective targeted cancer therapies, the oncolytic virus platform has been revisited. Clinical trials have demonstrated that oncolytic viruses are well tolerated, but their ability to eliminate tumor burden or effectuate cures is poor. This thesis attempts to address enhancing the oncolytic virus efficacy. The strategy is to modulate characteristics of the rhabdovirus by adding or swapping envelope genes of interest in order to enhance oncolytic activity, specifically by addressing tumor spread, immune evasion and safety. The fusogenic protein from reovirus is used to generate a recombinant VSV to enhance viral spread within the tumor. Similarly, recombinant VSVs are generated with envelope proteins either from other vesiculovirus members or from a beta-retrovirus, and used to demonstrate tumor infectivity in the presence of neutralizing antibodies. The recombinant VSV expressing the beta-retrovirus envelope protein demonstrates altered viral tropism, and like the replication incompetent VSV/FAST recombinant is not neurotoxic in animal models.

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John, thank you for granting me this opportunity and introducing me to the exciting field of oncolytic viruses. To Harry, the OHRI and the rest of the crew that support the man – thank you for your insight, time and resources.

To my Bethany Lynn, and my soldiers, Ayden, Connor and Cole, I thank you once again for allowing me to pursue my curiosity in the hopes that someday everyone will benefit.

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List of Abbreviations

3D – 3 Dimensions
5FU – 5-Fluorocytosine
aa – Amino Acids
Cha – Chandipura
CAR – Coxsackie Adedovirus Receptor
CD – Cytosine Deaminase
CPE – Cytopathic Effect
CRT – Conformal Radiation Therapy
CTL – Cytotoxic T-Cells
ddH2O – double distilled water
DNA – Deoxyribonucleic acid
EEV – extra-cellular envelope virus (form of Vaccinia virus)
F – fusion glycoprotein from RSV
FBS – Fetal Bovine Serum
FL – Firefly Luciferase
G – glycoprotein
GaLV – Gibbon ape leukemia virus
GFP – Green Fluorescent Protein
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLP – Hind Limb Paralysis
HSV – Herpes-Simplex Virus
IC – Intracranial
IFN – Interferon
IHC – Immunohistochemistry
IL – Interleukin
IN – Intranasal
IND – Indiana
Isf – Isfahan
IV – Intravenous
IVIS – In Vivo Imaging System
JSRV – Jaagsiekte Sheep Retrovirus
kb – Kilobases
kd – Kilodalton
L – polymerase
M – matrix
Mar – Maraba
mg – Miligrams
ml – Milliliters
MOI – Multiplicity of Infection
mRNA – Messenger RNA
N – nucleocapsid
NHP – Non-Human Primate
NJ – New Jersey

OV – Oncolytic Virus
P – phsophoprotein
PBS – phosphate buffered saline
PFU – Plaque Forming Units
PS – phosphotidylserine
RFP – Red Fluorescent Protein and is mRFP1 in this work
RNA – Ribonucleic acid
RSV – Respiratory syncytia virus
TK – Thymidine Kinase
ul – Microliters
VSV – Vesicular Stomatitis Virus
WT – Wild Type
YTDIEM – Tyrosine, Threonine, Aspartic acid, Isoleucine, Glutamic acid, Methionine

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General Introduction

The field of oncolytic viruses is reemerging. Advances in understanding the biology of the virus and the virus-cancer cell interaction have allowed this platform to progress beyond clinical trials. However, the efficacy of the oncolytic virus as measured in terms of regression, remission or cure of human cancer, must significantly improve. Intra-tumor spread of the virus is restricted, furthermore, repetitive dosing to enhance the oncolytic activity is limited due to the production of antibodies which neutralize some oncolytic viruses; and while the side effects of most oncolytic viruses are relatively benign, this must continuously be ensured.

The vesiculoviruses of the rhabdovirus family have potent oncolytic activity and have demonstrated cures in several animal models. Although still in the pre-clinical phase, members of the vesiculoviruses display oncolytic characteristics much like the oncolytic viruses currently used in human clinical trials[252,369]. Human clinical trials have identified barriers to current oncolytic virotherapy, namely inefficient viral spread within tumors, neutralization by host antibodies and safety. The theme of this body of work is to express exotic viral envelope proteins in a recombinant vesicular stomatitis virus in order to obtain oncolytic efficacy in circumstances under which the parent vesicular stomatitis virus may be toxic or ineffective. Three of the resulting outcomes: the effect on viral spread, viral evasion of the host immune system, and elimination of neurotoxicity – form the individual chapters herein and are preceded by a detailed overview of the field.

Background

Oncolytic Viruses are Biological Cancer Killing Machines.

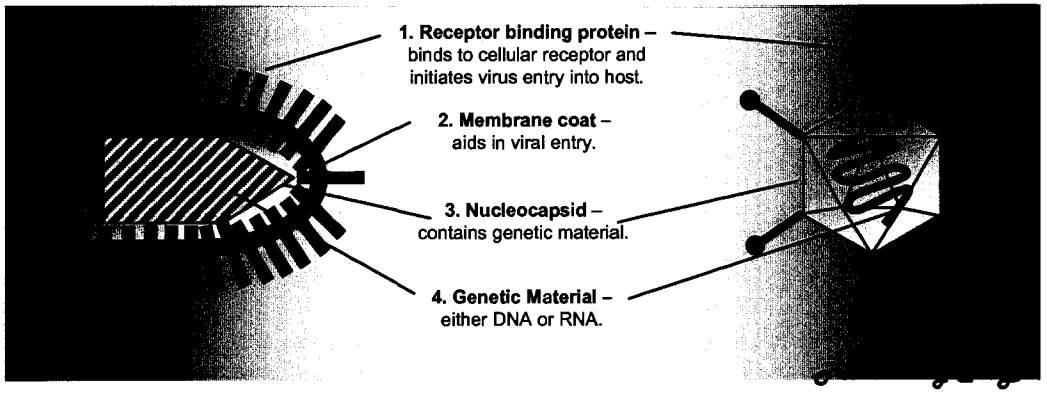
Oncolytic is a term meaning "the destruction of a tumor mass or cancer", and viruses which have the ability of infecting and killing tumor cells and leaving normal cells unharmed are known as oncolytic viruses (OV)[5,60,116,231,280,306,311,358,368]. There are naturally occurring oncolytic viruses while other viruses have been converted into oncolytic virus in the laboratory by genetic engineering or selection[311,354] (Table 1). Oncolytic viruses, like most viruses, consist of some basic components (Fig.1). They have a protein coat called a capsid that in some species of viruses is covered by an additional lipid bi-layer, called a membrane coat or envelope. These outer layers bear proteins that bind to cellular receptors, the coat also serves to protect the genetic material and some other viral proteins[127,159]. The genetic material can encode a plethora of genes depending on the type of virus, but in general a virus will encode 3 groups of genes: those that help produce the structural proteins from which the virus is assembled, those that help with replication, and those that encode virulence factors which help the virus overcome its host's defense systems[159,389]. It should be noted that some viruses also take advantage of the host proteins to help with their own replication [200,257]. Before focusing on the details of the life cycle of a vesiculovirus, an overview of the types of oncolytic viruses is required.

Table 1. Oncolytic viruses and summary of clinical trials.

Virus (Family)	***Tumor Selection Mechanism***	*Commercial Name*	*Trial Status*	*Target Cancer*	***References***
(Adenovirus)	Infect cells expressing CAR and serotype switching reduces non-cancer infection. In addition, deletion of the E1B gene confines replication to cancer cells.	ONYX-015 CV706 AD5-CD/TK H101	I,II,III H101 Approved in China 15 trials 8 with chemo	HNSCC, oral SCC, GBM, pancreas, ovarian, HCC, hepatobiliary mets, CRC, sarcoma, liver, CRC liver mets, lung mets, mets, prostate	61,89,124,125,135,139,153-155,158,163,195,259,262,267-269,303,304,397,403,419,420,424
Herpes Simplex Virus (Herpesvirus)	Deletion of specific genes confines replication to rapidly dividing cells.	G207 1716 Onco-VEX NV1020	I, II 6 trials	GBM, melanoma, subcutaneous mets, CRC liver mets	160,240,243,279,343
Vaccinia (Poxvirus)	Seeping out of vasculature at the tumor site allows this virus access to cancer cells.	Dryvax JX-594 NYCBOH	I, II 3 trials	Bladder, melanoma, mesothelioma	146,247,261
Newcastle Disease Virus (Paramyxovirus)	Infects interferon deficient cells and induces TNF-α which enhances anti tumor immune response.	PV 701 OV 001 MTH-68	I, II 3 trials	GBM, CRC, HNSCC, anal	5,82,122,233,285,311
Coxsackievirus (Picornavirus)	Infects tumors that over-express the DAF receptor.	CA21	I 1 trial	Melanoma	357
Reovirus (Orthoreovirus)	Replicates in cells that over-express oncogenic RAS and/or interferon deficient cells.	Reolysin	I 1 trial	Palpable cancerous tumors	260
Measles (Paramyxovirus)	Binds and infects cells with the CD46 and SLAM receptor.	WT MV-CEA	I	Non-Hodgkin's lymphoma, Hodgkin's lymphoma, leukemia	231
Mumps (Paramyxovirus)	Uses sialic acid-containing receptors for attachment and also has neuraminidase activity.	NA	Case series	Breast, cervical	11,231
West Nile Virus (Flavivirus)	The αvβ3 integrin and the lectins DC-SIGN and DC-SIGNR are potential receptors.	NA	Case series	Non-Hodgkin's lymphoma	231,359
Vesicular Stomatitis Virus (Rhabdovirus)	Infects interferon deficient cells.	NA	Pre-clinical animal models	NA	368,369
Myxoma (Poxvirus)	Replicates in STAT and/or interferon deficient cells.	NA	Pre-clinical animal models	Rhabdoid tumors	364,407,418
Influenza (Orthomyxovirus)	Deletion of specific gene allows for replication in interferon defective cells.	NA	Pre-clinical animal models	NA	264
Spumavirus Foamy Virus (Retrovirus)	Integrates into non active gene regions, otherwise no clear tumor cell preference.	NA	Pre-clinical	GBM	391
Moloney murine* leukemia virus (Retrovirus)	Selectively transduces rapidly dividing cells. Replication incompetent virus used for clinical trials, and replication competent used at the pre-clinical phase.	NA	I, II & pre-clinical animal model	GBM	68,391

HNSCC, head and neck squamous cell carcinoma; SCC, squamous cell carcinoma; HCC, hepatocellular carcinoma; CRC, colorectal carcinoma; Mets, metastatic disease; GBM, glioblastoma multiform; DAF, decay accelerating factor; CAR, coxsackie adenovirus receptor; SLAM: signaling lymphocyte activation molecule (CD150); TNF-α, tumor necrosis factor alpha; NA, not available or not applicable. *Unlike the other oncolytic viruses, the Moloney murine leukemia virus is not oncolytic per se but is used as a vector to introduce oncolytic suicide genes.

Figure 1. Basic components of an oncolytic virus.
Components shared by most viruses include the receptor binding protein which binds to the host cell, whereas the membrane coat and the nucleocapsid enclose the genetic material. Depicted are the prototypical vesiculovirus (left) and adenovirus (right).


1. Receptor binding protein – binds to cellular receptor and initiates virus entry into host.
2. Membrane coat – aids in viral entry.
3. Nucleocapsid – contains genetic material.
4. Genetic Material – either DNA or RNA.

Oncolytic Viruses – Meet The Players.

As previously mentioned, there are many different oncolytic viruses, varying in multiple characteristics such as size, shape, type of genetic material, number of genes, pathogenesis, and oncolytic activity to name but a few differentiating characteristics[159,200,210,315,322,404]. To date, the identified oncolytic DNA viruses consist of Adenovirus, Herpes-Simplex Virus (HSV) and Vaccinia virus[46,194,230,231,280,306], all of which are at risk of having their genome integrated into that of the host and induce cellular oncogenic transformation[270]. On the other hand, identified oncolytic RNA viruses consist of Coxsackie A21, West Nile virus, Reovirus, Newcastle Disease Virus, Measles, Mumps and Rhabdoviruses[46,194,230,231,280,306], and have not been reported to induce cellular transformation, unlike retroviruses[270]. Otherwise, non-oncolytic RNA viruses, such as the picornaviridae, dicistroviridae, flavivirus, and potyvirus have been reported to integrate their genome into the host without inducing cellular transformations[80,241,376].

Vesiculoviruses

The family of Rhabdoviridae belong to the order of the *Mononegavirales* which also include members of the *Paramyxoviridae*, *Filoviridae* and *Bornaviridae*. The family is divided into 6 genera: *Nucleorhabdovirus, Cytorhabdovirus, Novirhabdovirus, Ephemerovirus, Lyssavirus and Vesiculovirus*[263]. Rhabdoviruses have a broad distribution in nature, infecting plants, invertebrates and vertebrates. Of the more than 160 Rhabdoviruses, the

vesiculoviruses consist of at least 14 and they are clustered in two main geographic regions, the Americas and the Eastern hemisphere[64,127,404]. Infections are restricted to biting insects which act as the major reservoir and vector; however, infection of mammals does occur but is usually self-limiting and often results in a dead end transmission of the virus[64,222,404] however there have been reports of transmission between mammals[222,283].

Vesicular stomatitis virus (VSV) (New Jersey and Indiana serotypes)[76] Isfahan, Chandipura and Maraba are species within the *Vesiculovirus* genus and present a platform of potentially therapeutic oncolytic viruses[49,404]. VSV, the prototype vesiculovirus and the focus of this body of work, was first isolated in the Western hemisphere and is spread by biting insects in subtropical regions where it causes a relatively mild disease in farm animals characterized by lesions on the oral mucosa and in humans has been reported to cause influenza like illness[222,404]. When used to infect lab mice in experimental models, VSV displays a strong neurotropism and neurovirulence and can lead to lethal encephalitis, especially following intranasal administration - a characteristic not seen in cows, horses or pigs[64,74,222,227].

Although VSV is the platform on which this work is focused, the glycoprotein components of other vesiculoviruses are utilized and so a brief description these viruses follows. Chandipura was isolated in humans suffering from a febrile illness, it has also been isolated in outbreaks of encephalitis in

children, involving 329 cases with 183 deaths, from Andhra Pradesh State, India in 2003[55,302] and has also been isolated in sandflies[90]. Isfahan virus has been identified in sandflies, gerbils and humans in several regions of Iran. Its animal pathogenicity, growth rate, cytopathic effect, and plaque morphology are similar to other VSV serotypes[380]. Maraba was isolated from phlebotomine sand flies (Lutzomyia spp.) collected in the Amazon basin of Brazil, the pathogenicity of the virus in mice and Vero cells is similar to that of VSV-Indiana and VSV-New Jersey[84,147].

Vesiculoviruses have a compact genome of ~11-12 kb comprising of 5 or 6 genes which are all contained on a single negative sense RNA genome. The genes consist of the nucleocapsid (N) gene, the phosphoprotein (P) gene also known as the non-structural protein (NS), the matrix (M) gene, the glycoprotein (G) gene, and the large (L) protein or polymerase gene[18,19,127,379,404]. In some rhabdoviruses, such as fish infectious hematopoietic necrosis virus, bovine ephemeral fever virus, rabies virus, and sonchus yellow net virus an additional sixth gene is present and preliminary studies suggest they encode for a nonstructural gene, a nonstructural glycoprotein, a pseudogene and the sc4 nonstructural gene respectively[162,170,209,339,404]. Similarly, the P gene also contains an alternate start codon which encodes for a C and C' protein which are expressed in the cell cytoplasm but not in the virion. The functions of the C and C' proteins are unclear, but may play a role in virus transmission or pathogenesis in hosts since the kinetics of virus replication are unaffected when this gene is

eliminated[127,165,207,360,362,404]. During transcription of the RNA genome into the mRNAs by the RNA polymerase, there is a pause between the genes at the intergenic "stop-start" region[336], causing a 30% decrease in transcription efficiency at each junction and resulting in a graded production of mRNA following the gene order such that N>P>M>G>L[127,185,398,404].

The vesiculoviruses are particularly well suited as oncolytic virus candidates for the following reasons: their life cycle is rapid and produces high titers [14,115,222,227], they are relatively easy to purify and concentrate[143], are amenable to reverse genetics[44,115,338], can accommodate transgenes up to at least 4.5 kb in length within their genome[156] and have not been reported to undergo genetic recombination events[20,308,379]. Furthermore, VSV and Maraba have demonstrated efficacy as oncolytic viruses in in vivo animal tumor models when administered systemically[252,369]. As mentioned, vesiculoviruses have a broad tropism, moreover VSV has also been demonstrated to infect many human cancer cells [14,20,379] and is sensitive to interferon, thereby limiting replication to interferon deficient cells, namely up to 80% of tested cancer cells[14,20,368,369]. Vesiculoviruses are also highly immunogenic[115] a quality which is reported to enhance boosting in vaccine applications[227,318] as well as enhance anti-tumor immunity[115,276]. Vesiculoviruses and in particular VSV infections are not endemic to the human population, with a low seroprevalence generally restricted to enzootic regions or in people with a high risk of exposures such as animal handlers, veterinarians or laboratory personnel[14,53,64,114,190,404]. Fail safe mechanisms also exist to limit

possible systemic toxicity or outbreaks with the use of neutralizing antibodies via passive immunization or with the use of systemic interferon[227].

The broad tumor tropism and immunogenic qualities of a vesiculovirus, factors which enhance its oncolytic efficacy, are also its pitfalls. Neurotropism results in neurotoxicity in animals and has been observed with VSV in rodents when administered IV,IN or IC[64,74]; in non-human primates when administered IC[64]; as well as causing encephalitis in humans[298]. Maraba also has the same pathogenecity as VSV causing fatal encephalitis when administered IC in mice[147], whereas Chandipura has caused encephalitis and subsequent death in humans[55,302]. The ability of the vesiculoviruses to replicate in the CNS is unclear. Most studies using VSV have identified an inability of the neurons to produce type I and II interferon in a timely manner to inhibit virus replication despite the fact that neurons are responsive to type I IFN and that exogenous type I and II IFN strongly inhibits VSV replication in neurons both in vitro and in vivo[202,387]. Furthermore, in vivo data confirms a lack of interferon response in the central nervous system in comparison to peripheral nervous system when the virus is delivered intranasally in a mouse animal model[386].

In addition to the neurotropism, the immunogenic property of vesiculoviruses which may help it induce a greater anti-tumor immunity[43,115,227,276,318,369] also favor production of anti-viral antibodies - a barrier

to receiving subsequent doses of the virus[292,293]. These adverse qualities in part impede the use of vesiculoviruses in human clinical oncolytic virus trials[64].

Entry, Replication and Spread

The lifecycle of an oncolytic virus, using VSV as an example, can be broken down into 3 distinct events (Fig. 2). First, is the entry into the host cell which can be mediated by a receptor to which the virus binds before it enters, a time called adsorption. In the case of the vesiculoviruses, the glycoprotein (G protein) is the only protein on the surface of the virion. There are approximately 1200 G proteins per virion which are loosely arranged in 400 trimeric spikes at the surface of the virion or in the cell membrane of infected cells[79,94,95,187,205,404,425,426]. The size of the G protein varies between 511(VSV IND, Accession NP_041715) 523(Isfahan, Accession CAH17547) 530(Chandipura, Accession P13180) to 553(Maraba, Fig. 3) amino acids depending on the vesiculovirus serotype. The broad tropism of the vesiculovirus is in part due to the G protein as exemplified by the fact that VSV G has been used to pseudotype other vectors to enhance their tropism[52,277].

Figure 2. Life cycle of an oncolytic virus.
The life cycle of the oncolytic virus consists of entry into the cell, often mediated by a receptor, followed by the release of its genetic contents. Some replication takes place within the cytoplasm or the nucleus, followed by assembly of the viral components. When ready to exit the host, the virus either buds out through the cell membrane or lyses the entire cell allowing the release of all virus particles. Both exit strategies will eventually kill the host cell.

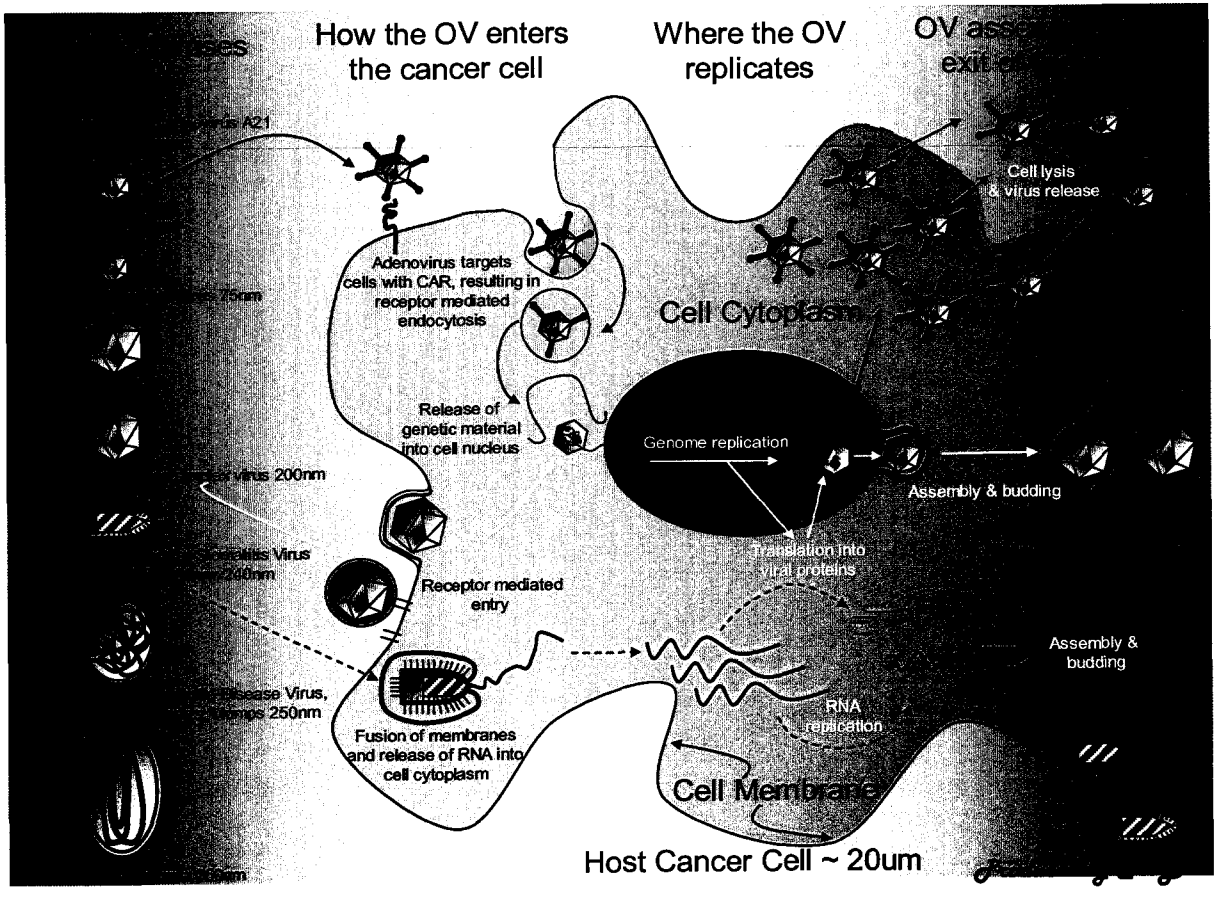

How the OV enters the cancer cell
Where the OV replicates
Cell lysis & virus release
Adenovirus targets cells with CAR, resulting in receptor mediated endocytosis
Cell Cytoplasm
Release of genetic material into cell nucleus
Genome replication
Assembly & budding
Translation into viral proteins
Receptor mediated entry
Assembly & budding
RNA replication
Fusion of membranes and release of RNA into cell cytoplasm
Cell Membrane
Host Cancer Cell ~ 20um

Figure 3. Nucleotide sequence of the Maraba glycoprotein.
The Maraba G nucleotide sequence has not yet been reported in the literature.

ATGAAAAAAACTAACAGGGTTCAAACACTCTTGATCGAGGTATTGAGACTTTTTCTCTTTTGTTTCT
TGGCCTTAGGAGCCCACTCCAAATTTACTATAGTATTCCCTCATCATCAAAAAGGGAATTGGAAGA
ATGTGCCTTCCACATATCATTATTGCCCTTCTAGTTCTGACCAGAATTGGCATAATGATTTGACTG
GAGTTAGTCTTCATGTGAAAATTCCCAAAAGTCACAAAGCTATACAAGCAGATGGCTGGATGTGC
CACGCTGCTAAATGGGTGACTACTTGTGACTTCAGATGGTACGGACCCAAATACATCACGCATTC
CATACACTCTATGTCACCCACCCTAGAACAGTGCAAGACCAGTATTGAGCAGACAAAGCAAGGAG
TTTGGATTAATCCAGGCTTTCCCCCTCAAAGCTGCGGATATGCTACAGTGACGGATGCAGAGGTG
GTTGTTGTACAAGCAACACCTCATCATGTGTTGGTTGATGAGTACACAGGAGAATGGATTGACTCA
CAATTGGTGGGGGGCAAATGTTCCAAGGAGGTTTGTCAAACGGTTCACAACTCGACCGTGTGGC
ATGCTGATTACAAGATTACAGGGCTGTGCGAGTCAAATCTGGCATCAGTGGATATCACCTTCTTCT
CTGAGGATGGTCAAAAGACGTCTTTGGGAAAACCGAACACTGGATTCAGGAGTAATTACTTTGCT
TACGAAAGTGGAGAGAAGGCATGCCGTATGCAGTACTGCACACAATGGGGGATCCGACTACCTT
CTGGAGTATGGTTTGAATTAGTGGACAAAGATCTCTTCCAGGCGGCAAAATTGCCTGAATGTCCT
AGAGGATCCAGTATCTCAGCTCCTTCTCAGACTTCTGTGGATGTTAGTTTGATACAAGACGTAGAG
AGGATCTTAGATTACTCTCTATGCCAGGAGACGTGGAGTAAGATACGAGCCAAGCTTCCTGTATC
TCCAGTAGATCTGAGTTATCTCGCCCCAAAAAATCCAGGGAGCGGACCGGCCTTCACTATCATTA
ATGGCACTTTGAAATATTTCGAAACAAGATACATCAGAGTTGACATAAGTAATCCCATCATCCCTCA
CATGGTGGGAACAATGAGTGGAACCACGACTGAGCGTGAATTGTGGAATGATTGGTATCCATATG
AAGACGTAGAGATTGGTCCAAATGGGGTGTTGAAAACTCCCACTGGTTTCAAGTTTCCGCTGTAC
ATGATTGGGCACGGAATGTTGGATTCCGATCTCCACAAATCCTCCCAGGCTCAAGTCTTCGAACA
TCCACACGCAAAGGACGCTGCATCACAGCTTCCTGATGATGAGACTTTATTTTTTGGTGACACAG
GACTATCAAAAAACCCAGTAGAGTTAGTAGAAGGCTGGTTCAGTAGCTGGAAGAGCACATTGGCA
TCGTTCTTTCTGATTATAGGCTTGGGGGTTGCATTAATCTTCATCATTCGAATTATTGTTGCGATTC
GATCACGAATTCTGGATCCGATACGTAACGCTCTGCAGCTGCGGGTTGCATTAATCTTCATCATTC
GAATTATTGTTGCGATTCGCTATAAATACAAGGGGAGGAAGACCCAAAAAATTTACAATGATGTCG
AGATGAGTCGATTGGGAAATAAATAA

The broad tropism is also thought to be due to the interaction of the G protein with a ubiquitous receptor such as phosphatidylserine (PS)[227,249,334], although this has recently been debated and PS may only be a component of the receptor or there may be an altogether different receptor[67,333].

The G protein is a Class I bitopic integral membrane protein[37,177,316,404], meaning it has a signal sequence which is cleaved thereby positioning the amino terminus in the extra-cellular side of the membrane and leaving the short 29aa carboxy terminus on the cytoplasmic side of the membrane, and embedded in the bi-layer is a 20aa hydrophobic transmembrane region[177,401,404,414]. The ectodomain comprises more than 85% of the protein (446aa), and it binds to a receptor[115,227,313] which mediates virus entry as evidenced by the fact that removal of the G protein by protease treatment significantly reduces the infectivity[35], furthermore recombinant vesiculoviruses lacking the G protein are unable to produce replication competent virions[375]. Binding to the receptor initiates a clathrin mediated uptake and the virion is engulfed in an endosome[374]. The acidic environment of the endosome (pH <6.2) triggers a reversible conformational change of the G protein allowing for increased exposure of a fusion domain potentially located between residues 118-139[30,63,78,104,121,141,296,297].

The fusion of the viral membrane to that of the endosome[104] allows for the release of the nucleocapsid, and M protein into the cytoplasm[307]. The M protein inhibits cellular transcription[36], disrupts Rae1 (RNA export 1 homologue)

function thereby inhibiting mRNA export out of the nucleus[108,109] preventing cellular production of interferon, the cell's antiviral response[36,66,161,188,203]. The M protein also depolymerizes actin, tubulin and vimentin causing cell rounding leading to cell death[39,237,352,422] and the M protein is also found to associate with the mitochondria where it regulates apoptosis[133,228]. The G protein progresses to the perinuclear region where some evidence suggests it may also affect transport of mRNA from the nucleus[83]. The G protein can induce syncytia formation when expressed in cells, and like the M protein is also toxic and contributes to cell death[52,58,277]. The L and P polymerase complex binds to the negative sense RNA genome which is covered by the N protein[188]. The L and P proteins form the RNA-directed RNA polymerase which starts transcription at the 3' end commencing with a 48 nucleotide leader sequence followed by the five monocistronic mRNAs which are subsequently capped and polyadenylated[404]. At some point after initiation of translation of viral proteins, the N, P and L complex form the replicase complex[152] which produces a positive sense viral genome to serve as a template for minus stranded genomes[187,222].

With regards to the production of the G protein, the ectodomain is translated and translocated into the endoplasmic reticulum where 2 N-linked glycans are added, disulfide bonds are formed between 6 pairs of cysteine residues and homotrimerization occurs. The protein is then transported to the golgi and then on to the cell surface, the entire process only taking 30 minutes[54,95,239,320]. A YTDIEM aa sequence motif contained in the cytoplasmic

tail of VSV G has been identified as being important for the enhanced ER export rate[342], and shows homology with many cytoplasmic targeting signals that are able to direct targeting to a variety of cellular locations such as the basal membrane and to coated pits[381]. At the level of the plasma membrane, the G proteins are organized into microdomains in regions that correspond to virus budding[3,47].

The third step in the life cycle is assembly and exiting the cell. Vesiculoviruses assemble in regions localized near the membrane bound G proteins, an area referred to as a "virus factory". It is in these dense regions where the M protein has been found to interact with the nucleocapsid (negative sense RNA bound to N, P and L proteins) forming a bullet-like shaped skeleton around the nucleocapsid[21,22]. The membrane bound G protein is then thought to interact with the M or N protein[187]. Although the M protein can mediate budding of virions from the infected cell without the G protein [161,191], components of both the cytoplamic tail and the membrane proximal region of the ectodomain of the G protein have been identified as important in increasing the efficiency of virus budding, perhaps by increasing the cell membrane curvature or they may enhance the recruitment of cellular factors that help with membrane fission[187,312,335]. The importance of the cytoplasmic domain is substantiated by the fact that it is required for the incorporation of foreign envelope proteins into VSV[278]. As budding progresses it causes extensive damage to the cell leading to lysis of the host[404]. It should however be noted that not all viruses bud from the cell

membrane, others such as adenovirus are passively released when the host cells lyse (Fig. 2)[347].

Vesiculoviruses spread by budding from the plasma membrane of the infected cell and disseminating to other host cells or organisms. The budding of the virion in itself does not cause the formation of syncytia, however in a low pH (<6.0) environment, cells infected with VSV or transiently expressing the G protein do form syncytia[415], a method by which other viruses are known to spread[99,100,105]. As previously reviewed, VSV G enters the cells via receptor mediated endocytosis, a pH dependant entry mechanism whereby the pH in the endosome induces a conformational change exposing the fusion domain within the G protein. The crystal structure of the low pH form of the G protein reveals that is has three stranded coiled-coils at the trimer axis and a long three stranded β sheet, characteristics of both class I and II fusion proteins respectively[313], thereby putting this fusogenic protein into a class of its own[314,367,373,410]. Fusogenic membrane glycoproteins are immunostimulatory, likely because they induce cell necrosis rather than immunosuppressive apoptosis[24,253,416]. Another method by which VSV G is implicated in the stimulation of the host immune response, is via the formation of tubulovesicular structures carrying viral DNA which is responsible for initiating a potent innate immune response via plasmacytoid dendritic cells[289].

The neurovirulence of VSV has been attributed to the neurotropism of the G protein allowing for the infection of neurons and inducing neurotoxicity. Attenuation of the neurovirulence has been achieved by infecting laboratory animals with recombinant VSV after rearrangement of the viral gene order, expressing the G protein from other serotypes, truncating portions of the cytoplasmic tail from the G protein and by inducing mutations within the M gene[65,70,118,119,246,369,412]. Although attenuated, most recombinants resulted in neurotoxicity except for the combination of an M gene mutation with the addition of the HIV-1 gag gene. This combination resulted in a non-neurotoxic VSV recombinant when administered intracranially at doses of 1e4 PFU which can be increased to 1e7 PFU when coupled with a G gene truncation of the cytoplasmic tail[65].

How to Create a Designer Vesiculovirus

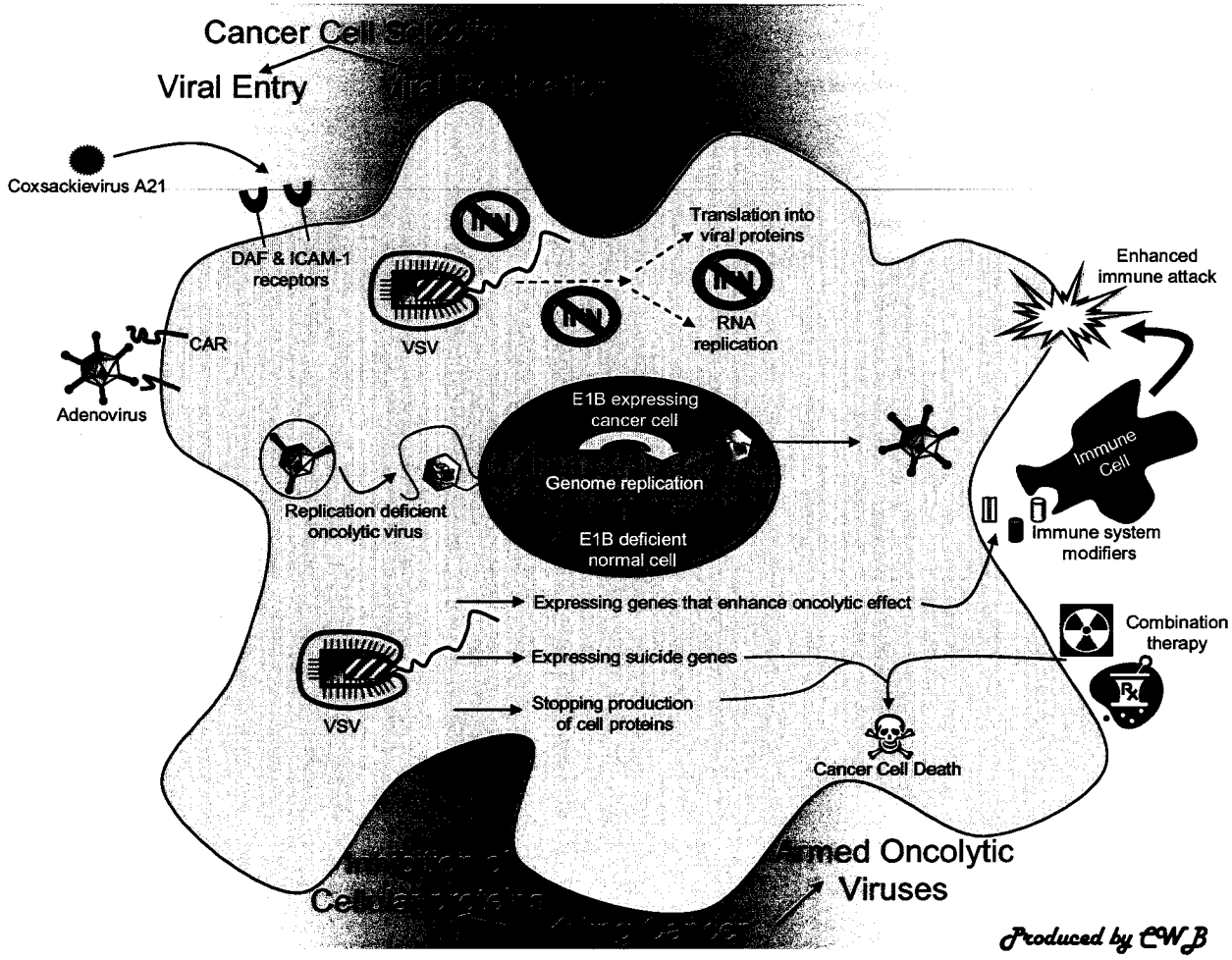
Although VSV infection does result in the death of the host cancer cell by necrosis and by inducing apoptosis[17,132,134,169,345] thereby effectuating cures in some animal tumor models[252,293,369], there may be circumstances under which the generation of an attenuated virus or the expression of additional genes may produce a more effective oncolytic agent. This section will provide a broad review of the engineered oncolytic viruses followed by a more detailed explanation of strategies used to engineer vesiculoviruses with enhanced oncolytic activity.

Targeting the Tumor Cell

Some naturally occurring oncolytic viruses, predominantly RNA viruses like VSV, will inherently infect tumors without having been genetically altered. A basic understanding of the mechanisms by which an oncolytic virus selectively infects a tumor cell over a normal human cell is fundamental in determining which types of oncolytic virus are suited for a particular type of cancer. Tumor specificity can be determined at many levels, anywhere from viral entry to viral replication (Fig. 4). The mechanisms whereby VSV confers tumor selectivity are reviewed.

VSV does not establish tumor selectivity at the level of viral entry, given that VSV is thought to bind to phosphatidylserine, a ubiquitous receptor[227,249,334]. This is in contrast to other oncolytic viruses, such as coxsackie, which selectively target tumor cells based on the tumor surface molecules ICAM-1 (Intercellular adhesion molecule-1) and DAF (Decay Accelerating Factor) which serve as the receptors for the virus to bind and enter the tumor cells[280]. Different oncolytic virus species have been engineered to bind specifically to tumors (Fig. 4). For instance, the natural receptor for adenovirus is the

Figure 4. Oncolytic virus tumor specificity and virus killing mechanisms.
Oncolytic viruses selectively infect and kill cancer cells. Selectivity can be found at the level of viral entry, as demonstrated with coxsackie A21 binding to ICAM-1 and DAF receptors located on cancer cells[311]. Enhanced selectivity is demonstrated with a genetically modified adenovirus receptor shaft binding more specifically to CAR (coxsackie adenovirus receptor) on tumor cells[42]. Replication of vesicular stomatitis virus (VSV) is restricted to cancer cells because of a defective interferon pathway. Similarly, the E1B deletion keeps adenovirus replication in tumor cells where they complement the E1B function. Killing specificity is also multilevel: VSV and adenovirus inhibit the cellular production of essential proteins thereby initiating programmed cell death of the cancer cell. Furthermore, OV's can be armed with various types of genes, such as suicide genes like TK and 5FU and immune system modifiers as described in the text. Combining OV therapy with other therapies, such as radiation, chemotherapy, cytokines and immune suppressants could result in a synergistic killing effect.


Cancer Cell
Viral Entry
Coxsackievirus A21
DAF & ICAM-1 receptors
CAR
Adenovirus
VSV
Translation into viral proteins
RNA replication
Enhanced immune attack
Immune Cell
Immune system modifiers
E1B expressing cancer cell
Genome replication
E1B deficient normal cell
Replication deficient oncolytic virus
Expressing genes that enhance oncolytic effect
Expressing suicide genes
Stopping production of cell proteins
VSV
Combination therapy
Cancer Cell Death
Armed Oncolytic Viruses
Produced by CWB

CAR receptor but it is expressed on many normal tissues and often only poorly expressed on tumor cells[30]. An attempt to target adenoviruses more specifically to tumors was accomplished by genetically modifying the viral fiber-knob protein that normally binds CAR[266]. A process called "serotype-switching" re-targets adenovirus away from normal tissues and towards binding partners expressed preferentially on the tumor cell surface[42,271,280,324]. Serotype-switching is accomplished by switching serotype determinants between serologically distinct adenoviruses[197]. Furthermore, similar results may be obtained by genetically inducing a mutation in the fiber-knob, or by generating chimeric fiber-knobs[136,266,405]. Serotype-switching is also a strategy that is used to generate recombinant viruses that can evade neutralization by pre-existing antibodies[310]. In the case of VSV, serotype-switching has been performed for the purposes of developing better vaccine vectors for boosting of the immune response[318], however this strategy has not yet been described for the purposes of enhancing the oncolytic activity of vesiculoviruses. It is important to note that unlike VSV, adenovirus or reovirus which are significantly impaired by neutralizing antibodies, measles and HSV are not affected by antibodies when delivered directly within the tumor, and antibodies do not interfere with the systemic viral delivery of NDV [32,168,280] or the extra-cellular envelope virus (EEV) form of vaccinia[181,199,356] to the tumor.

For VSV, tumor selectivity is determined at the level of viral replication by a defective innate anti-viral mechanism. In a healthy cell, the innate immune

response mechanisms are initiated upon host cell recognition of viral RNA by cellular receptors, such as dsRNA-dependent protein kinase[208], Toll-like receptors (TLR3, TLR7 and TLR8), RIG-1, Mda-5[411] and or TLR7 and TLR8[341]. Upon recognition of the viral RNA, transcription factors, such as NF-κB and IRF3 are activated[341] and in turn mediate transcriptional changes within the host cell, such as the upregulation of cytokines (interferon beta), chemokines and mediators of adaptive immune responses which function to limit virus replication, recruit immune cells and direct the subsequent adaptive immune responses[340]. VSV[20,368] as well as reovirus[186,329,348] are examples of interferon sensitive viruses. Fortunately, 80% of human cancer cell lines tested have a defective interferon system[369] thereby allowing VSV to replicate unabated.

Tumor selectivity can also be engineered at the level of viral replication (Fig. 4). In the case of VSV, it has been demonstrated that it is sensitive to interferon and thereby replicates in cells with defective interferon pathways, namely cancer cells. Although VSV leaves normal tissues relatively unaffected, it is still detected in normal cells and kills nude mice within 6-10 days when a single dose of 1e8 PFU of virus is administered directly in the tumor. However no toxicity was noted if the mice were co-treated with 4 doses of exogenous interferon, protecting mice for the duration of the study without sequelae for up to at least 45 days[368]. Given the toxicity with wild type VSV, a recombinant VSV expressing the IFN-ß gene has significantly improved the specificity and safety profile of VSV by 60% compared to wild type VSV, whereby Balb/c mice treated

with one dose of 1e8 PFU of virus administered intravenously were asymptomatic for the entire duration of the study lasting 120 days[276]. In a similar approach, sequencing of interferon inducing temperature sensitive VSV mutants, identified unique M gene mutations. Specifically, in one mutant (AV1) methionine 51 was replaced by arginine (M51R), and in another mutant (AV2) valine 221 was replaced by phenylalanine (V221F) and serine 226 was replaced by arginine (S226R). Subsequently a recombinant with a deleted methionine in position 51 of the M protein (ΔM51) was generated and found to be as attenuated as the temperature sensitive mutants, and all three M mutants still retained their oncolytic activity and were able to effectively decrease tumor burden in animal models[369]. The M mutants were well tolerated when administered intravenously in Balb/c mice at 5e8 PFU/dose for 6 doses and were reportedly asymptomatic for the duration of the study lasting 32 days. Similarly, mice also tolerated intranasal instillation at 5e7 PFU/dose for 6 doses and were also asymptomatic for the duration of the study lasting 80 days[88,110,120,254,332,369]. The ΔM51 mutant was well tolerated systemically and could be given in doses up to 10000 fold more than wild type VSV before inducing toxicity in mice[7,295,369]. When administered intranasally in Balb/c mice, the 50% lethal dose (LD50) was 3e8 PFU for ΔM51 compared to 1e4 for wild type VSV. Similarly when given intravenously in CD1 mice the LD50 was 8e9 PFU for ΔM51 versus 1e8 PFU for wild type VSV[369]. To further demonstrate the neurotoxicity of VSV, 5ul of 2e7 PFU of either WT or ΔM51 was administered intracranially in 8 week old female Balb/c and within 6 days mice develop hind limb paralysis. Interestingly if mice are intravenously

primed with ΔM51 VSV at 1e8 pfu 24h prior to intracranial innoculation with 2e7 pfu of ΔM51 VSV (in 5 μl PBS) the ΔM51 primed mice survive while all WT and PBS primed mice developed hindlimb paralysis, demonstrating that systemically administered mutant ΔM51 can protect against a lethal intracranial dose of VSV[29]. It is important to note that although the ΔM51 mutant does refine the targeting of VSV, it also significantly changes other related factors, such as: 1) The toxicity of the ΔM51 when administered systemically is thought to be due to increased TNFα production, something that is not observed after administration of WT VSV[295]; 2) Unlike VSV which inhibits host gene expression, ΔM51 induces expression of proapoptotic genes; 3) The apoptotic mechanisms initiated via ΔM51 is through the death receptor pathway via caspase 8 rather than the mitochondrial pathway via caspase 9 induced by WT virus[133,134].

Similar tumor selective replication mechanisms have been engineered in other oncolytic viruses. For example, when the E1B region in the adenovirus genome is deleted thereby restricting viral replication to tumor cells which complement the function of the deleted E1B region, namely mRNA export [34,275,280,400]. Herpes simplex virus (HSV-1), a large DNA virus, must have one or more of its genes deleted so that it can selectively replicate in tumor cells. When the RR or γ34.5 gene is deleted, HSV-1 can only replicate in rapidly dividing cells such as tumor cells[41,48]. Another method used to confer specificity, is to ensure that the viral genes will only be expressed in the tumor specific cell type[201], for example the addition of a prostate specific promoter in the adenovirus genome has

allowed the virus to replicate in prostate cancer cells in patients and effectively decrease PSA levels, a prostate tumor specific marker[85,355].

This overview of oncolytic virus tumor targeting emphasizes the importance of understanding the underlying targeting mechanisms, as one advantage of the oncolytic virus platform is the potential of engineering multi-targeted strategies to enhance oncolytic efficacy.

Killing Tumors by Targeting the Sweet Spot

There are various strategies employed by oncolytic viruses to kill the host tumor cell. Some mechanisms used by viruses to target intracellular tumor cell molecular pathways are hereby summarized (Fig. 4). Strategies employed by adenovirus and VSV include inhibiting cellular protein production or function [274,369,404]. In the case of VSV this is performed by the M protein which blocks cellular mRNA transport and prevents the antiviral response[8,113,164,369,402]. Another intimately linked strategy includes the initiation of cell death either indirectly by structurally destroying the cell via the budding of virus from the cell membrane or directly via apoptosis [242,274,384]. With respect to VSV, again it is the M protein which has been implicated in the apoptotic pathway, in the case of ΔM51 it is initiated through the death receptor pathway via caspase 8 rather than the mitochondrial pathway via caspase 9 as for WT VSV virus[133,134]. Some viruses, such as adenovirus and HSV-1, may even be so kind as to inhibit tumor cell death until the virus is ready to exit, thereby maximizing the amount of virus produced

per infected tumor cell [274,404]. Other strategies employ the use of extracellular signals, for example, NDV causes infected tumor cells to become more sensitive to the viral induced secretion of an inflammatory signal (TNF-α) by peripheral mononuclear blood cells which is cytotoxic to the now over-sensitized tumors cells [234]. The secretion of inflammatory signals also stimulates the innate immune response, attracting neutrophils and antigen presenting cells to the tumor, thereby further enhancing tumor cell death. The phenomenon whereby even uninfected tumor cells are eradicated is referred to as the bystander effect[123] and greatly enhances the effectiveness of the oncolytic virus. Furthermore, if the adaptive immune response recruits cytotoxic T-cells and develops anti-tumor antibodies, this will not only complement the ongoing tumor slaughter but provide a long term anti-tumor immunity, thereby eliminating the chance of recurrence[26].

Host-Tumor-Virus Arena.

When designing an oncolytic virus there are as many viral, if not more, host and tumor factors to consider. Although, characteristics of the viruses, such as tumor selection and killing mechanism can be modified, all of this must put into the context of a dynamic host-tumor-virus environment. The oncolytic virus often initiates a cascade of intra and extra-tumoral events which may either enhance or impede oncolytic activity[27,365]. Similarly the tumor may have also modified its surroundings such as its architecture and blood supply[224] as well as the local immune cells so as to prevent detection by the immune system[86]. The current understanding of the role of the immune system, tumor vasculature and

tumor architecture in the framework of uninfected and infected tumors are hereby reviewed.

Cancer immunosurveillance is a task whereby the body's own immune system recognizes and eradicates cancer cells[50,51,106,382]. Immunosurveillance was demonstrated by immunizing animals against specific types of cancer. Immunediting theory incorporates the evidence demonstrating how cancer cells are able to modify local immune factors, suppress any co-required "danger" signal(s) and escape immunosurveillance, thereby explaining how cancers are still able to develop in immune competent individuals[102,166,250,281,294]. Several tumor based mechanisms for evasion of CTL-mediated eradication have been described[166]. Tumor-associated immune cells have been demonstrated to produce immunosuppressive cytokines such as IL-10 and TGFβ to dampen adaptive antitumor immunity. In addition, tumor associated T-regulatory cells suppress the proliferation and effector function of cytotoxic T-cells[86]. Similarly, it has been demonstrated that intra-tumoral oncolytic virus infections can be neutralized by monocytes[129,130,211]. Furthermore, systemic delivery of oncolytic virus is also impaired by binding to white blood cells[238], activation of the complement cascade[183,189] and or by the production of anti-viral antibodies thereby impairing the overall oncolytic effect[59,292,293].

The route of administration influences the amount of oncolytic virus delivered to the tumor. Intra-tumoral injections are thought to be the best method

of delivery[365], however the volume injected and tumor type dictate how much virus is actually retained[25], and this method of delivery is not practical for wide spread metastatic disease or safe for inaccessible tumors. Although an intravenous route of delivery is more appealing for a systemic metastatic disease, it also elicits a very strong antibody response against the virus[293,365,366]. Specifically with respect to VSV, antibodies recognize at least 11 antigenic determinants on the G protein, 4 of which neutralize infectivity in both the Indiana and New Jersey serotypes[45,193]. Although non-neutralizing antibodies do not impede viral infection in vitro, they are thought to have a more pertinent role in vivo whereby they can elicit neutralization through an antibody mediated complement dependent lysis of virions or infected cells, or even through antibody dependent cell cytotoxicity[131,216]. Furthermore it should be noted that the glycosylation sites on the G protein do not significantly contribute to the epitope formation in the Indiana serotype[193], but have been found to provide stability to the disulfide bonds that determine the epitopes of the New Jersey serotype[151].

Contrary to the immune system which hinders the oncolytic virus, both the adaptive and the innate immune responses have also been implicated in enhancing tumor oncolysis. Tumor infiltrative neutrophils enhance tumor necrosis after oncolytic virus infection[43]. Likewise, activation of an adaptive immune response through CD8 T cells which mediate direct tumor cell lysis and release potent inflammatory cytokines, has been demonstrated as pivotal in the formation of an anti-tumor immunity and preventing recurrences[38,43,244,369]. Altogether, this

provides evidence that the immune system is intimately linked to, and forms part of, the balance between tumor formation and tumor eradication.

Given that the immune system can be a most potent ally in the fight against cancer, strategies such as expressing immunomodulatory genes within recombinant oncolytic viruses such as IL-2[172], IL-4[291], IL-12[363,394,395], IL-18, IL-21[172], GM-CSF[175,300], IFN-β[276] and IFN-γ[379] have been devised to enhance oncolytic virus therapy and more importantly geared toward facilitating an adaptive antitumor immune response. However it should also be noted that these approaches also manipulate the innate and adaptive response to the virus[365].

Another approach to enhancing an anti-tumor immune response is through the expression of fusion proteins. The intra-tumoral expression of viral fusogenic proteins has demonstrated improved tumor vaccination[24,173]. The mechanism by which fusogenic membrane proteins are thought to enhance anti-tumor immunity is by providing the necessary "danger" signal(s) that are lacking which in turn stimulate the dendritic cells to present foreign antigens and stimulate tumor specific cytotoxic T cells followed by an influx of natural killer cells and macrophages resulting in reduction of tumor growth in both injected and non-injected tumors in the same animal[173,250]. Examples of fusogenic proteins that have demonstrated an enhanced anti-tumor immune response include VSV G, the F protein from the respiratory syncytial virus, measles virus H and F proteins, and the hyperfusogenic version of the GaLV glycoprotein[23,24,105,171,172,174].

Furthermore, as previously mentioned, measles and NDV are able to spread in the presence of neutralizing antibodies[32,280] and coincidentally Measles and NDV spread via syncytia formation[103,213] suggesting that this mechanism of spread provides shelter from the neutralizing antibodies which are less able to mitigate virus spread within an infected tumour but may be more effective at neutralizing the virus during tumor to tumor spread[27].

In theory, infection by a single potent rapidly replicating virus particle can potentially destroy an entire tumor, however trials have demonstrated that viral spread throughout the tumor is limited to a small number of cells with little or no spread from the primary site of infection[195,280]. As such we need a better understanding of the dynamics of viral spread in the host[251,421]. Some studies suggest that the replication and spreading of the virus within tumors is likely to be compromised by the restrictive properties of solid tumor architecture and micro-environment such as the extra-cellular matrix, hypoxia, high interstitial tumor pressure, and low pH[282,346]. The spreading of VSV infection in vitro is known to be pH sensitive resulting in loss of infectivity of the virion in pH environments of 6.8 – 6.4[141,245,415], the same pH range that has been documented to be found in solid tumor cores[150,256,282,299,344,371,372,377]. Yet despite the reported poor efficiency of oncolytic virus infection at the tumor site, cures have been achieved in animal models. In a mouse colon cancer tumor model, very little IV administered VSV is actually delivered to the tumor in part because of the inflammatory process which clots off the microcirculation to the tumor thereby eliminating further delivery of

the oncolytic virus. However, the inflammatory cascade that ensues attracts neutrophils which destroy the remaining tumor. If the neutrophils are removed from the equation the virus is able to spread within the tumor thereby demonstrating the important role of the innate immune response in preventing oncolytic virus intra-tumor spread yet enhancing tumor killing[43]. This provides another example whereby a better understanding of the host-tumor-virus microenvironement will aid in the development of oncolytic agents that are able to stimulate endogenous anti-tumor mechanisms resulting in synergized tumor eradication.

Engaging an Armed Dealer

Several classes of genetic payloads have been used to arm non-replicating viral-based gene delivery vehicles such as tumor suppressors, antioncogenes, prodrugs, antiangiogenic and immuno-modulatory genes[166]. Armed oncolytic viruses are recombinant replicating viruses that have been generated to express genes that enhance their oncolytic effect, examples include cytokines, chemokines, fusion proteins as well as suicide genes (Fig. 4).

As previously mentioned, the immune system is a most potent ally in the fight against cancer[26], which is why immune system modifiers such as cytokines and chemokines have been recruited to generate more effective oncolytic viruses and have demonstrated significant enhanced oncolytic effects compared to their parent or "un-armed" virus. Recombinants expressing IL-2[5,172], IL-4[291], IL-

12[363,394,395], IL-18, IL-21[172], IFN-β[276], IFN-γ[379] and GM-CSF[5,175,300] have been created and demonstrate enhanced oncolytic effect in animal models. In the case of Granulocyte Macrophage – Colony Stimulating Factor (GM-CSF), it is one of the most potent stimulators of a specific and long-lasting anti-tumor immunity and is important in the maturation of antigen-presenting cells to induce T-cell activation and provide an anti-tumor immune response[2,5,96]. Recombinant adenovirus expressing GM-CSF and B7-1, a co-stimulatory molecule and member of the B7 family of cytokines, has demonstrated decreased tumor burden and increased survival in animal models due to an enhanced antitumor response[62]. Furthermore, recombinant vaccinia and HSV virus expressing GM-CSF have also demonstrated efficacy in animal tumor models as well as increased inflamation, lymphocyte and macrophage infiltration within patient tumors in clinical trials[5,175,196,248].

Oncolytic viruses armed with fusogenic membrane glycoproteins have also demonstrated enhanced oncolytic effects and include VSV G, the F protein from the respiratory syncytial virus, the measles virus H and F proteins, and the hyperfusion version of the retroviral GaLV envelope glycoprotein[23,24,105,171,172,174,353]. The enhanced oncolytic effect is due to a more potent anti-tumor immune response, instigated by syncytiosomes which load dendritic cells more efficiently with tumor antigens, a process termed cross-presentation[24]. The enhanced oncolytic effect with membrane fusion proteins is reported to kill tumors more efficiently than suicide genes[23].

Suicide gene therapy is a strategy whereby genes which code for enzymes that convert innate chemicals into toxic drugs are used to enhance the oncolytic effect. Examples of pro-drug activating enzymes are the HSV-tyrosine kinase (TK) which converts valganciclovir to the toxic ganciclovir, and the E.coli cytosine deaminase (CD) gene which converts the anti-fungal agent 5-flurocytosine into the chemotherapy agent 5-FU[5]. Recombinant VSV virus armed with the suicide gene product thymidine kinase (TK) demonstrated that VSV expressing TK, but not GFP, was able to exert enhanced antitumor activity against metastatic disease. Following intravenous administration of the recombinant VSV-tk, valganciclovir is administered systemically but is only converted to its lethal form in VSV-tk infected tumor cells expressing the TK enzyme [258]. After VSV-tk treatment, immunocompetent Balb/c mice bearing mammary adenocarcinoma exhibited prolonged survival against lethal lung metastasis[111]. Furthermore, adenovirus engineered to express a fused CD/TK gene has demonstrated increased response in patients with prostate cancer[124,125]

A more recent strategy involves the expression of short hairpin RNA (shRNA) which suppresses specific gene production. Adenovirus armed with shRNA against the angiogenic factor VEGF demonstrated a significant decrease in tumor blood supply and a greater anti-tumor effect[423].

To add yet another dimension, combination therapies are also being employed as a strategy to enhance the oncolytic effects, and evidence suggests this combination strategy may also help prevent formation of drug resistant tumors[331]. For instance, combining various oncolytic viruses[215], with chemotherapeutic agents[5], cytokines[5], radiation[4,125,361] and immunosuppressants[129,182,211,406] or combinations thereof, have resulted in oncolytic synergies. Understanding the various tumor targeting and oncolytic mechanisms will help harness the oncolytic virus's potential, and allow for the development and administration of a safe and effective cancer therapy.

A potential issue regarding armed viruses, similar to that encounterd in gene therapy, is the potential activation of an immune response to the transgene being expressed[225,396], resulting in a ineffective therapy and or inability for repeated treatments.

It's Alive – Rescuing Recombinant Vesiculoviruses

The generation of recombinant viruses has allowed for significant advances in the field of virology and of oncolytic viruses, therefore a brief review of the vesiculovirus rescue system follows. Given the negative sense RNA genome of the vesiculoviruses, additional undertakings had to be overcome to allow for the recovery of VSV from DNA[214,336,413]. Unlike positive sense RNA viruses, the negative sense RNA viruses cannot initiate an infectious cycle after deproteinized RNA is introduced into appropriate cells[16]. To be infectious, the

RNA must be encapsidated by the N protein in the presence of the viral RNA polymerase L and cofactor P[69]. In order to accommodate this in an in vitro setting, a plasmid encoding the viral anti-genome flanked by T7 promoter and T7 terminator sequences is co-transfected with expression plasmids (pBluescript SK (+) with T7 promoter) encoding N, P and L proteins into BHK-21 cells expressing the T7 RNA polymerase[214,413]. The antigenome RNA is then encapsidated by the N protein forming the ribonucleoprotein (RNP) which is then replicated to form the genome RNP from which all viral mRNA's are transcribed initiating the infectious cycle[115]. A hepatitis delta virus ribozyme is critical for replication and allows for autolytic cleavage of the 3' end of the transcript thereby forming the necessary exact terminus of the VSV transcript[214,284,413]. Expression of the T7 polymerase in stable cell lines[128] or with a recombinant vaccinia virus[214,413] results in rescued virus. The efficiency of recovered virus using this method is low, occurring approximately in one out of 2e7 cells. Given a direct correlation with a 10 fold drop in virus recovery for every kilobase added to the genome, the rate limiting step is thought to be due to the encapsidation process, as this must occur to protect the RNA before the L and P proteins initiate replication[214]. Another important discovery that sanctions the expression of inserted transgenes, is the unique intergenic sequence found between each of the five genes. This region encodes a stop/start sequence which signals the termination of transcription of the previous gene and initiates the transcription of a next gene[336]. Put altogether, this system permits the use of recombinant DNA technology to genetically engineer and rescue recombinant oncolytic vesiculoviruses.

A Brief Oncolytic History & Summary of Oncolytic Virus Clinical Trials

Viral treatment of cancer has been described since the beginning of the twentieth century [28,194,391] with the first reported case of leukemia remission occurring after a flu like syndrome thought to be caused by the influenza virus[93,194,230]. Similarly, remissions were reported after infections with chickenpox (varicella-zoster virus)[194], measles[40] and Newcastle disease virus (NDV)[81]. Interestingly, after the onset of vaccinations, patients with cancer who were vaccinated with attenuated rabies, smallpox or measles virus vaccines would on occasion have responses[230].

As it was becoming apparent that viruses had potential anti-cancer effects, the first rudimentary clinical trials were performed as early as the 1950's[176], and a number of case series reported that viruses, such as adenovirus[368], mumps virus[10] and Egypt 101 isolate of West Nile virus[358] among others, could be used against cancer[194,280,358]. The early testing of viruses as a cancer therapy peaked in the 1950's and 60's and although oncolytic viruses were able to kill tumor cells, treatments did not seem to alter the course of the disease[194,311,323] and toxicities such as encephalitis were occurring[358]. Given these limitations, the exploitation of this viral phenomenon would lag behind conventional surgery, radiotherapy, chemotherapy and immunotherapy. Viral treatments of cancer fell out of favor and the number of clinical trials decreased dramatically in the 1970-1980's[194,324]. A more detailed historical perspective is reviewed by J.C. Sinkovics, 2005 [354].

Despite current conventional therapies, 5 year survival rates for advanced cancers and the most common cancers, lung and colorectal, are less than 60%. In addition the number of cases of cancer are increasing and this year in Canada there will be 160000 new cases and 73000 deaths due to cancer alone[1]. With a need for a better cancer therapeutic, a resurgence of viral therapy has occurred over the past decade, coupled with a better understanding of the viral biology and the onset of gene therapy, viruses are now engineered with safety features and enhanced abilities to selectively infect and kill tumor cells[34,368].

The renaissance of clinical trials with oncolytic viruses started in 1996 with adenovirus[138] and numerous trials have since been conducted with other oncolytic viruses. Most of these viruses have undergone phase I trials in order to determine dose range, safety and side effects. Others oncolytic viruses have undergone phase II testing to determine effectiveness. To date, only adenovirus has had a phase III trial to confirm its effectiveness and has since been approved for clinical use in China [140]. Adenovirus is the most extensively tried oncolytic virus and has been administered through various routes to various cancers with and without metastasis and even in combination with chemotherapeutic agents. Outcomes with adenovirus are variable, and despite widespread tolerance and safety there has been one fatality[5]. However, currently adenovirus H101 has been approved by the Chinese State Food and Drug Administration for use in

combination with chemotherapy in the treatment of late-stage refractory nasopharyngeal cancer[140].

Indicators for successfully passing from one phase to another are adverse events such as toxicities and side effects, and effectiveness as measured by survival rate, tumor size, remission, recurrence or cures. Other key measures pertaining to oncolytic virus treatments include virus production at the tumor site or elsewhere, as well as the production of antibodies against the virus. Pertinent findings from individual trials are summarized in Table 1, and their significance in determining the future directions of the field are collectively reviewed in this section.

HSV, first tested in recurrent glioma patients, is generally well tolerated and has shown some response. Similarly mumps and reovirus were also well tolerated. Patients treated with reovirus had low response rates, however in most cases the disease had not progressed during the course of the study[260]. The use of NDV administered to patients suffering from high grade gliomas, a disease where few survive beyond a year after diagnosis, has yielded promising results in a trial with 4 of 14 patients reporting significant clinical improvements resulting in a good quality of life with 5 to 9 year long-term survival[82]. However more recent trials with NDV have resulted in some dose limiting toxicities, although these were eliminated when the virus was given at a lower initial dose, a phenomenon called desensitization[212,232], and treatments resulted in a near-complete response in

one case. Unfortunately despite all safety precautions with NDV, a patient suffered fatal respiratory failure due to rapid tumor lysis [5]. Most recently, clinical data from a Phase 1 / 2 trial of a targeted and immuno-stimulatory armed vaccinia virus demonstrated efficacy in 10 of 13 patients with advanced liver tumors. In addition to the virus being well tolerated, seven patients survived double their life expectancy – another landmark achievement for oncolytic virus therapy[198].

As previously mentioned, a combination strategy is more likely to succeed given the complex biology of cancer[274], as such, some oncolytic virus trials have used adenovirus in combination with chemotherapy which resulted in almost all of the trials reporting responses[5]. One trial was conducted using an E1B deleted adenovirus expressing two pro-drug activating enzymes, CD and TK, and was administered to prostate cancer patients. Patients then received the pro-drugs 5-fluorocytosine and valganciclovir in combination with 70 to 74 gray (Gy) of 3 dimensional conformal radiation therapy (3D-CRT). This trial reported success as PSA levels had decreased for all patients[125].

In summary, after a total of approximately 500 patients have been treated with 6 different virus strains, we can conclude that although toxicities varied, oncolytic virus administration is generally very well tolerated in comparison to other cancer therapeutics[230,251] and in some cases toxicities could be decreased even further with slow infusion of the virus[212,232]. The optimum viral dose has yet to be determined and is dependent on the type of oncolytic virus and route of

administration. Responses varied significantly and the criteria that defined a response were also quite variable, however synergy between oncolytic virus and combination therapies such as chemotherapy and 3D-CRT resulted in increased responses in some cases.

Key points of focus for future trials include safety, identifying barriers to optimizing responses and establishing universal measures of response or effectiveness of treatment. These trials have provided us with virus species specific characteristics, some of which will be modified and enhanced in the laboratory, however many of these factors will also be solved with more trials.

Back to the Bench – Clinical Trial Take Home Messages

Clinical trials have demonstrated that oncolytic viruses are better tolerated than many other types of cancer treatments, with flu like symptoms being the most common effect [251].Yet clinical responses have not been dramatic. The lack of efficacy is the key challenge, and is likely multifactorial in nature [251].

The lack of efficacy, meaning the inability to completely destroy the tumor and cure the patient of their disease, can be due to many viral and host factors. As such we need a better understanding of the dynamics of viral spread in the host[251,421]. Present oncolytic virus administration is generally well tolerated, as such, more virus could be administered in order to enhance viral spread in the tumor, however currently there are biological limitations as to how much virus

can be produced without incurring enormous costs[251,391,421]. Routes of administration also influence how much virus is delivered to the tumor, with intra-tumoral injections being more successful but limited to larger accessible tumors. Ideally systemic administration of the oncolytic virus would allow for the virus to seek out tumor cells of varying sizes and location[116]. A few barriers that have been identified specific to systemic delivery are the liver, as well as components of the immune system such as anti-virus antibodies, complement and white blood cells[59,183,189,230,238]. The function of the liver is to filter toxins, as such, liver clearance of the virus is a natural process. Future work will focus on minimizing liver clearance of the virus potentially by altering a viral factor or cloaking the virus[292,293,324] so that it is not recognized by the liver. A similarly strategy, called serotype switching[324], could be used to avoid oncolytic virus recognition by anti-virus antibodies which neutralize the oncolytic virus before is able to infect the tumor. Perhaps understanding how vaccinia, NDV and Measles virus are able to evade antibody or complement neutralization may help with systemic viral delivery to the tumor[32,231,280]. Interestingly, immune compromised patients have had the highest rates of remission in early oncolytic virus trials, as such immune suppressing the host may help increase the efficacy and spread of the oncolytic virus within the tumor[324]. Efficacy would also increase with a virus which has multiple methods of action for tumor selection and tumor killing thereby simultaneously targeting different tumor pathways and making it a more potent virus [251,274].

It is also important to develop tools that will allow us to more effectively monitor and quantify the oncolytic virus activity in human tumors. It has already been proposed that detection of viral genomes in the blood would be more representative of the amount of virus produced[5,230]. Oncolytic virus engineered to express marker genes, such as carcinoembryonic antigen (CEA), allows for peripheral detection of virus presences via the detection of CEA in the blood[286,287]. But it is equally important to monitor real time virus replication in the tumor as well as in other organs[251,324,421]. Real-time monitoring of oncolytic virus biological activity and distribution within the tumor and patient may only be possible with imaging technology such as single-photon-emission computed tomography (SPECT) or positron-emission tomography (PET). Conventional CT and MRI imaging has only been able to convey information regarding tumor location, size, shape and tissue density, but these do not necessarily correlate with oncolytic virus activity[230]. With the development of a recombinant oncolytic measles virus expressing the thyroidal sodium iodide symporter (NIS), infected cells concentrate radioactive iodine from the blood stream thereby allowing infected cells to be detected using SPECT or PET imaging[92,145,311,324].

Although relatively safe to date, oncolytic viruses are biological entities which are susceptible to mutation and reversions[87], and unlike any other therapy there is also the chance of viral spread by the patient to the healthy population. Arming the virus to make it more potent and capable of eliciting a strong anti-tumor immunity and thereby further inhibiting its ability to be detected by the

immune system may seem counterintuitive in an era of expected avian influenza pandemics[60]. Given the uncertainty of mutations, the possibility of integration of viral genomes into the host genome, a risk of autoimmune diseases due to improper immune modulation, and spread of mutated oncolytic virus to the general population, the establishment of a strict governing body is required[60,391]. For example, it would be prudent for every oncolytic virus species brought to clinical trial to have a proven fail-safe mechanism[324] such as a virus specific neutralizing antibody or specific drug-like inhibitor of oncolytic virus replication such as interferon for VSV. Furthermore, because of the risk of patients infecting other people, immediate contacts should also be monitored for the possibility of virus spread[421].

In summary, despite a perceived inability to treat systemic disease[305] these trials have provided us with invaluable information about the interaction of the virus with the patients and the cancer, and have identified areas of focus which will spearhead the current field of oncolytic virus research. The future challenges consist of enhancing efficacy, developing standards to measure the effectiveness of oncolytic viruses and maintaining safety (Table 2).

Table 2. Clinical barriers, solutions and goals for the oncolytic virus platform.

• Clinical Challenges	• Strategies Tackled in the Lab	• The Ultimate Designer Oncolytic Virus
1.Enhancing OV Efficacy Enhance anti-tumor immunity Increasing viral load to tumor • Avoid infecting healthy cells • Avoid antiviral antibodies	• Understanding the host-tumor-virus environment • Combining therapies • Discovering other candidate viruses • Expressing fusion proteins and immune system modifiers Altering viral target and evading host immune system: • Cloaking the virus • Carrier cells delivering virus • Serotype switching • Using a decoy virus proteins • Modifying the host immune system	1. Infect only tumor cells 2. Replicate and kill tumor cells 3. Does not integrate its genetic material into the host genome (common to retroviruses and some DNA viruses) 4. Genetically stable such that it is identical between progeny 5. Easy to genetically modify 6. Stable in the circulatory system so that it can be administered intravenously 7. Can be mass produced 8. Safety mechanism whereby it can be inhibited by an antiviral 9. Infect all tumor cell types 10. Does not initiate an antibody response against itself 11. Not endemic to the population (minimize having antibodies against the virus) 12. No side effects
2. Monitor and Quantify OV Activity	• Marker genes to estimate viral numbers (i.e. CEA) • Real time imaging of OV spread with SPECT / PET	
3. Preventative Measures • Virus mutations • Integration into host genome • Autoimmunity • Avoiding public spread	• Generate fail safe mechanism • Monitor patient contacts • Governing body enforcing regulations	

Back to the Future – The Next Generation of Oncolytic Viruses

To improve on the oncolytic virus strategy, this thesis attempts to address enhancing oncolytic virus efficacy, a current roadblock identified in clinical trials. The overall strategy is to modulate characteristics of the rhabdovirus by adding or swapping envelope genes of interest in order to enhance oncolytic activity. The fusogenic protein from reovirus is used to generate a recombinant VSV to enhance viral spread within the tumor. Similarly, recombinant VSVs were generated with envelope proteins either from other vesiculovirus members or from a beta-retrovirus, and used to demonstrate tumor infectivity in the presence of neutralizing antibodies. The recombinant VSV expressing the beta-retrovirus envelope protein is also used to demonstrate altered viral tropism and does not demonstrate any neurotoxicity in animal models.

Hypothesis

Expressing heterologous viral proteins in place of the VSV G protein in a recombinant vesicular stomatitis virus permits oncolytic efficacy in circumstances under which the parent vesicular stomatitis virus may be toxic or ineffective.

Objectives

The first objective is to express a fusogenic reovirus protein p14 FAST to enhance the spread of the recombinant vesicular stomatitis virus in cancer cells and obtain oncolytic efficacy in a mouse tumor model.

The second objective is to produce recombinant VSV bearing viral envelope proteins from other vesiculovirus members (Maraba, Chandipura and Isfahan) or from a beta-retrovirus (Jaagsiekte Sheep Retrovirus) and demonstrate that the recombinant virus can be delivered systemically, and evade pre-existing anti-VSV serum and allow for successful tumor infection in an in vivo mouse model.

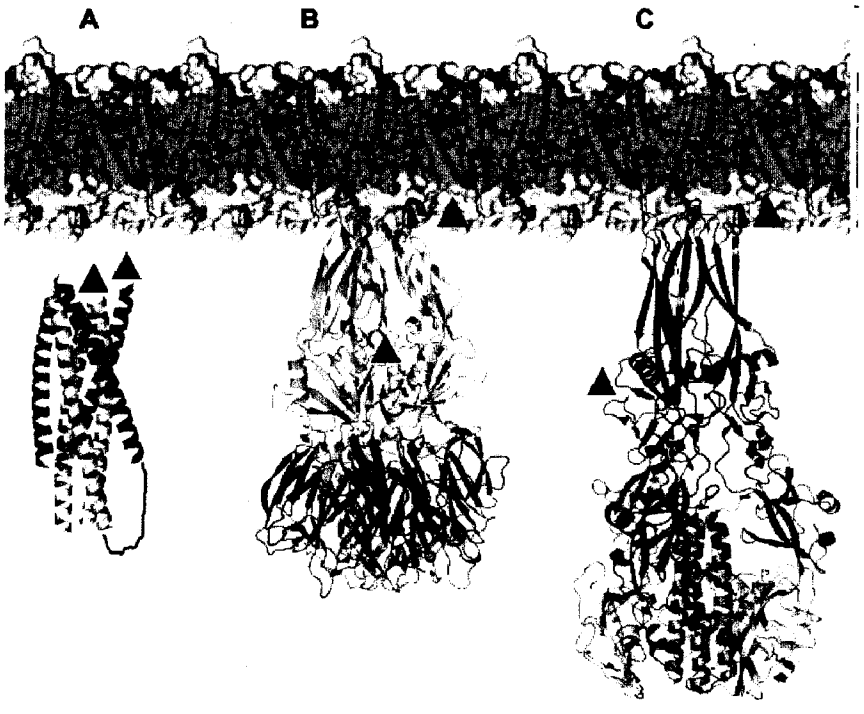
The third objective is to produse recombinant VSV bearing a viral envelope protein from a beta-retrovirus (Jaagsiekte Sheep Retrovirus) and demonstrate that the recombinant virus is no longer neurotropic or neuro-toxic.

Chapter 1: The p14 FAST protein is a neurovirulence factor and an oncolytic agent.

Introduction

Fusogenic proteins have been used to enhance oncolytic effects and have been inserted into recombinant oncolytic viruses where they enhance viral spread, inflict toxicity to infected cells and the resulting syncytia stimulate an anti-tumor immunity leading to an overall increased tumor-killing capacity[23,24,349,391]. Glycoproteins from some enveloped viruses have evolved to combine the function of receptor binding and membrane fusion. Upon binding to their receptor or within the acidic confines of the endosome, the glycoprotein undergoes a conformational change resulting in the exposure of a fusion domain which then interacts with and disrupts the surrounding membrane[410]. Three classes of viral fusion proteins have been identified based on post fusion states. Class I have trimers of hairpins containing a central helical coiled-coil structure such as that found in hemagglutinin on the influenza A virus, Ebola glycoprotein (gp2) or RSV F. Class II fusogenic proteins also have trimers of hairpins but they are composed of beta structures such as Semliki forest virus E1 or Dengue 2 virus E glycoproteins. Class III fusion proteins also have trimers of hairpins and a central helical trimeric core like class I, but each fusion domain exposes two fusion loops at the tip of a beta sheet like class II proteins; examples include the HSV gB and VSV G glycoproteins[410] (Fig. 5).

Figure 5. Different classes of viral fusion proteins.
Structural motifs of viral fusion proteins depicting class I, II and III fusion proteins in their post-fusion conformations. (A) HIV-1 gp41; (B) Flavivirus fusion protein E and (C) VSV glycoprotein G. Reproduced with permission from Weissenhorn et al.2007[410]. Elsevier liscence number 1958230406156.



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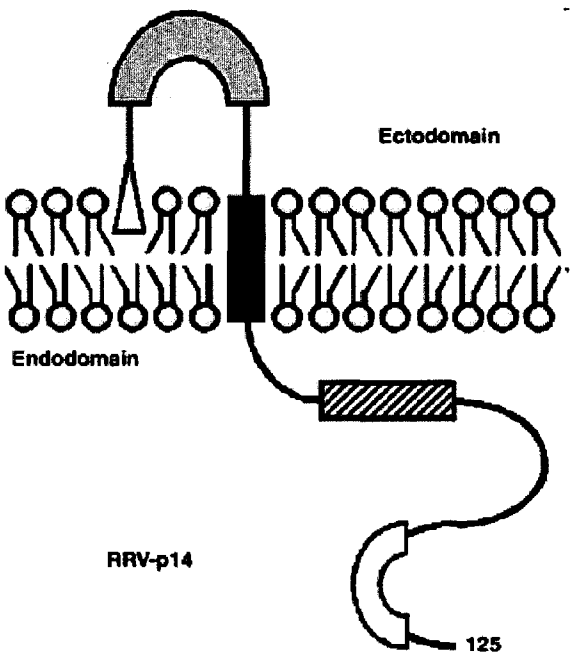
Recently p14 a Reovirus fusogenic peptide, FAST (Fusion Associated Small Transmembrane), was identified as being the least complex protein-based membrane fusion machine, being both necessary and sufficient for membrane fusion as demonstrated using proteoliposomes[385]. The *Orthoreovirus* genus is one of nine accepted genera within the family Reoviridae, a family of double-stranded RNA (dsRNA) viruses having icosahedral non-enveloped capsids[263]. Members of the *Orthoreoviridae* genus share similar capsid morphologies, host ranges, biological properties, replication strategies, electropherotypes and protein profiles[390]. Some of these viruses, particularly amongst the avian reoviruses (ARV) but also atypical mammalian and reptilian isolates, possess the unusual ability to induce syncytium formation[97]. These non-enveloped viruses express non-structural fusogenic proteins that are nonessential for viral replication in vitro and have been postulated to be virulence factors that facilitate rapid dissemination in vivo[98,101].

The p14 FAST protein of reptilian reovirus is a small, 125 amino acid, class III, bitopic protein with proportional ecto and endo domains. The 36 residue amino terminus is myristylated and contains a 20 residue hydrophobic patch, both are obligate requirements for membrane fusion activity[71,73] (Fig. 6). The 68 residue endodomain contains a polybasic and polyproline region of no known function[385]. The protein mediates extensive cell-cell fusion when expressed in cultured cells rather than virus-cell membrane fusion, and only needs to be expressed in the donor set of membranes undergoing fusion[71,385]. The p14 FAST

Figure 6. Hypothetical protein structure of FAST.

Structural motifs in the p14 FAST protein. The 125-residue p14 protein is depicted in its functional N exo / C cyt membrane topology. Structural motifs include an N-terminal myristate (open triangle) that may interact with the external leaflet of the bilayer, a moderately hydrophobic patch (shaded rectangle) that shares certain features with fusion peptide motifs, a transmembrane domain (black rectangle), a membrane-proximal polybasic region (hatched rectangle), and a C-proximal polyproline motif (open rectangle). Reproduced with permission from Top et al. 2005[385]. Nature Publishing Group license number 1958231067812.



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protein mediates cell-cell cytoplasmic membrane fusion from a subset of membrane microdomains leading to apoptosis and membrane instability[72,327]. Unlike enveloped virus fusion glycoproteins, the FAST protein does not contain any coiled-coil domains, fusion motifs or large multimeric ectodomains, thereby placing this unique fusion protein in a class of its own[71]. FAST does not appear to undergo a large energy releasing triggered conformational change to expose a fusion domain such as class I and II enveloped virus fusion proteins[71]. FAST proteins lack specific receptor-binding activity, and use ubiquitous molecules such as cadherins or other adhesion molecules to promote close membrane apposition during the fusion process[328] thereby permitting a broad tropism.

Fusogenic viruses are usually enveloped, and the fusogenicity is often associated with a glycoprotein that is also required for viral entry[410]. It is difficult to examine the role of a syncytial phenotype in virulence and pathogenesis when such a property is required for efficient viral entry. I have added the p14 FAST gene to the vesicular stomatitis virus (VSV) genome, a non-fusogenic prototypical member of the *Rhabdoviridae* family. By adding this fusogenic function to VSV I have made a virus that does not require the syncytial phenotype for entry but utilizes this ability to enhance its spread in vivo. Furthermore I evaluate its role as an oncolytic agent.

This novel fusogenic virus, VSV/FAST, displays normal replication in vitro but enhanced spread and virulence in vivo. VSV/FAST more rapidly and

consistently reaches high titres in the brains of infected mice where it mediates extensive tissue damage. Thus the p14 FAST protein of reptilian reovirus is a bona fide virulence factor that enhances viral spread in vivo. Furthermore the fusogenic effect in VSV also acts as an independent oncolytic factor generating efficacy in mice bearing subcutaneous or pulmonary tumors.

Material and Methods

Generation of VSV/FAST Recombinants:

PCR was performed on a plasmid containing the p14 FAST cDNA using the following primers: CGCTCGAGACCACCATGGGGAGTGGACCCTCTAATTTC and CGGCTAGCCTAAATGGCTGAGACATTATCGAT, in order to add an upstream XhoI site and a downstream NheI site. The resulting PCR product was cloned into the XhoI/NheI sites of pVSV-XN and pVSV-XN Δ M51. Following sequencing, the genome plasmids were used to rescue infectious virus. The procedure for rescuing these infectious VSV/FAST recombinants was similar to that previously described[214]. Briefly, BHK cells expressing the T7 RNA polymerase (BHK-T7) were grown to approximately 85% confluency in sterile polystyrene 24-well microtiter plates (Costar 3526, Corning, Corning, NY, USA). BHK-T7 cells in 400ul of serum free medium per well were transfected with each well receiving an additional 100ul of serum free medium containing 2ul of Lipofectamine 2000 (Invitrogen), 0.5ug of the genome plasmid and 0.5ug of the support plasmids containing the elements of the VSV ribonucleoprotein (125ng pBS-VSV N, 313ng pBS-VSV P, 63ng pBS-VSV L). After 5 hours of tranfection, the serum free media was replaced with 500ul of media containing 10% FBS. Wells in which cells were observed to have CPE 72 – 96h after transfection were passaged, amplified and filtered through 0.2μm filters. Rescue efficiency with this method is <1.5%.

The replication incompetent VSV/ΔG/mRFP1/FAST recombinant was generated using the above generated pVSV-X(FAST)N genome vector, removing the G gene and replacing it with the mRFP1 transgene (Accession number AF506027). The details of this cloning are as follows: mRFP1 was originally flanked by XhoI and NheI sites and subsequently blunt end ligated into a pT7Blue-3 vector (Novagen). After confirmation of orientation, the mRFP1 gene was removed from the pT7Blue3 vector using the MluI and SalI restriction sites and then subcloned into the MluI and XhoI sites of a pT7Blue3 vector containing the following stop-start sequence (GGTATGAAAAAAACTAACAGATATCACG) between the XhoI and NheI sites. The mRFP1-Stop-Start gene was then removed by digesting at the MluI and XbaI sites and blunt end ligated back into pT7Blue-3. After confirmation of orientation, the mRFP1-Stop-Start gene was removed by digesting with MluI and SalI and ligated into the MluI and XhoI sites of the previously generated pVSV-X(FAST)N genome vector, thereby replacing the G gene contained within the MluI and XhoI sites.

The replication competent ΔM51VSV/mRFP1/FAST was generated using the mRFP1-Stop-Start gene flanked with MluI and SalI, previously described, and blunt ligating this back into pT7Blue3 and confirming it in the reverse orientation; this allowed the gene to be flanked by SalI sites. Subsequently digesting with SalI

and inserting into the XhoI site of pVSV-XN Δ M51 genome produced ΔM51VSV/mRFP1/FAST. Proper orientation was confirmed.

Rescue of the VSV/ΔG/mRFP1/FAST and ΔM51VSV/mRFP1/FAST recombinants was performed using a novel method. Using 90% confluent BHK-T7 cells in 6 well plates (Costar 6 Well Microplates, Corning, Corning, NY, USA) which were incubated with Vaccinia-T7 at an MOI of 1 and after 1h of adsorption time, the inoculum was removed and 1.5ml of serum free media was added to the wells. Each well of cells was transfected with an additional 500ul of serum free media containing 5ul of Lipofectamine 2000 (Invitrogen), 2ug of the genome plasmid and 2.5ug of the support plasmids containing the elements of the Maraba ribonucleoprotein (1ug pCI-Neo-Maraba N, 1.25ug pCI-Neo-Maraba P, 0.25ug pCI-Neo-Maraba L). It should be noted that for rescuing the replication incompetent virus 0.25ug/well of the pMD2G-VSVG (Tronolab, Lausanne, Switzerland) support plasmid was added to the transfection. After 5 hours of transfection, the serum free medium was replaced with 2ml of medium containing 10% FBS. Wells in which cells were observed to have CPE as early as 24h after transfection were filtered, passaged, filtered amplified and filtered again through 0.2μm filters. Rescue efficiency with this protocol is 100%. All rescued recombinants were plaque purified 3 times, amplified, filtered through 0.2μm filters and titrated on Vero cells.

Viruses and Cells:

BHK-T7 (gift from Karl-Klaus Conzelmann) and Vero, HEK 293T, 4T1, CT26, Colo 205, NCI-H747, SW620, HT-29, NCI-H520, NCI-H23, NCI-H226, A549, DU 145, MDA-MB-435S, U-2 OS, and SK-ES-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA 20108 USA). SNB19 cells were obtained from the National Institute of Health (Bethesda MD, USA), GM38 cell from the National Institute of General Medical Sciences Mutant Cell Repository (Camden, NJ), and the human Ewing sarcoma 91304 cell line originated from Ottawa Health Research Institute (Ottawa, Canada). Cells were propagated in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal calf serum (Cansera, Etobicoke, Ontario, Canada). BHK-T7 were grown in the presence of G418 (Invitrogen) at a final concentration of 0.2mg/ml and used for virus rescues.

For production of virus used in animal experiments HEK 293T or Vero cells were infected at an MOI of 0.01. Culture supernatants were collected 24 hours later and cleared by centrifugation and filtration through a 0.2μm filter. Virus was pelleted and then banded on a continuous 5% - 40% sucrose gradient made in PBS. Banded virus was extensively dialyzed against PBS, aliquoted and stored at -80°C. Stocks were titered on Vero cells.

In vitro Tissue culture Experiments:

HEPES buffered medium was used to maintain a pH range of 6.4 to 6.8 in low pH condition and pH 7.3-7.5 in normal pH condition for tissue culture medium supplemented with 10% FBS. The pH was confirmed before and after the experiment using a calibrated pH meter (Accumet pH meter 915, Fisher Scientific). Where indicated, 7.4 → 7.4 indicates virus was adsorbed on cells at pH 7.4 and then rinsed 3 times with medium pH 7.4 followed by incubation in medium at pH 7.4, whereas 7.4 → 6.8 indicates virus was adsorbed on cells at pH 7.4 and then rinsed 3 time with medium at pH 6.8 followed by incubation in medium at pH 6.8; and 6.8 → 6.8 indicates that virus was adsorbed on cells at pH 6.8 followed by 3 rinses at pH 6.8 and incubated at pH 6.8. All adsorbtion times were for 45 minutes in a 37°C incubator with 5% CO_2.

Where indicated, cells were pretreated with interferon alpha-2b (Intron A, Schering Corporation, Kenilworth, NJ 07033) at a concentration 150 international units per ml for a total of 6 hours and then replenished with fresh cell culture media.

Crystal violet staining was performed on adherent cells after removing culture media, incubating with a 1% crystal violet stain in 100% methanol for 10 minutes, followed by 3 washes in ddH_2O.

In- Cell Western:

Cells were fixed with a 1:1 mixture of acetone and methanol for 10 minutes, followed by blocking in 20% v/v Fish Gel TBS for 1 hour at RT, then incubated with 1:5000 rabbit polyclonal VSV, washed 4x with FG/TBST, secondary Anti rabbit 680 (Molecular Probes) is applied at 1:2000 (Alexa Fluor 680 goat anti-rabbit IgG (H+L) absorbs at 680 and emits at 700nm fluorescence). Washed again 4 times with FG/TBST. Cells were then immediately scanned using the Odyssey Li-cor and measurements were recorded in mean fluorescent units.

Mouse Experiments:

Female, 8 to 10 week-old Balb/c, Balb/c nude and CD1 nude mice were obtained from Charles River Laboratories (Wilmington, MA) and B6 nude mice were obtained from Taconic (Hudson, NY). All experiments were conducted with the approval of the University of Ottawa or McMaster University Animal Care and Veterinary Services. Mice were anesthetized with 3% induction and 2% maintenance of isoflurane for intranasal (IN) instillation of 10ul or 50ul of virus and allowed to recover under oxygen. Intravenous (IV) administration of 100ul of virus was performed through the tail vein. Intratumoral injections were performed using a 50ul volume with a 27 gauge x ½" needle. Mice were monitored daily and euthanized at indicated time points or upon signs of morbidity by carbon dioxide narcosis. Tissues were collected from euthanized mice and immediately placed at minus 80 degrees Celsius. For the in vivo biodistribution assay single organs were halved before freezing, otherwise only

one bilateral organ was used for virus titration. Viral content of homogenized tissues was determined by plaque assay on Vero cells. All tissues had undergone only one freeze thaw cycle and tissue weight was measured after homogenization.

For the mouse tumor models, mice were either injected subcutaneously with 1e6 CT26 cells or intravenously with 1e6 human Ewing sarcoma 91304 cells. Treatments began when subcutaneous tumors were visible and measure at least $13mm^3$ or greater, whereas lung tumor bearing mice began treatments after 1 week of tumor implantation, a time determined from previous experiments. Tumor volumes in cubic millimeters were determined using digital calipers and the formula (lager measurement/2) x $(\text{smaller measurement})^2$.

Immunohistochemistry:

Tissues were harvested as described, placed in OCT mounting media (Tissue-Tek, Sakura Finetek, Torrance, CA, USA) and sectioned in 4um sections with a microtome cryostat (Microm HM500 OM Cryostat). Sectioned tissues were fixed in 4% paraformaldehyde for 20 minutes and used for hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC). IHC was performed using reagents from a Vectastain ABC kit for rabbit primary antibodies (Vector Labs, Burlingame, CA), according to instructions provided. Primary antibodies used were polyclonal rabbit antibodies against VSV (gift of Earl Brown). Briefly, endogenous peroxidase activity was blocked by incubating with 3% H_2O_2 followed by blocking of non-specific epitopes with 1.5% normal goat serum, then

by blocking with avidin and biotin. PBS washes were performed between all blocking and incubating steps. Sections were incubated with anti-VSV antibody (1:5000, 30 minutes) followed by anti-rabbit biotinylated secondary antibody. The avidin:biotinylated enzyme complex was added and the antigen was localized by incubation with 3,3-diaminobenzidine. Sections were counterstained with hematoxylin. Slides were scanned on a Nikon Coolscan.

Analysis of tumor perfusion:

Mice were injected intravenously with 100 μl of a 50% solution of 100 nm diameter orange fluorescent microspheres (Molecular Probes, Burlington, Canada). Five minutes later, animals were sacrificed and the tumors harvested, covered in OCT media and then immediately snap frozen on dry ice and then placed at -80°C until ready for sectioning as previously described. Tumor perfusion was analyzed by visualizing fluorescent microspheres in the vasculature of 10 μm unfixed frozen sections using a ScanArray Express microarray scanner with a standard Cy3 laser (Packard Bioscience).

Detection of Anti-VSV T cells:

Performed by Kyle Stephenson. Balb/c mice were infected with 1e7 PFU of either WT VSV/GFP or VSV/FAST intravenously. Blood samples were taken five and seven days post-infection and spleens were removed seven days post infection. Peripheral blood mononuclear cells and splenocytes were stimulated for 6 hours with the MPY peptide, a CD8 epitope of VSV-N. Golgi plug (BD

Results

Generation of VSV/FAST recombinant viruses

The p14 FAST open reading frame (Gift from Roy Duncan, Dalhousie University, NS Canada) was PCR-amplified from pcDNA-p14 FAST to add flanking XhoI and NheI sites. The resulting PCR product was subcloned into pVSV-XN and the pVSV-XNΔM51 genome using these sites (Fig. 7). Following sequence confirmation, infectious VSV/FAST and ΔM51VSV/FAST virus was rescued. Two additional VSV recombinants were also generated in similar fashion. ΔM51VSV/mRFP1/FAST includes an mRFP1 transgene, and a replication incompetent VSV/ΔG/mRFP1/FAST that lacks the G gene.

VSV/FAST recombinants results in syncytia formation in vitro

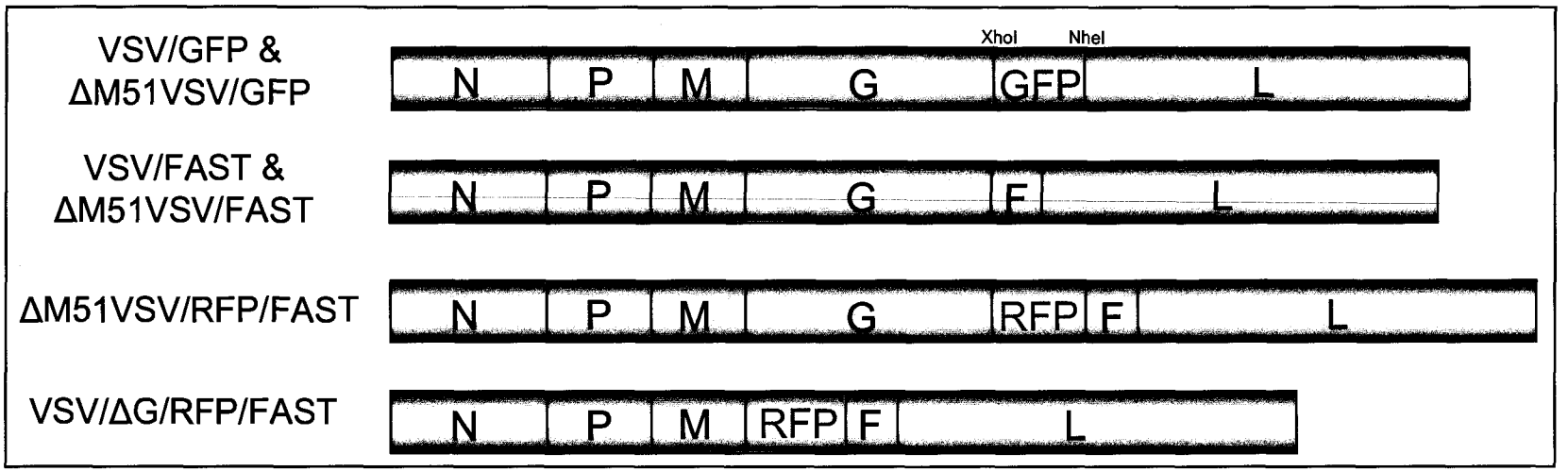
Infection of Vero (Fig. 8), HEK 293T and BHK-T7 cells (not shown) with all the replication competent FAST recombinant viruses resulted in the formation of large syncytia unlike the control VSV recombinant, thereby confirming successful viral rescue and FAST transgene expression.

Plaques and cytotoxicity of recombinant FAST viruses

I noted that VSV/FAST, Δ51VSV/FAST and Δ51VSV/mRFP1/FAST generated plaques at least as large as VSV and Δ51VSV/GFP (Fig. 9), if not larger at higher titers (Fig. 9C,H). Even the replication incompetent Δ51VSV/ΔG/mRFP1/FAST generated plaques (Fig. 9K arrows), unlike the

Figure 7. Generation of VSV/FAST.
(A) The p14 FAST gene from a fusogenic reptilian reovirus was subcloned into the pVSV-XN and pVSV-XNΔM51 genome plasmid between the glycoprotein (G) and polymerase (L) gene, as GFP had been inserted previously, and rescued as VSV/FAST and ΔM51VSV/FAST respectively. In addition, a construct was generated with the red fluorescent protein (mRFP1) gene upstream of the FAST gene to form the ΔM51VSV/mRFP1/FAST recombinant, and a glycoprotein deleted construct gave rise to the replication incompetent VSV/ΔG/mRFP1/FAST recombinant virus. F denotes the FAST gene in this figure. (B) Western blot performed by Brian Lichty and Kyle Stephenson. Western blot analysis of infected cell lysates and sucrose banded virus reveals that FAST is expressed in VSV/FAST infected cells and is present in the viral particle. Vero cells were infected with virus overnight and cellular lysates were run (lanes 1-4) or virus was banded on a sucrose gradient and 1x108 PFU were run per lane (lanes 5-7). Blots were probed for VSV proteins (left), FAST protein (middle) or GFP (right). Lanes: (1) VSV/GFP, (2) mock, (3) VSV/FAST, (4) ΔM51VSV/FAST (5) VSV/GFP, (6) VSV/FAST and (7) ΔM51VSV/FAST.

A.


VSV/GFP &
ΔM51VSV/GFP
XhoI
NheI
N
P
M
G
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L
VSV/FAST &
ΔM51VSV/FAST
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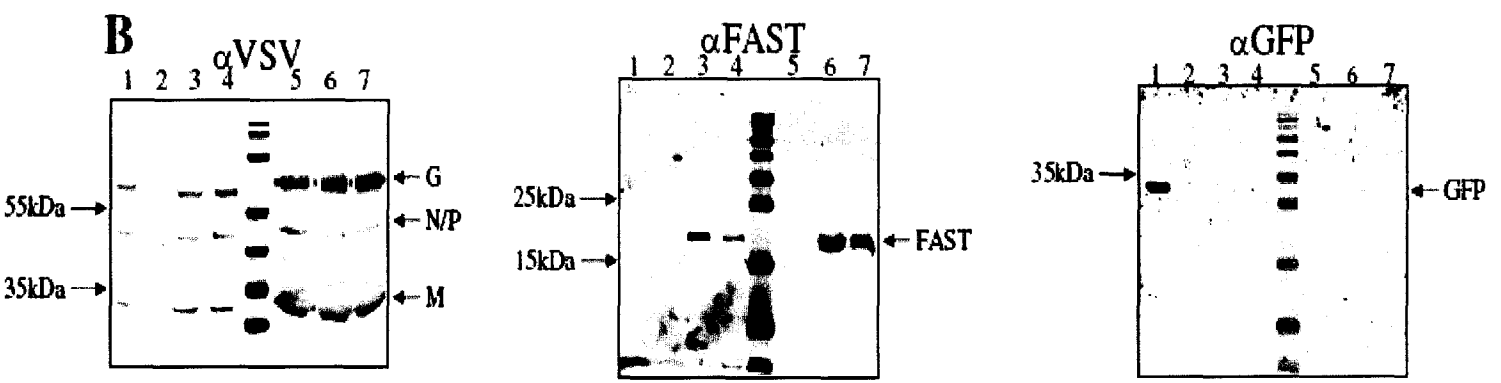

B
αVSV
1 2 3 4 5 6 7
55kDa
35kDa
G
N/P
M
αFAST
1 2 3 4 5 6 7
25kDa
15kDa
FAST
αGFP
1 2 3 4 5 6 7
35kDa
GFP

Figure 8. VSV/FAST recombinants form syncytia in vitro.
Confluent Vero cells were infected with the replication competent FAST recombinants at a multiplicity of infection (MOI) of 1. Photomicrographs taken at 24 hours (A) and 48 hours (B) after infection and show the large syncytia induced by the recombinant virus. Bottom row images are taken using a microscope with fluorescent lighting and appropriate GFP and RFP filters. Remaining images are taken in bright field. Magnification is 8x.

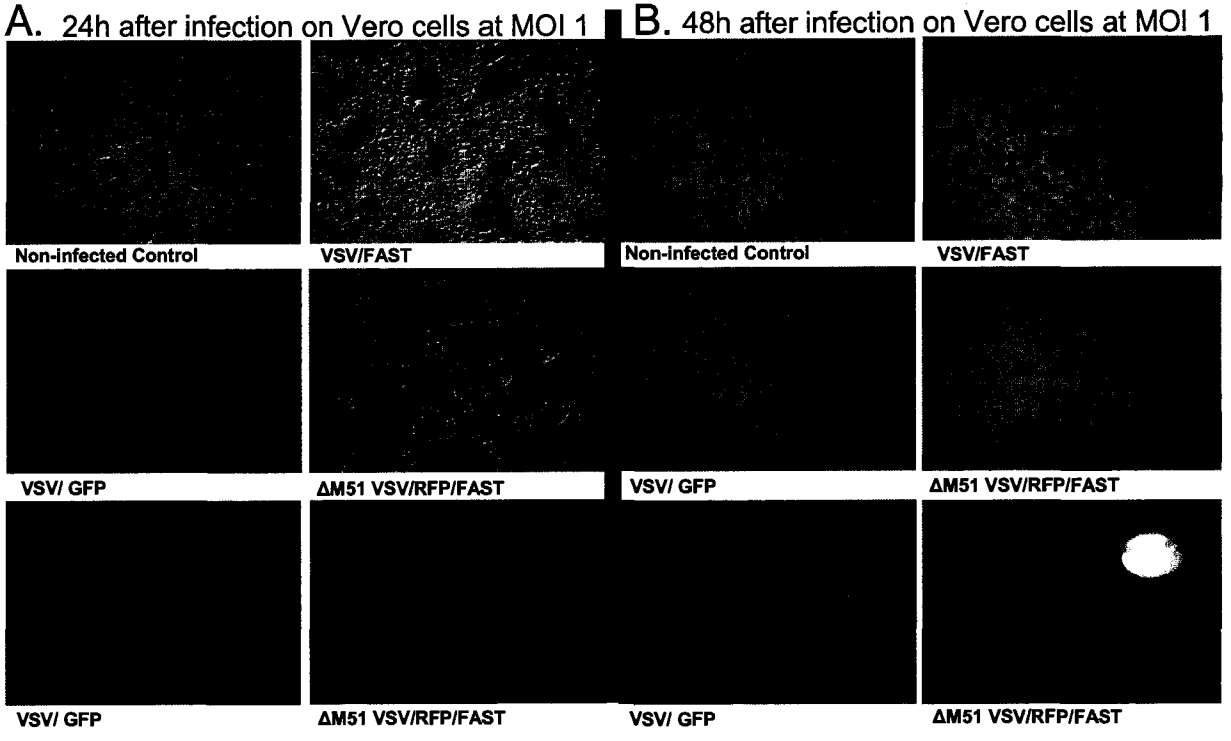
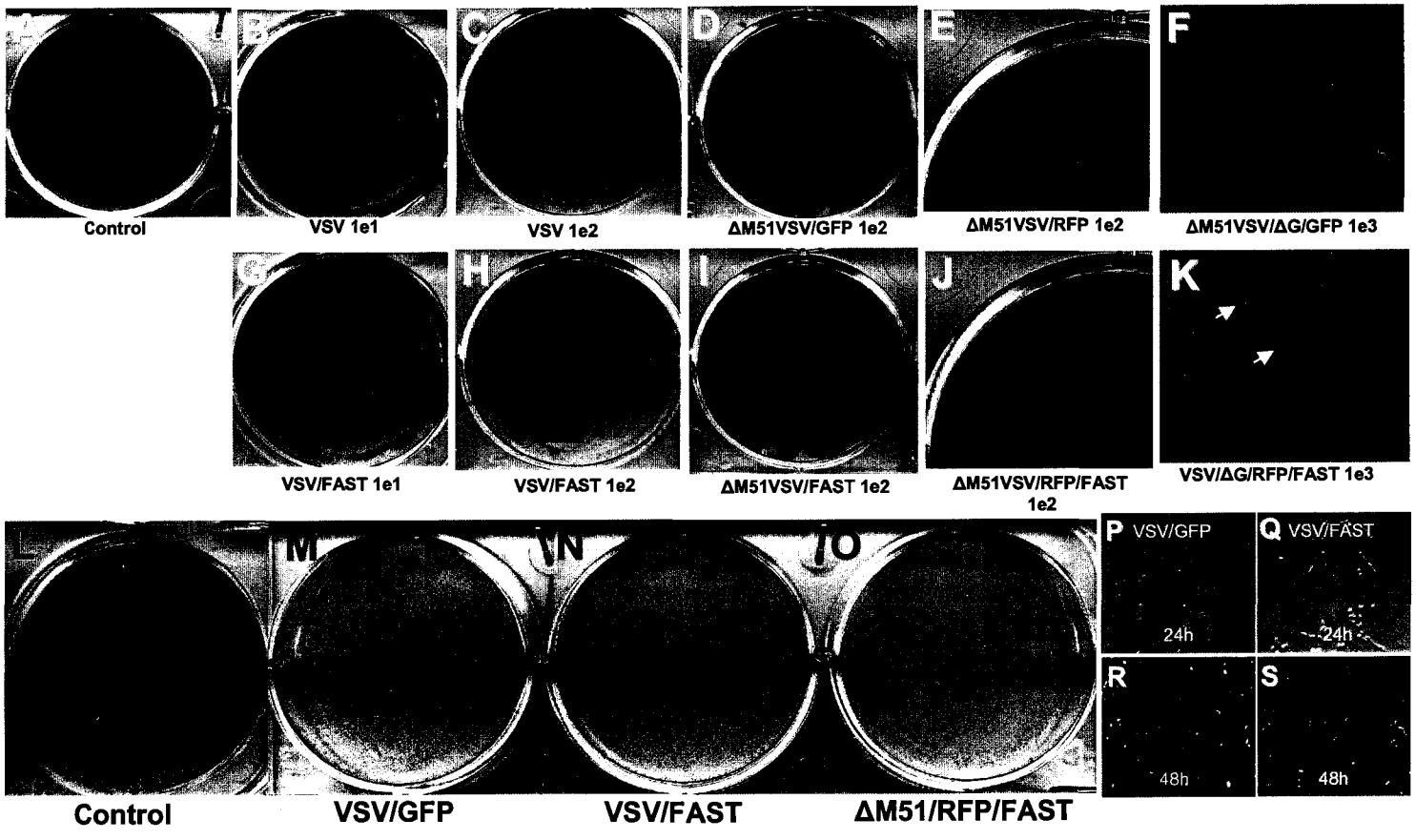

A. 24h after infection on Vero cells at MOI 1
B. 48h after infection on Vero cells at MOI 1
Non-infected Control
VSV/FAST
Non-infected Control
VSV/FAST
VSV/ GFP
ΔM51 VSV/RFP/FAST
VSV/ GFP
ΔM51 VSV/RFP/FAST
VSV/ GFP
ΔM51 VSV/RFP/FAST
VSV/ GFP
ΔM51 VSV/RFP/FAST

Figure 9. Plaques and cytotoxicity of recombinant FAST viruses.
(A-K)Plaque morphology was observed by infecting a monolayer of Vero cells with the indicated PFUs. After a 30 minute adsorption, an agarose overlay was applied. Plaques were observed 48 hours later. Note larger cleared zones in VSV/FAST plaques (G & H). Arrows (K) indicate plaques observed on cells infected with VSV/ΔG/mRFP1/FAST. (L-O)To evaluate CPE, a monolayer of Vero cells was infected at an MOI of 1 with the indicated viruses and crystal violet staining was performed 48 hours later. (P-S)To determine the effect of the recombinant VSV/FAST virus infection in non-confluent Vero cell cultures, cells seeded at low density were infected and observed at 24 and 48 hours and reveal similar CPE compared to the parent VSV/GFP virus.


A
Control
B
VSV 1e1
C
VSV 1e2
D
ΔM51VSV/GFP 1e2
E
ΔM51VSV/RFP 1e2
F
ΔM51VSV/ΔG/GFP 1e3
G
VSV/FAST 1e1
H
VSV/FAST 1e2
I
ΔM51VSV/FAST 1e2
J
ΔM51VSV/RFP/FAST 1e2
K
VSV/ΔG/RFP/FAST 1e3
L
M
N
O
Control
VSV/GFP
VSV/FAST
ΔM51/RFP/FAST
P VSV/GFP
24h
Q VSV/FAST
24h
R
48h
S
48h

replication incompetent Δ51VSV/ΔG/GFP (Fig. 9F). Larger clear zones and the presence of syncytia characterized the VSV/FAST plaques (Fig. 9G). At an MOI of 1 VSV/FAST and ΔM51/mRFP1/FAST generated equivalent cytopathic effects (CPE) as compared to VSV/GFP virus (Fig. 9L-O). Without a confluent monolayer of cells, the VSV/FAST recombinant does not induce large syncytia, but still results in CPE characteristic of the parent VSV/GFP virus (Fig. 9P-S).

FAST expression does not alter/impair VSV replication in vitro

As the VSV/FAST recombinants rapidly induced the formation of large syncytia in infected monolayers, and the FAST protein has been demonstrated to induce apoptosis following cell-cell fusion[327], I set out to determine whether this fusogenic phenotype impaired VSV replication *in vitro*. To this end I performed a one-step growth curve in Vero cells and saw that the growth kinetics for VSV/FAST and VSV/GFP were identical (Fig. 10), similarly the average highest achievable titers of the individual replicating recombinants was within a log of the parent strain (Table 3).

Syncytia formation enhances spread of VSV/FAST

To determine if the FAST protein contributed to the spreading of the recombinant VSV independently of the G protein, a low pH media (pH 6.4) was used as it has been demonstrated that the spread of VSV is significantly attenuated in a low pH environment[141,245,415]. The recombinant FAST virus was allowed to infect a confluent monolayer of Vero cells at pH 7.4, after a 45min

Figure 10. Growth of VSV/FAST in vitro.
Confluent monolayers of Vero cells were infected with either VSV/GFP or VSV/FAST at an MOI of 5.0 for 45 minutes in minimal media after which monolayers were washed three times. Samples were collected at 0, 4, 8, 12 and 24 hours post-infection and viral titres were determined by plaque assay on Vero cells using standard techniques. Three samples were obtained for each time point. T=0 represents titer after 45 minute adsorption and 3 washes with 500ul of PBS. Error bars represent SEM.

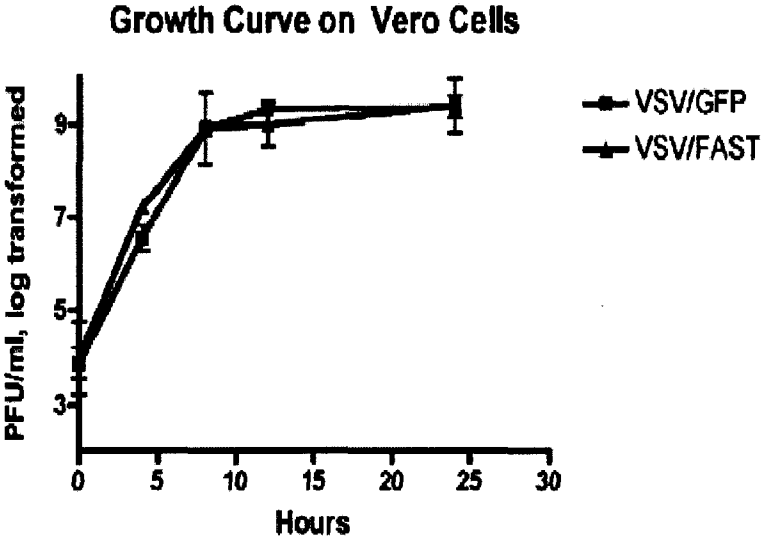

Growth Curve on Vero Cells
PFU/ml, log transformed
9
7
5
3
0
5
10
15
20
25
30
Hours
VSV/GFP
VSV/FAST

Table 3. Maximum achievable titers for parent and recombinant viruses.
Results are averages of 3 independent eperiments. The asterisk refers to the titer per ml of culture medium obtained after virus rescue of the glycoprotein deficient recombinant VSV/ΔG/mRFP1/FAST virus. Titers are per ml after concentration except for where indicated by the asterisk.

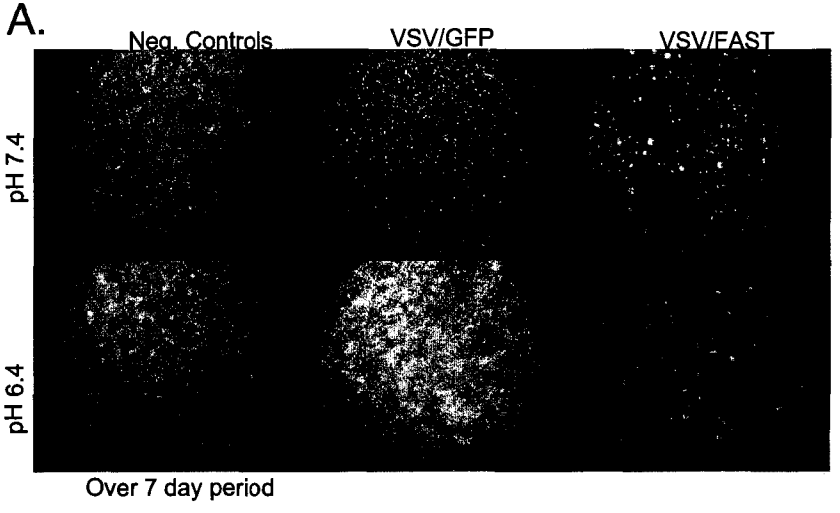
Recombinant	Max Titer
VSV/ GFP	1.7e11
VSV/ FAST	1.5e10
ΔM51VSV/ GFP	1e11
ΔM51VSV/ FAST	3.3e10
ΔM51VSV/ RFP FAST	3.3e11
VSV/ΔG/ RFP/FAST	6e4 *

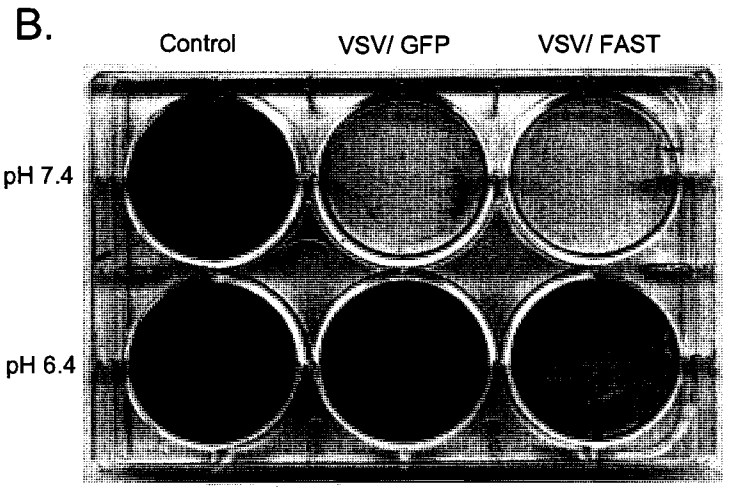
period of adsorption the cells were washed three times with PBS and then media at pH 7.4 or 6.4 was applied to the cells. Over a 7 day period it was observed that the parent VSV virus was unable to spread and form plaques in the low pH environment, unlike the recombinant VSV/FAST (Fig. 11a) which also resulted in more CPE (Fig. 11b). To further determine if the recombinant virus was in fact spreading in the low pH environment, an in cell western analysis was performed using the above mentioned pH conditions, followed by probing the remaining bound cells at 24, 48 and 96h after adsorption with an anti-VSV polyclonal antibody followed by secondary conjugated to a fluorescein probe (Fig. 11C). When scanned, the fluorescent information was converted to pixel count and ploted in a graph (Fig. 11D), confirming that the recombinant VSV/FAST spreads more effectively than parent VSV in a low pH environment.

Syncytia formation is independent of VSV G and increases CPE

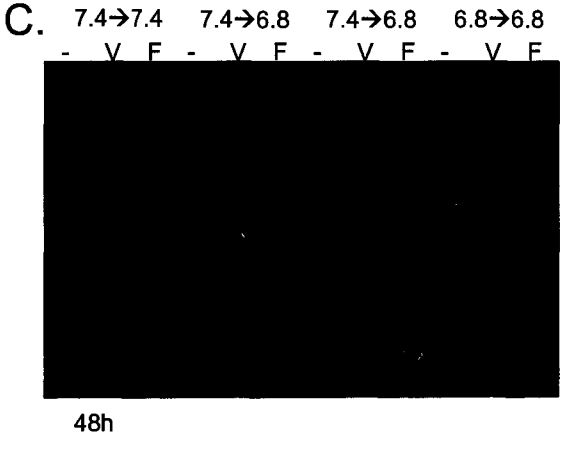
To further confirm that syncytia formation is a characteristic unique to the FAST protein and not the G protein, I generated a replication incompetent VSV/ΔG/mRFP1/FAST virus. Large syncytia are observed when VSV/ΔG/mRFP1/FAST infects a monolayer of cells (Fig. 12A & B) in comparison to the replication incompetent ΔM51VSV/ΔG/GFP. In addition, significant CPE are seen in the cells infected with VSV/ΔG/mRFP1/FAST after 48 hours in comparison to the replication incompetent ΔM51VSV/ΔG/GFP (Fig. 12B). The degree of CPE generated by VSV/ΔG/mRFP1/FAST in comparison to

Figure 11. FAST allows VSV to spread in a low pH environment.
(A)After seven days in a low pH environment, VSV/FAST is able to form plaque like clearings in the over-confluent monolayer of Vero cells, unlike VSV/GFP. (B)Crystal violet stain was performed on cells in panel (A) and confirmed the increased absence of crystal violet cells in the low pH VSV/FAST well compared to the parent VSV/GFP virus at pH 6.4. (C) In cell Western was performed on cells that were infected at pH 7.4 and allowed to incubate at the indicated pH. (D) Graphic representation of quantified data from in cell Western done at 24, 48 and 96h time points. Data between VSV and VSV/FAST at pH 7.4 and VSV/FAST 7.4→6.8 was not significantly different using a two way anova for repeated measures ($p>.05$). However all points were significantly different when compared to VSV 7.4→6.8 with a $p<0.001$. Samples were done in octoplicates, with 7.4→6.8 samples duplicated (hence 16 samples) which were averaged. Data represent a 1 time experiment. (-)=no virus; V=VSV; F=VSV/FAST.





Effect of a Low pH Environment on the Spread of VSV/FAST in comparison to VSV



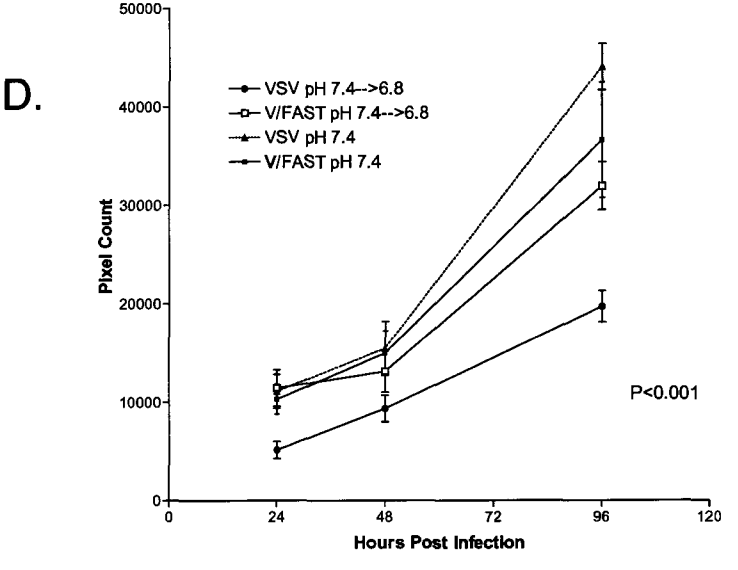
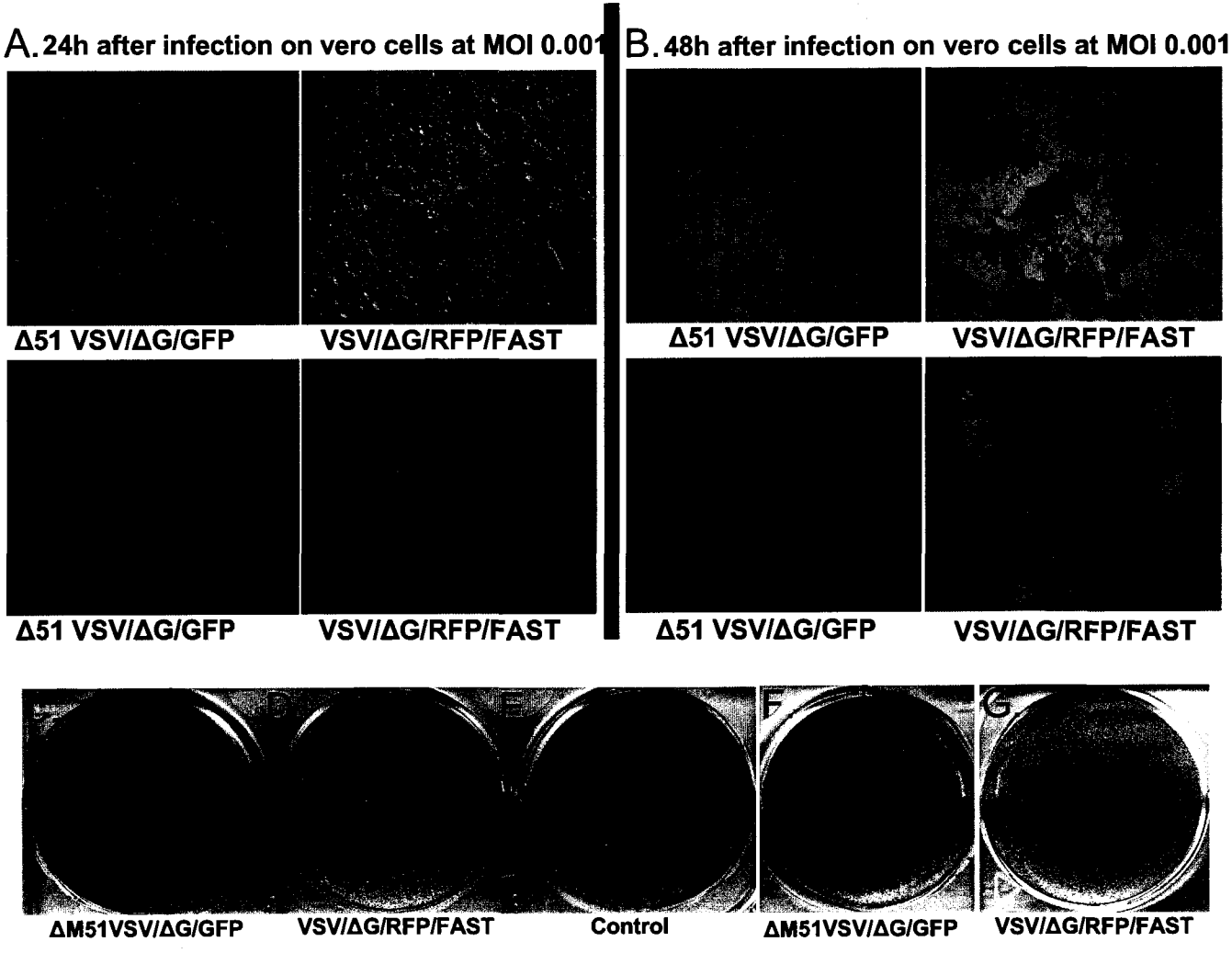


Figure 12. Syncytia formation is independent of VSV G and increases CPE. Confluent Vero cells were infected at MOI 0.001 and imaged at 24 (A) and 48 (B) hours. (A)In comparison to the replication incompetent VSV/GFP, there are large syncytia in the VSV/ΔG/mRFP1/FAST infected cells. (B)Significantly more CPE is noted at 48h in the cells infected with VSV/ΔG/mRFP1/FAST. (C-G)To further confirm increased CPE by the VSV/ΔG/mRFP1/FAST recombinant, a monolayer of Vero cells was infected with an MOI 1 and crystal violet staining was performed at 72h (C-E) and 96h (F-G). Top and bottom row images taken with bright field/white light microscope. Middle row images taken with a fluorescent microscope using appropriate RFP and GFP filters.


A. 24h after infection on vero cells at MOI 0.001
B. 48h after infection on vero cells at MOI 0.001
Δ51 VSV/ΔG/GFP
VSV/ΔG/RFP/FAST
Δ51 VSV/ΔG/GFP
VSV/ΔG/RFP/FAST
Δ51 VSV/ΔG/GFP
VSV/ΔG/RFP/FAST
Δ51 VSV/ΔG/GFP
VSV/ΔG/RFP/FAST
ΔM51VSV/ΔG/GFP
VSV/ΔG/RFP/FAST
Control
ΔM51VSV/ΔG/GFP
VSV/ΔG/RFP/FAST

the replication incompetent VSV is further confirmed using crystal violet staining 72 hours after infection (Fig. 12C-E), and is even more evident after 96 hours (Fig. 12F-G)

VSV/FAST Recombinant viruses have a similar tropism to the parent virus

To determine if FAST was able to alter the tropism of the recombinants, I performed an infectious screen on a subset of both normal and neoplastic cell lines derived from both human and animal origin. As expected, the recombinant FAST virus had the same tropism as the parent VSV. The cells which were not infected by the parent were not infected by the recombinant, similarly for permissive cells (Fig. 13). It could again be noted however, that the recombinants resulted in a slightly higher degree of CPE (Fig. 13B).

FAST is not a significant functional component of the VSV/FAST membrane

The FAST protein is found to be expressed in the recombinant virion (Figure 7B). In order to demonstrate if it was a functional component of the virion, I infected Vero cells at MOI 1 with either the VSV/ΔG/mRFP1/FAST or ΔM51VSV/ΔG/GFP replication incompetent recombinants (Fig. 14A). After 48h, the supernatant was collected, filtered through a 0.2 μm filter and further incubated in media in the presence of anti-VSV neutralizing antibodies, and then passed onto a separate well of Vero cells. No evidence of subsequent infection up to 96h with the passaged replication incompetent FAST or ΔM51VSV/ΔG/GFP virus was observed(Fig. 14B). This experiment was also repeated in the absence

Figure 13. Recombinant FAST virus has the same tropism as the parent virus.
A panel of cell lines consisting of immortalized animal cells, animal and human carcinomas and human sarcomas was infected with the ΔM51VSV/FAST or ΔM51VSV/GFP. (A) Cell lines which are resistant to ΔM51VSV/GFP infection are also resistant to ΔM51VSV/FAST infection as indicated by (-). (B) Although ΔM51VSV/FAST infects the same cell lines as the parent virus, there is less residual crystal violet staining 48h after infection at an MOI of 1, indicating more extensive CPE.

A.

18 Cell Lines Tested	VSV	FAST
Vero		
CT26		
4T1		
DU145		
747D		
BHK-21		

B.

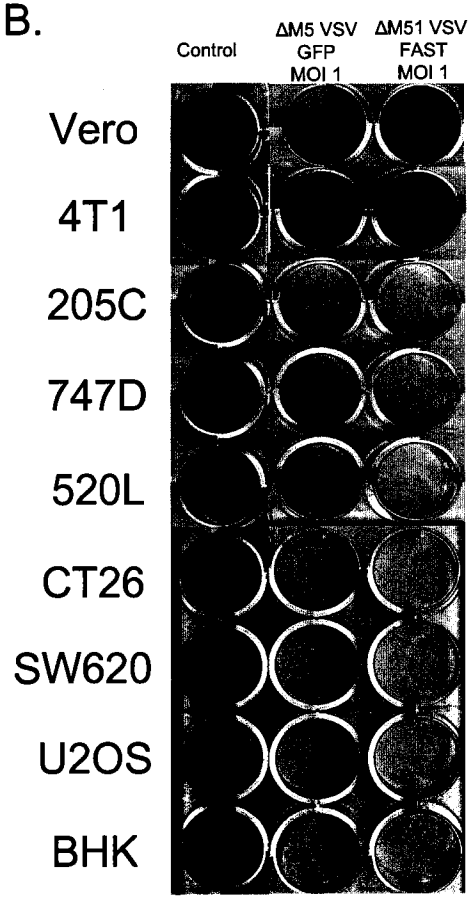
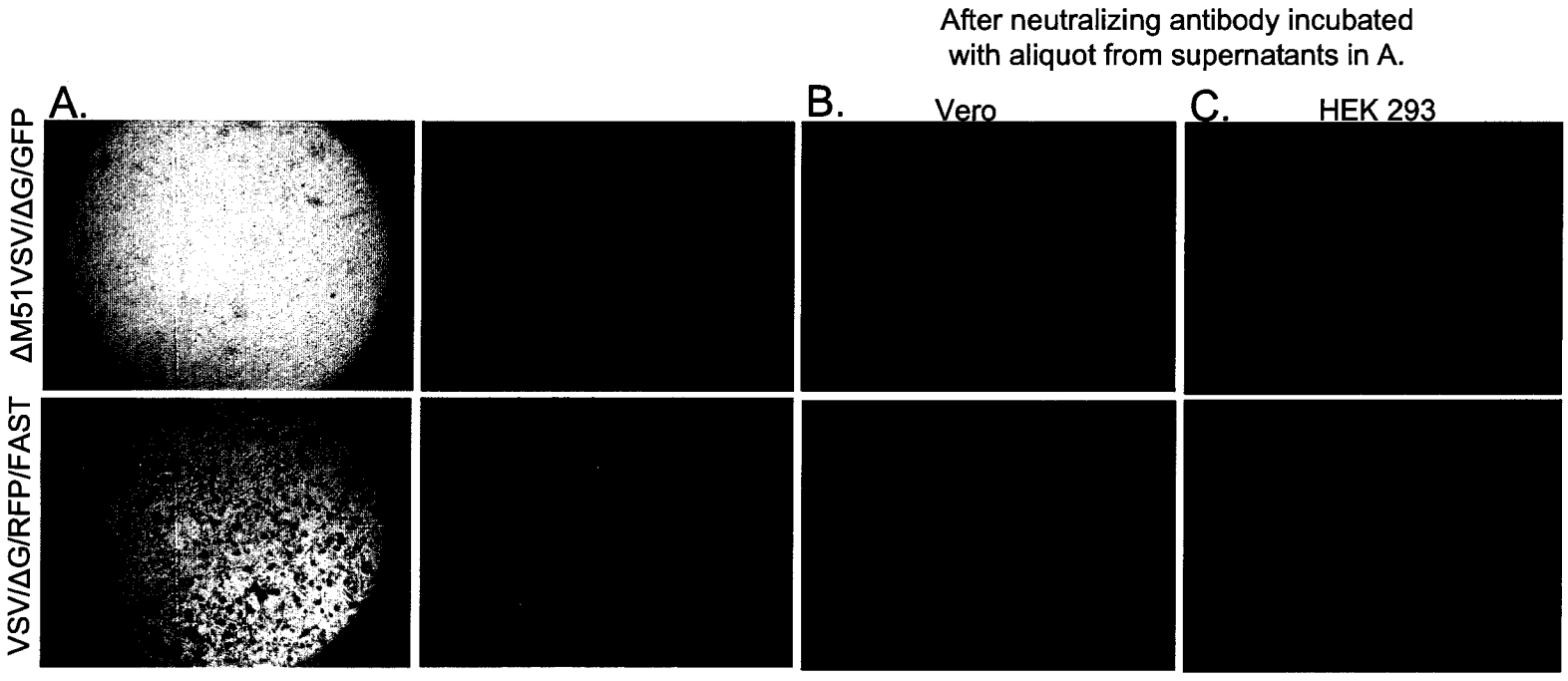


Figure 14. FAST is not a significant functional component of the virion.
To determine if FAST was incorporated as a component of the membrane in the VSV virion, Vero cells and HEK-293 cells were infected with VSV/ΔG/mRFP1/FAST at MOI of 1(A). Supernantant was collected 48h later and filtered through a 0.2μ filter and the filtrate was then pre-incubated with neutralizing antibody before being passaged onto confluent Vero cells (B) or passaged onto HEK-293 cells (C) and observed under a fluorescent microscope for infection for up to 96h.

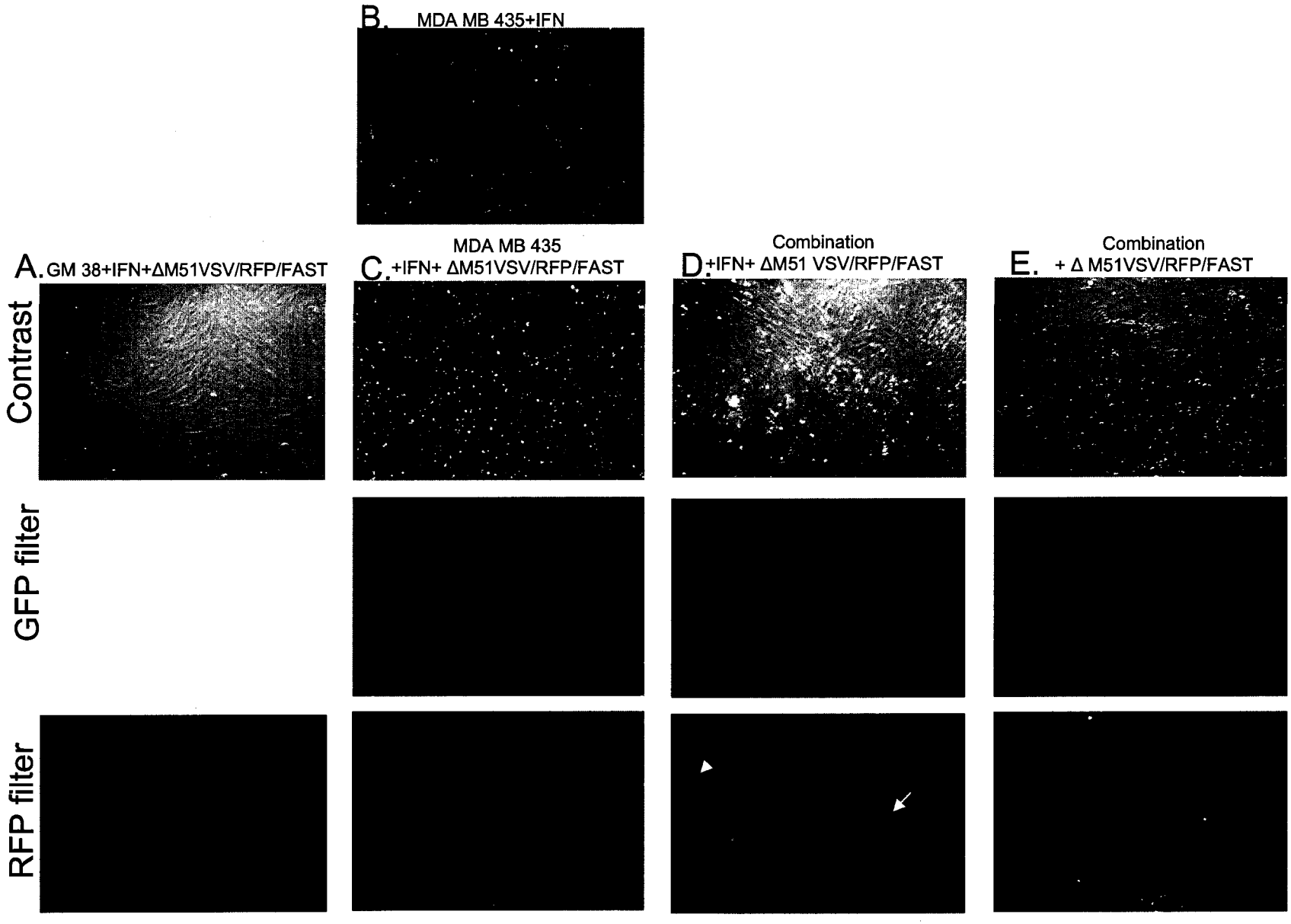

After neutralizing antibody incubated
with aliquot from supernatants in A.
A.
B.
Vero
C.
HEK 293
ΔM51VSV/ΔG/GFP
VSV/ΔG/RFP/FAST

of anti-VSV neutralizing antibodies on HEK 293T cells with the same results (Fig. 14C).

Recombinant VSV/FAST virus is sensitive to interferon

Given that FAST can help VSV spread in the presence of a low pH environment and also in the absence of the G protein; I proceeded to determine if the ΔM51VSV/FAST recombinant was sensitive to interferon. To investigate this possibility in vitro, I used GM-38 human dermal fibroblast cells which can produce interferon and respond to exogenous interferon and are not susceptible to infection by the VSV/FAST recombinant (Fig. 15A). In addition wells were seeded at low density with MDA-MB-435 breast cancer cells and allowed to form colonies (Fig. 15B). The MDA-MB-435 cells endogenously express the GFP, and are not responsive to interferon nor produce interferon and are susceptible to VSV/FAST infection (Fig. 15C). GM-38 were then added to fill in the gaps between the colonies of MDA-MB-435 cells and form a co-culture resulting in a monolayer of heterogeneous cell types (Fig. 15D-E). Wells were pre-treated with interferon for 6h and then Δ51VSV/mRFP1/FAST was added at an MOI of 1(Fig. 15D). Cells were visualized at 24 and 48h and it was observed that the breast cancer cells (green) are predominantly infected with the recombinant FAST virus (Red) (Fig. 15D). Occasionally normal fibroblast are noted to be infected (Fig. 15D arrow), however this is a very infrequent event in comparison to a co-culture which is not pre-treated with interferon (Fig. 15E).

Figure 15 Recombinant ΔM51VSV/mRFP1/FAST is sensitive to interferon.
To determine if ΔM51VSV/mRFP1/FAST is sentitive to interferon, co-cultures of interferon expressing and sensitive cells (GM-38) (A) and human breast carcinoma cells expressing GFP (MDA-MB-435) (B) were pre-treated with interferon and infected with the FAST recombinant(D). (E) More GM-38 cells are infected with the FAST recombinant in the absence of interferon in comparison to D. Arrow indicates cell that was not expressing GFP but expressed mRFP1. Arrow head indicates cell that poorly expressed GFP and mRFP1. MDA MB 435 GFP were seeded at low density (100 cells per well) 5 days prior to overlay with 5e6 GM38, 48h later they were treated with IFN as indicated. This experiment has been repeated three times in 2 independent experiments all with similar results.


B. MDA MB 435+IFN
A. GM 38+IFN+ΔM51VSV/RFP/FAST
C. MDA MB 435 +IFN+ ΔM51VSV/RFP/FAST
D. Combination +IFN+ ΔM51 VSV/RFP/FAST
E. Combination + Δ M51VSV/RFP/FAST
Contrast
GFP filter
RFP filter

VSV/FAST is more virulent in vivo

To investigate the impact of FAST expression on VSV spread and replication in vivo, Balb/c mice were infected intranasally with a range of doses of VSV/GFP or VSV/FAST. Animals were monitored for signs of hind-limb paralysis (HLP) and euthanized at the first clear signs of paralysis. Mice developed HLP at day 7 post-infection with VSV/FAST at doses of 1e7 PFU (1 of 5) and 5e7 PFU while at a dose of 5e8 three of four mice had HLP at day 5 and the remaining mouse developed HLP at day 7 (Table 4). Mice receiving 1e7 or 5e7 PFU of VSV/GFP intranasally failed to develop signs of HLP and survived treatment. It should be noted that the mice surviving infection with VSV/GFP showed no overt sequelae unlike the two mice that did not develop hindlimb paralysis following infection with 5e7 PFU with VSV/FAST which did not appear to recover fully. These mice both displayed weight loss, hunched posture, ruffled fur, sunken/shut eyes, erratic movement and isolation.

Interferon-inducing mutants of VSV have been shown to be highly attenuated in vivo. These viruses have mutations in the matrix protein making them unable to block the production of interferon by infected cells[109,369,402]. IN administration of up to 5e9 PFU of ΔM51-VSV/FAST resulted in no toxicity, a dose well above the lethal dose for VSV/FAST (Table 4). Thus the fusogenic phenotype does not overcome the severe attenuation associated with interferon-induction in vivo.

Table 4. Development of hindlimb paralysis after intranasal VSV/FAST.
Various gradient doses of VSV/FAST, ΔM51VSV/FAST and VSV/GFP recombinants were instilled intranasally to Balb/c mice in order to evaluate the impact in vivo. Mice were then observed for signs of morbidity. VSV/FAST is more virulent than the parent VSV/GFP, whereas ΔM51VSV/FAST is the most attenuated virus resulting in no observable morbidities. ND = not done.

	Virus		
Intranasal Dose	VSV/GFP	VSV/FAST	ΔM51-VSV/FAST
5e6	0/4	0/4	ND
1e7	0/5	1/5	ND
5e7	0/5	3/5	ND
5e8	1/5	4/4	0/5
5e9	ND	ND	0/5

In a parallel experiment groups of 5 Balb/c mice were infected intranasally with 5e7 PFU of either VSV/GFP, Δ51VSV/GFP (an interferon-inducing mutant[369]) or VSV/FAST. On day 6 the mice were euthanized, brains were collected and infectious virus load was measured by plaque assay. The mutant virus failed to enter the brain as expected while both of the wildtype strains did (Fig. 16), however VSV/FAST consistently produced higher titers of virus in the brain at this time-point. This may indicate that VSV/FAST more efficiently enters the brain when delivered by this route of administration.

Biodistribution of VSV/FAST in vivo

The systemic distribution of both VSV/FAST and VSV/GFP following intravenous (IV) delivery of 2e8 PFU was evaluated by performing an *in vivo* biodistribution assay. Measurement of infectious viral particles in various tissues highlighted a more extensive infection of the brain by VSV/FAST at all time-points (Fig. 17). Tissue titre data demonstrated that VSV/FAST consistently was present at higher titres, by 1, 4 & 1 logs at 24h, 48h and 96h respectively in the brains of infected mice in comparison to VSV/GFP (Fig. 17). In both VSV/FAST and VSV/GFP infected animals, the slope of the brain titres are still increasing after 48h in contrast to all other titred organs. Hind limb paralysis was noted in 1 of the 3 animals 96h after IV administration of 2e8 of VSV/FAST, whereas none of the VSV/GFP infected animals demonstrated any signs of neurotoxicity at this time point. Once again, VSV/FAST demonstrated an enhanced ability to rapidly generate high viral titers in the brains of infected mice.

Figure 16. VSV/FAST in the brain following intranasal instillation.
Groups of five Balb/c mice were infected intranasally with 5e7 PFU VSV/GFP, ΔM51-VSV/GFP or VSV/FAST. On day 6 brains were collected and tissue titers were determined by duplicate plaque assays. Although not significantly different, it should be noted that the VSV/FAST brain titers were more consistent in comparison to VSV/GFP.

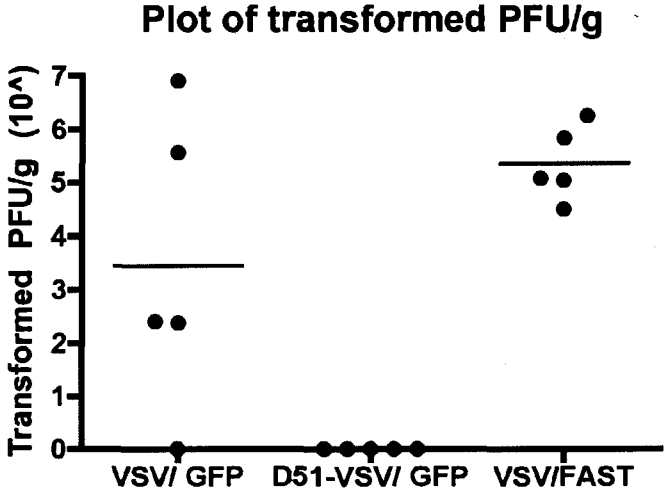
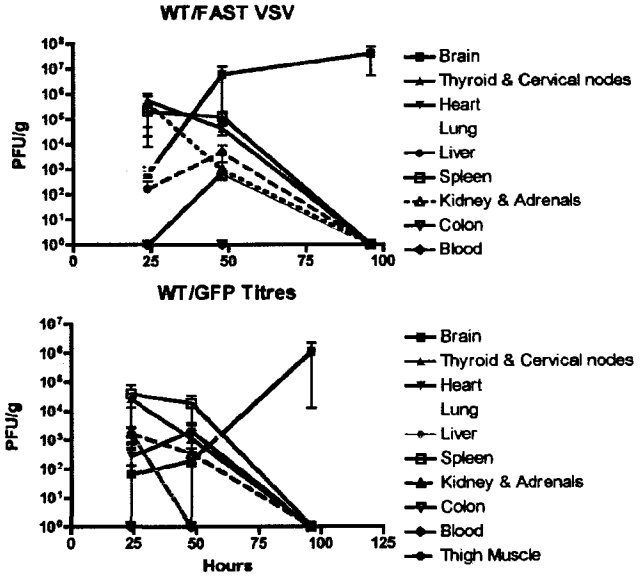

Plot of transformed PFU/g
Transformed PFU/g (10^)
7
6
5
4
3
2
1
0
VSV/ GFP
D51-VSV/ GFP
VSV/FAST

Figure 17. Biodistribution of VSV/FAST in vivo.
VSV/FAST is present at higher titres at 24, 48 and 96h in the brains of infected mice in comparison to VSV/GFP. Slope of titres of brain tissue were increasing for both VSV/GFP and VSV/FAST compared to all other tissues titred. Mice were injected IV with 2e8 PFU of either VSV/GFP or VSV/FAST and tissues from three animals were analyzed for each time point. Error bars represent standard deviations.


WT/FAST VSV
PFU/g
Brain
Thyroid & Cervical nodes
Heart
Lung
Liver
Spleen
Kidney & Adrenals
Colon
Blood
WT/GFP Titres
PFU/g
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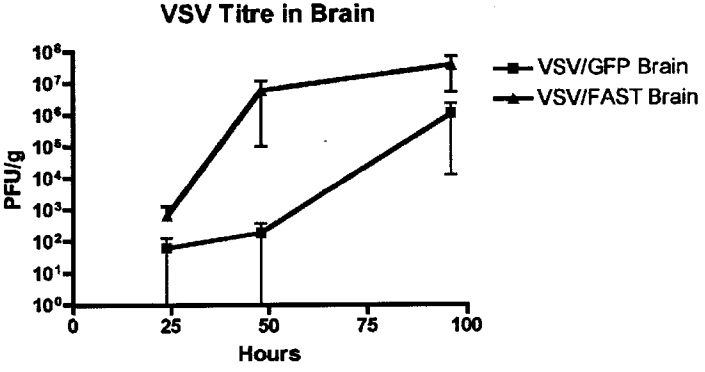
Thyroid & Cervical nodes
Heart
Lung
Liver
Spleen
Kidney & Adrenals
Colon
Blood
Thigh Muscle
Hours


VSV Titre in Brain
PFU/g
VSV/GFP Brain
VSV/FAST Brain
Hours

Distribution of VSV/FAST within the brain.

Brains were collected from Balb/c mice infected intranasally with 5e8 PFU of either the parental VSV/GFP or VSV/FAST viruses. K. B. Stephenson and B. Lichty performed immunohistochemistry on serial brain sections to determine the extend of infection on days 4 and 5 post-infection. This analysis demonstrated that the parental VSV/GFP virus moved more rapidly up the olfactory bulb and into the brain but the extent of the infected zone and the intensity of staining was greater at each time point for the VSV/FAST virus (Fig. 18).

VSV/FAST does not alter the CD8+ T cell response in healthy mice.

Given the lag between the VSV/FAST titer in the brain at day 4 and the onset of hindlimb paralysis at day 7, K. B. Stephenson and B. Lichty used VSV/FAST to investigate if VSV/FAST is able to evade acquired immunity. To this end the CD8+ T cell responses induced following infection with either VSV/GFP or VSV/FAST was examined. Following a single IV dose of 1e7 PFU, peripheral blood was collected at day 5 and both blood and spleens were collected at day 7 post-infection. The percentage of CD8+ T cells displaying IFNγ expression following exposure to the MPY peptide was similar in each case (Fig. 19). Thus, the FAST expressing virus appears to induce a similar magnitude of T cell response following infection at the 7 day mark.

Figure 18. Distribution of VSV/FAST in the brain after intranasal instillation.
This work was performed in collaboration with K. B. Stephenson and B. Lichty. Balb/c mice were infected intranasally with 5e8 PFU of either the parental VSV/GFP or VSV/FAST. Brains were collected for immunohistochemical staining on days 4 and 5 post-infection and stained with a pan-VSV polyclonal antibody. At day 4 post-infection virus was detected within the olfactory bulb (lower arrow, middle section, panel A) as well as the middle of the brain (upper arrow, middle section, panel A) in brains infected with VSV/GFP while the VSV/FAST virus was primarily confined to the olfactory bulb at this time-point (arrow, middle section, panel B) although some brains showed staining at distal sites even at this earlier time-point (arrow, rightmost section near the hippocampal sulcus, panel B). At day 5 post-infection small regions of staining were seen in olfactory bulbs at or near ventricles within the brains of mice infected with VSV/GFP (arrows, third section, panel C) while extensive staining was seen in the middle of brains, especially around the 3rd ventricle, in mice infected with VSV/FAST (first 5 sections, panel D). The intensity and extent of staining observed was consistently greater in VSV/FAST infected brains, successive sections from the same brain shown. The horizontal or axial sections shown for each brain are 8 micron sections thick and are 250 microns apart proceeding from the bottom to the top of the brain left to right.

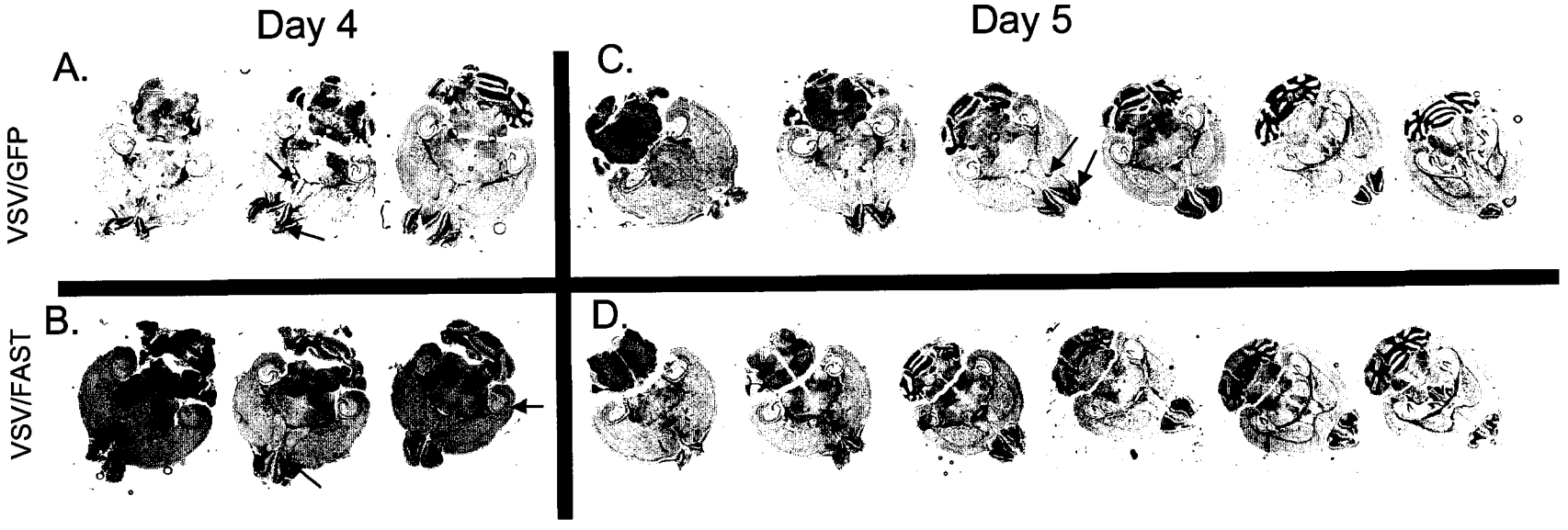
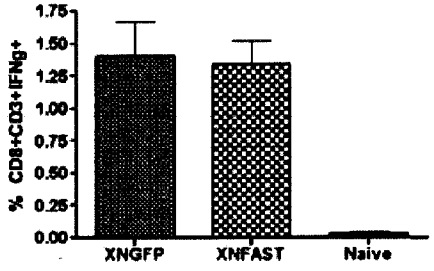
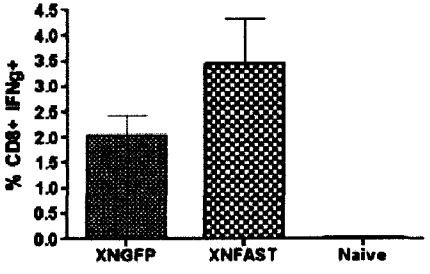

Day 4
Day 5
A.
C.
B.
D.
VSV/GFP
VSV/FAST

Figure 19. VSV/FAST does not alter the acquired immune response.
This figure was performed in collaboration with K. B. Stephenson and B. Lichty. Groups of five Balb/c mice were infected intravenously with $1x10^7$ PFU of either VSV/GFP or VSV/FAST. Five days post-infection peripheral blood was collected and VSV responsive CD8+ T cells were enumerated by intracellular staining for IFNγ production in response to the MPY peptide derived from the nucleocapsid of VSV Indiana. Seven days post-infection peripheral blood and splenocytes were collected and again VSV responsive CD8+ T cells were enumerated. No significant differences were detected.

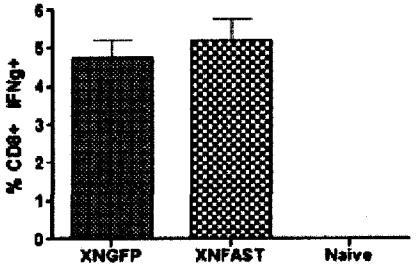
MPY stimulated blood 5 days post-infection



MPY stimulated blood 7 days post-infection



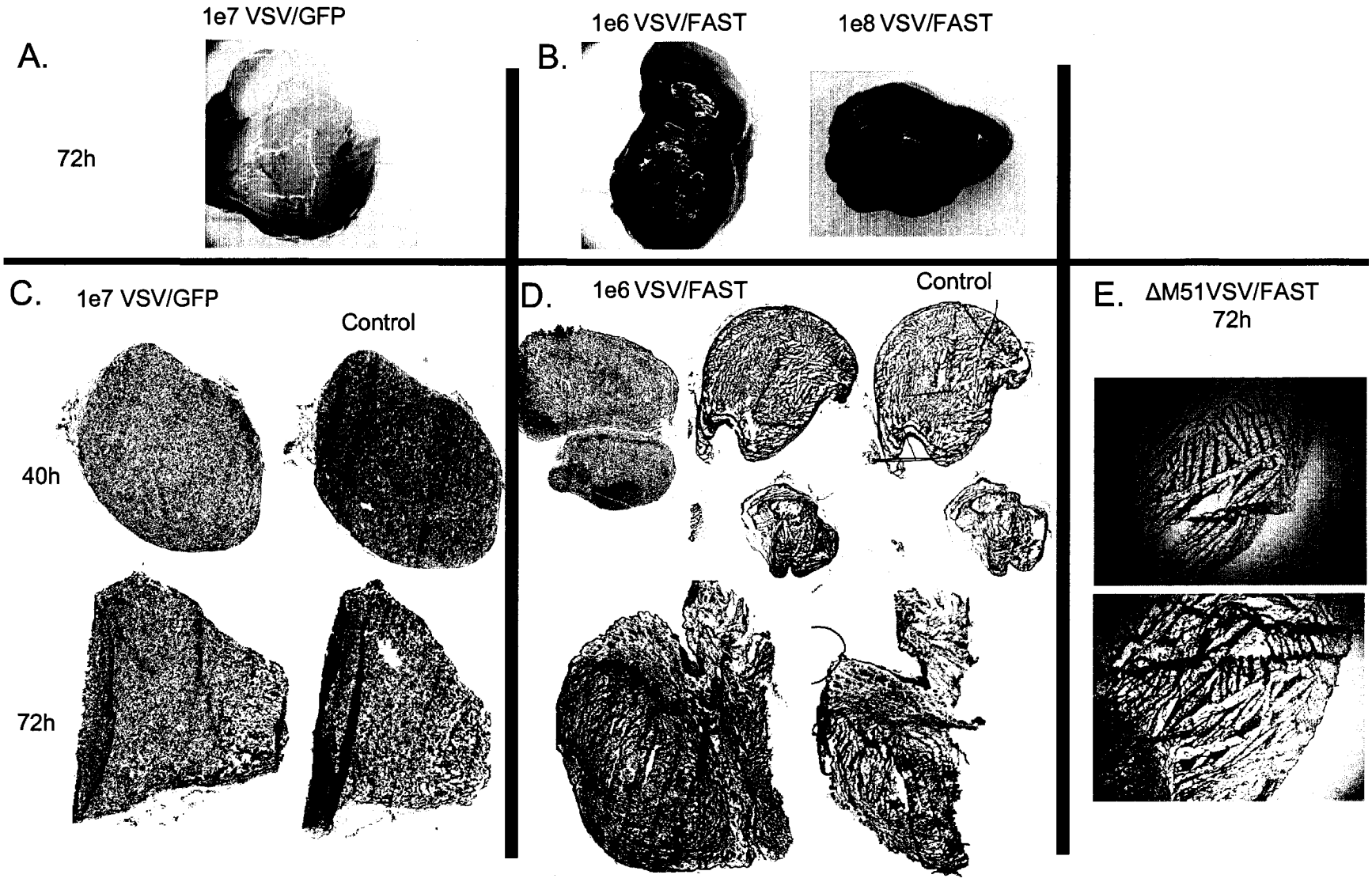
MPY stimulated splenocytes 7 days post-infection



Distribution of VSV/FAST within subcutaneous CT26 Tumors

Given the seemingly enhanced ability to spread in cancer cell lines in vitro as well as in vivo brain tissues, I decided to investigate the oncolytic potential of the recombinant FAST virus. Mice bearing subcutaneous syngeneic CT26 tumors were treated with two intravenous doses, VSV/FAST in comparison with VSV/GFP at the indicated PFU (Fig. 20). On gross examination of the tumors harvested at 72h, there was consistently more necrosis visible in VSV/FAST treated tumors (Fig. 20B). Tumors were also harvested at 40 and 72h, sectioned and IHC was performed. Staining with a polyclonal anti-VSV antibody at 40h demonstrated a larger area of spread of the recombinant VSV/FAST virus in comparison to the parent, affecting not only greater portions of the outer rim of the tumor but also regions well within (Fig. 20D). Furthermore, at 72h in some tumors a significant increase in spread of the recombinant FAST virus was noted, involving almost the entire tumor section (Fig. 20D). Note the degree of staining of the recombinant FAST occupies a larger area in comparison to the parent VSV/GFP despite being given at a 1 log lower dose. Similar IHC results were obtained for ΔM51VSV/FAST treated tumors (Fig. 20E).

Figure 20. IHC of CT26 tumors treated with the FAST recombinant.
To evaluate the intra tumor spread of VSV/FAST and ΔM51VSV/FAST in comparison to VSV/GFP, mice bearing subcutaneous CT26 tumors were treated with one dose of virus for those sacrificed at 40h and 2 doses (48h apart) for those sacrificed at 72h. (A-B)Whole tumors 72 hours after virus treatment. (B)Tumors treated with VSV/FAST exhibited a necrotic/hemorrhagic appearance in comparison to VSV/GFP treated tumors (A). (C-E)IHC was performed and revealed that at 1e6 PFU, VSV/FAST occupies a larger staining area and involves more of the perimeter of the tumor (D) at both time points in comparison to VSV/GFP given at 1e7 PFU (C). (E)Similar results were obtained with IHC of the tumors treated with the ΔM51VSV/FAST recombinant. Controls consist of sections lacking the incubation with the primary anti-VSV polyclonal antibody. These sections are representative of 3 independent experiments.


A.
1e7 VSV/GFP
72h
B.
1e6 VSV/FAST
1e8 VSV/FAST
C.
1e7 VSV/GFP
Control
40h
72h
D.
1e6 VSV/FAST
Control
E.
ΔM51VSV/FAST
72h

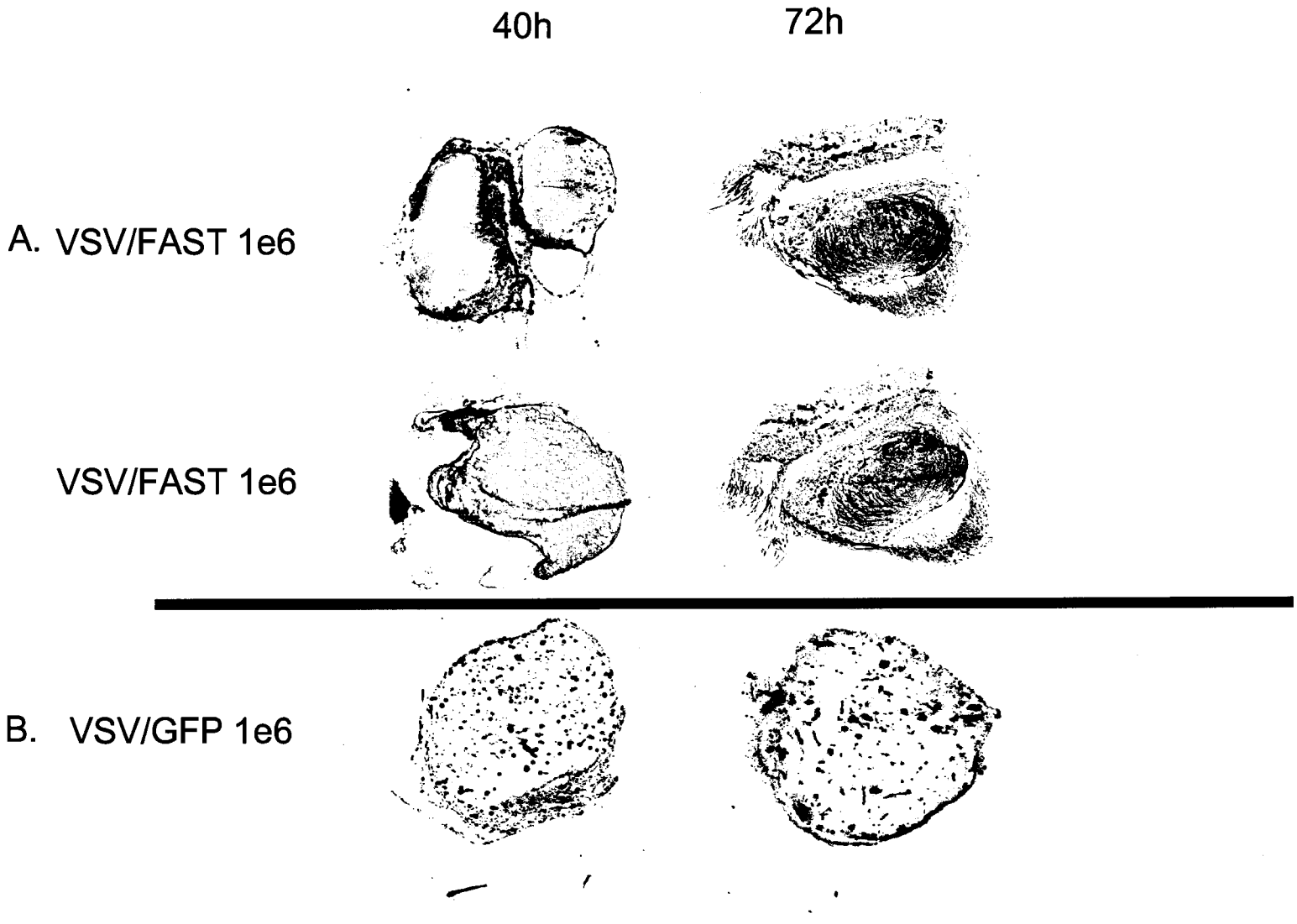
Tumor vasculature is disrupted by VSV/FAST

Tumor vascular shutdown occurs after initial infection of CT26 tumors with an intravenous dose of VSV of 5e8 PFU[43] and is followed by a slow reperfusion after 5 days (C. Breitbach verbal communication). To determine if the VSV/FAST recapitulated this vascular shutdown phenomenon, mice bearing subcutaneous CT26 tumors were infected intravenously with 1e6 VSV/FAST, a dose at which VSV/GFP does not induce vascular shutdown (Fig 21B). Prior to harvesting tumors, mice were injected with 100nm fluorescent microspheres to visualize perfusion. Tumors were harvested at 40 and 72 h after virus injection and sectioned and scanned to reveal localization of microspheres. At 40h no perfusion is noted within the tumors (Fig. 21A), suggesting a compromised flow consistent with previous findings using VSV/GFP but only when given at doses of 5e8 PFU. At 72h there is a significant increase in fluorescent microspheres within the tumor center (Fig. 21A), suggestive of hemorrhage rather than a slow reperfusion encountered in tumors treated with VSV/GFP 5 days after infection.

VSV/FAST recombinant is an effective oncolytic agent

Given the previous in vitro and in vivo evidence that FAST enhances spread at a lower dose when compared to VSV/GFP, it was left to be determined if FAST enhanced tumor oncolysis to the point of generating measurable efficacy in vivo. To investigate this possibility, immune competent Balb/c mice bearing subcutaneous CT26 tumors were treated intravenously with 3 doses of virus 48h apart, comparing 1e6 and 1e7 PFU of ΔM51VSV/FAST to 1e7 and 1e8 PFU of

Figure 21. Tumor vasculature is disrupted by VSV/FAST.
To evaluate the tumor perfusion status after infection with VSV/FAST, fluorescent microspheres were injected intravenously in mice bearing CT26 tumors prior to sacrifice at 40 and 72h time points. At 40h after infection with 1e6 PFU of VSV/FAST, few if any fluorescent microspheres (black) are observed within the tumor core. However, at 72h the fluorescent microspheres have returned to the tumor core. In comparison, the VSV/GFP treated tumors did not show any disruption of perfusion at this dose and at these time points.


40h
72h
A. VSV/FAST 1e6
VSV/FAST 1e6
B. VSV/GFP 1e6

ΔM51VSV/GFP. Tumors were measured at the indicated time intervals (Fig. 22). Although there was efficacy compared to untreated mice ($P<0.01$), there was no difference between the two treated groups (Fig. 22). Over the 26 day course of this study, tumor volumes continued to increase in all groups. To confirm the presence of virus within the tumor, a subset of tumors receiving 1e6 PFU of ΔM51VSV/FAST were harvested and titered at 48 and 72h and titers of 1e8 were obtained, similar to what is achieved when 1e8 PFU of ΔM51VSV/GFP is administered intravenously.

VSV elicits a potent anti-tumor immune response which is known to contribute to tumor eradication[43,115,227,276,318,369]. To evaluate the possibility that an active immune system may be obscuring the enhanced viral oncolysis of the ΔM51VSV/FAST in comparison to ΔM51VSV/GFP, both viruses were evaluated in an immune incompetent mouse model (Fig. 23). Mice bearing sub-cutaneous CT26 tumors were treated intravenously with 5e8 PFU of virus, for a total of 6 doses. Tumors in mice receiving ΔM51VSV/FAST were significantly smaller ($p<0.001$) for a prolonged period of time than those receiving ΔM51VSV/GFP or the untreated group (Fig. 23). It is important to note the eventual increase in tumor volume in both treated groups.

No significant difference is seen with either ΔM51VSV/FAST or ΔM51VSV/GFP when six 5e8 PFU treatments were given by direct intra-tumoral

Figure 22. ΔM51VSV/FAST is as effective as ΔM51VSV/GFP.
Given the oncolytic activity of VSV/FAST in vitro, animals bearing subcutaneous CT26 tumors were treated intravenously with the indicated viruses at the indicated dose. The data reveal a significant difference ($p<0.01$) between the treated animals and the untreated controls, but no significant difference was noted between the treated groups. Arrows indicate injection time points. Data consist of an average of 4 mice per experimental group, and 2 mice in the untreated control group. Similar findings were observed in a repeat experiment. Error bars are excluded due to overlap in the treatment group. Significance was calculated using a two way ANOVA for repeated measures followed by a post hoc Tukey's test.

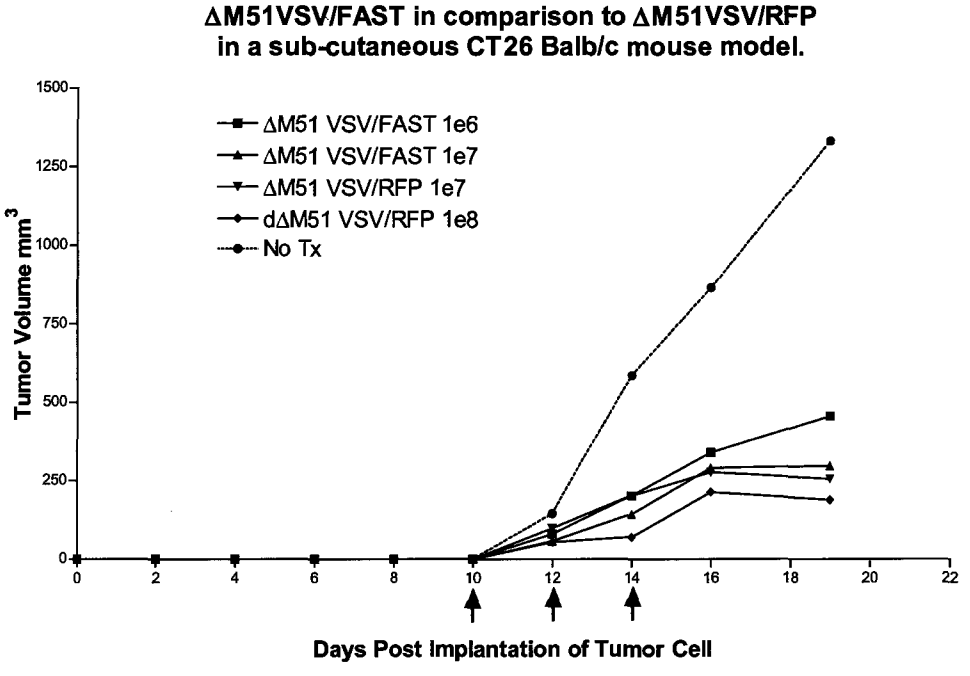
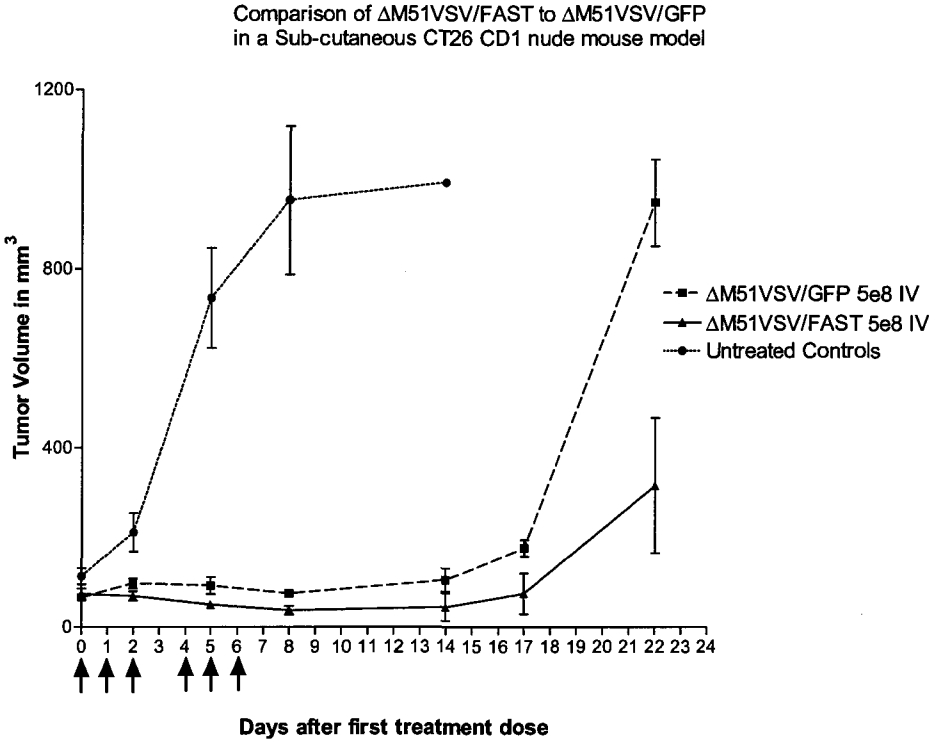

ΔM51VSV/FAST in comparison to ΔM51VSV/RFP
in a sub-cutaneous CT26 Balb/c mouse model.
ΔM51 VSV/FAST 1e6
ΔM51 VSV/FAST 1e7
ΔM51 VSV/RFP 1e7
dΔM51 VSV/RFP 1e8
No Tx
Tumor Volume mm3
1500
1250
1000
750
500
250
0
0
2
4
6
8
10
12
14
16
18
20
22
Days Post Implantation of Tumor Cell

Figure 23. Systemic ΔM51VSV/FAST is more effective in nude mice.
CD1 nude mice bearing sub-cutaneous CT26 tumors were treated with 6 intravenous doses of virus at 5e8 PFU/dose. The data reveal a significant difference ($p<0.001$) between the ΔM51VSV/FAST and ΔM51VSV/GFP treated animals or the untreated controls. Arrows indicate injection time points. Data consist of an average of 5 mice per group. Similar findings were observed in a repeat experiment. Error bars represent the standard error of the mean. Significance was calculated using a two way ANOVA for repeated measures followed by a post hoc Tukey's test.


Comparison of ΔM51VSV/FAST to ΔM51VSV/GFP
in a Sub-cutaneous CT26 CD1 nude mouse model
Tumor Volume in mm3
1200
800
400
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Days after first treatment dose
ΔM51VSV/GFP 5e8 IV
ΔM51VSV/FAST 5e8 IV
Untreated Controls

injection in CD1 nude mice bearing sub-cutaneous CT26 tumors (Fig. 24). It should be noted that mice in this group were sacrificed due to the increasing contralateral tumor burden.

FAST is an oncolytic agent

Given that it was not possible to clearly discern between the intrinsic effects of the VSV and that of the FAST transgene in inducing tumor oncolysis in the subcutaneous efficacy experiments, I proceeded to treat tumor bearing mice with the replication incompetent VSVΔG/mRFP1/FAST virus. Immune competent Balb/c mice bearing subcutaneous syngeneic CT26 tumors were treated with intra-tumor injections of virus at the time points indicated (Fig. 25). Tumors treated with VSVΔG/mRFP1/FAST were significantly smaller ($P<0.05$) than tumors treated with either replication incompetent ΔM51VSVΔG/GFP or PBS. Tumors treated with VSVΔG/mRFP1/FAST were not only inhibited from growing, but they eventually shrunk in size as time progressed, to the point where up to 42 days after onset of treatment 3 of 4 mice were completely cured of their tumors whereas 1 mouse had a re-growth of a tumor which had completely regressed.

To evaluate the oncolytic effect on another tumor model, immune incompetent mice bearing 91304 human Ewing sarcoma lung tumors were treated with intranasal instillation with VSVΔG/mRFP1/FAST and compared to

Figure 24. VSV/FAST is as effective as VSV/GFP as an oncolytic.
CD1 nude mice bearing sub-cutaneous CT26 tumors were treated with 6 intra-tumor injections of virus at 5e8 PFU/dose in 50ul. The data reveal a significant difference $p<0.001$ between the treated animals and the untreated controls, but not between the different recombinants themselves. Arrows indicate injection time points. Data consist of an average of 5 mice per group. Error bars represent the standard error of the mean. Significance was calculated using a two way ANOVA for repeated measures followed by a post hoc Tukey's test.

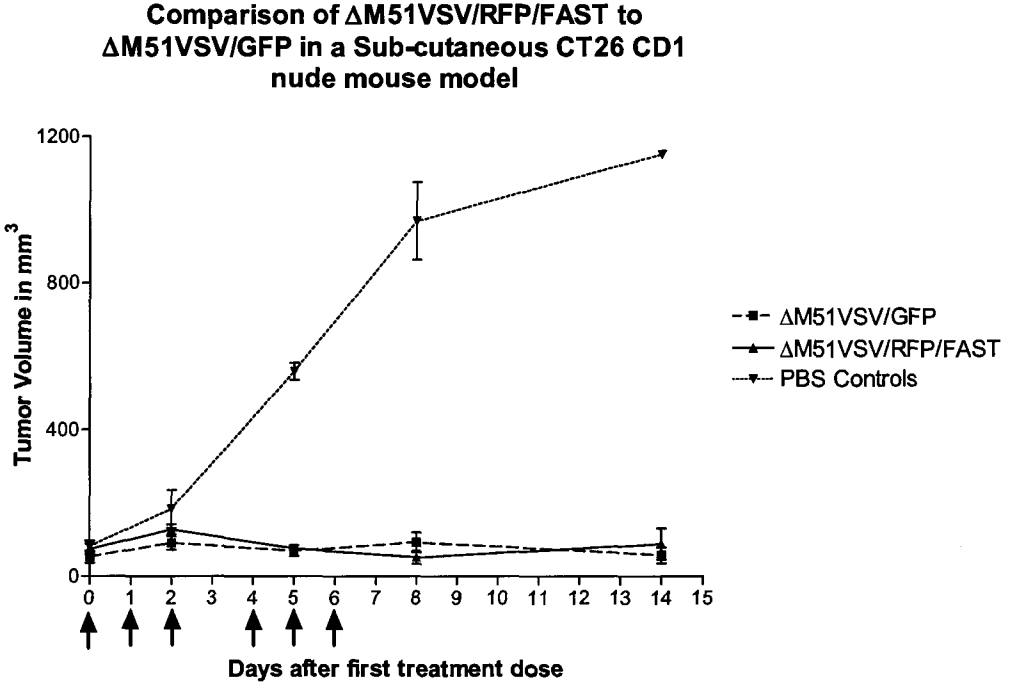
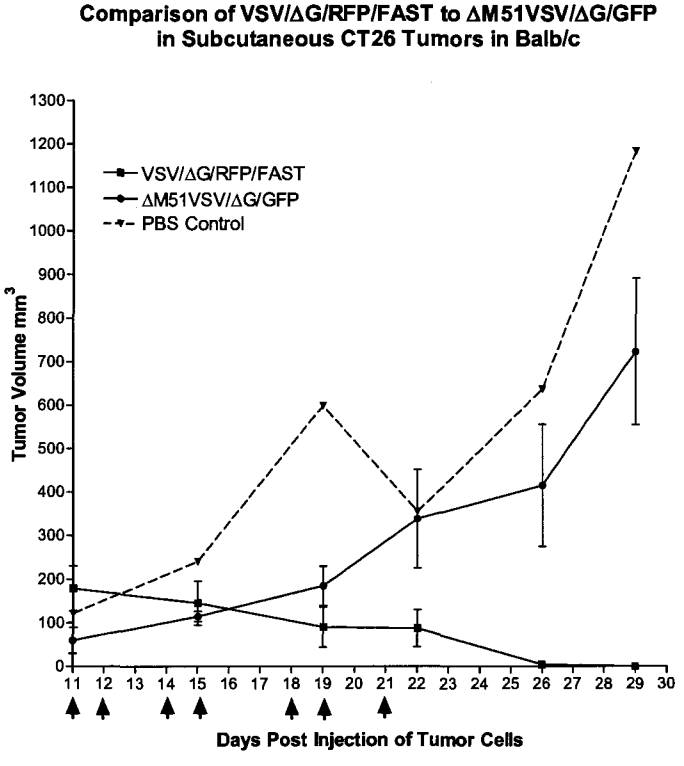

Comparison of ΔM51VSV/RFP/FAST to ΔM51VSV/GFP in a Sub-cutaneous CT26 CD1 nude mouse model
Tumor Volume in mm3
1200
800
400
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days after first treatment dose
ΔM51VSV/GFP
ΔM51VSV/RFP/FAST
PBS Controls

Figure 25. FAST is an effective oncolytic agent.
Tumors were injected directly with 50ul of either PBS, or 3e4 of virus for a total of 7 treatments. When administered IT in sub-cutaneous CT26 tumors in Balb/c mice, tumors treated with VSVΔG/mRFP1/FAST were significantly smaller ($p<0.05$) and regressed in comparison to ΔM51VSVΔG/GFP or PBS treated controls. Arrows indicate injection time points. Data consist of an average of 4 mice per group, except for 2 in the PBS treated control group. Error bars represent the standard error of the mean. Significance was calculated using a two way ANOVA for repeated measures followed by a post hoc Tukey's test.


Comparison of VSV/ΔG/RFP/FAST to ΔM51VSV/ΔG/GFP
in Subcutaneous CT26 Tumors in Balb/c
VSV/ΔG/RFP/FAST
ΔM51VSV/ΔG/GFP
PBS Control
Tumor Volume mm3
Days Post Injection of Tumor Cells
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

ΔM51VSVΔG/GFP treated and untreated controls. Outcomes were measured in terms of survival (Fig. 26), and although not significant based on 3 mice per group the data are suggestive of a trend in enhanced survival in mice treated with VSVΔG/mRFP1/FAST. In our experience, the 91304 cell line has resulted in 100% lung implantation in over 50 mice with this animal model (Fig. 27A-B), however to ensure this, random mice in this study were sacrificed and dissected to reveal tumor bearing lungs at day 15 (Fig. 27C-E) and at day 26 (Fig. 27F). Altogether, these data demonstrate that VSVΔG/mRFP1/FAST has oncolytic effects.

FAST stimulates an anti-tumor immune response in mice with tumors

Non-injected contra-lateral tumors in mice receiving VSVΔG/mRFP1/FAST were also smaller in size in comparison to the control tumors (Fig. 28A) a phenomenon not observed in immune compromised animals (Fig. 28B), coupled with the fact that their titers were negative for virus suggests that FAST may have an immunostimulatory role. To investigate this further, splenocytes were isolated from VSVΔG/mRFP1/FAST cured mice and adoptively transferred into Balb/c mice which were subsequently challenged 24h later with 3e5 CT26 and 1e5 4T1 cells subcutaneously. Over a 3 week period, 2/3 mice receiving the splenocytes from the VSVΔG/mRFP1/FAST mice did not develop CT26 tumors in comparison to the controls which developed 4T1 tumors (Table 5), thereby supporting the role of FAST as a potent anti-tumor immunostimulant.

Figure 26. FAST in a Human Ewing sarcoma mouse lung model.
B6 mice bearing 91304 human Ewing sarcoma lung tumors were treated with intranasal instillation of 3e4 PFU/dose of VSVΔG/mRFP1/FAST. Arrows indicate injection time points. Data consist of an average of 3 mice per group, except for 2 in the no-treatment control group. Survival was calculated using Kaplan Meier survival curves. P values were calculated using log rank test.

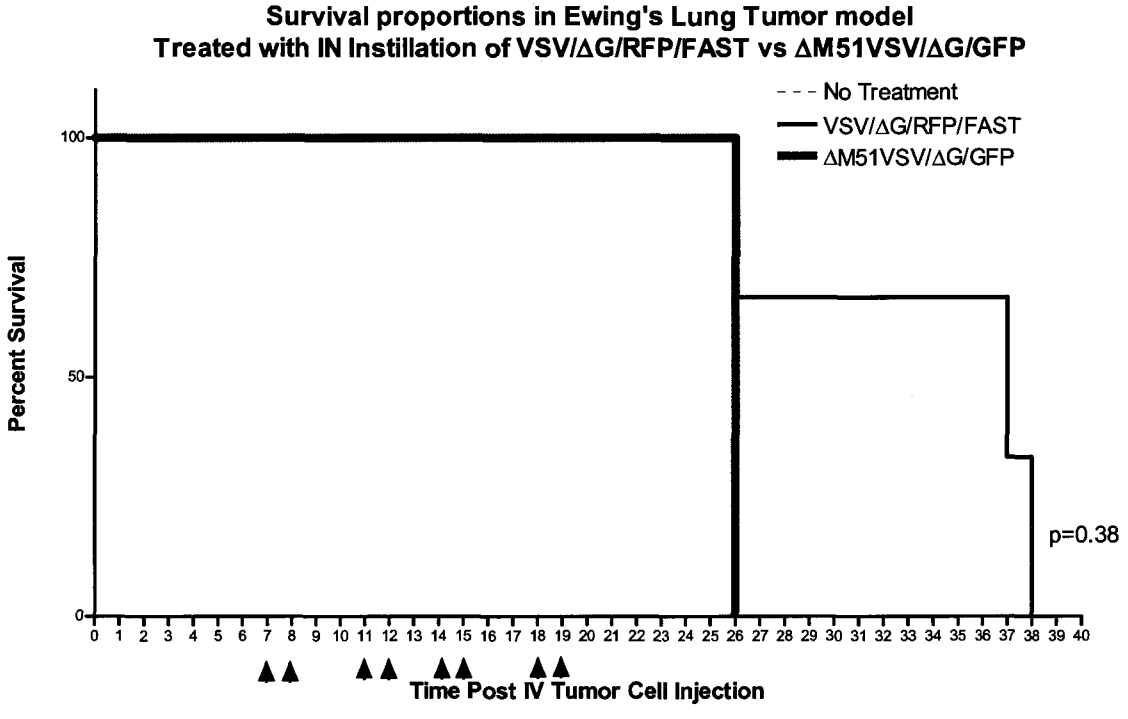

Survival proportions in Ewing's Lung Tumor model
Treated with IN Instillation of VSV/ΔG/RFP/FAST vs ΔM51VSV/ΔG/GFP
No Treatment
VSV/ΔG/RFP/FAST
ΔM51VSV/ΔG/GFP
Percent Survival
100
50
0
p=0.38
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Time Post IV Tumor Cell Injection

Figure 27. Confirming intranasal instillation of VSV/ΔG/mRFP1/FAST.
(A)Mice injected intravenously with 1e6 human 91304 Ewing sarcoma cells develop lung tumors within 14 days in comparison to un-injected controls(B). (C-E)To confirm the delivery of intranasal instillation of 50ul of 3e4 PFU of virus to B6 mice bearing 91304 human Ewing sarcoma lung tumors, mice were sacrificed after 3 treatments, lungs were dissected and imaged. (C)Tumors treated with ΔM51VSVΔG/GFP did not show any green fluorescence. (E-F) Tumors treated with VSVΔG/mRFP1/FAST demonstrated red fluorescence, which was not present in ΔM51VSVΔG/GFP treated lungs(D). (G)At 26 days post tumor implantation, all ΔM51VSVΔG/GFP treated and all untreated mice were euthanized because of tumor burden.

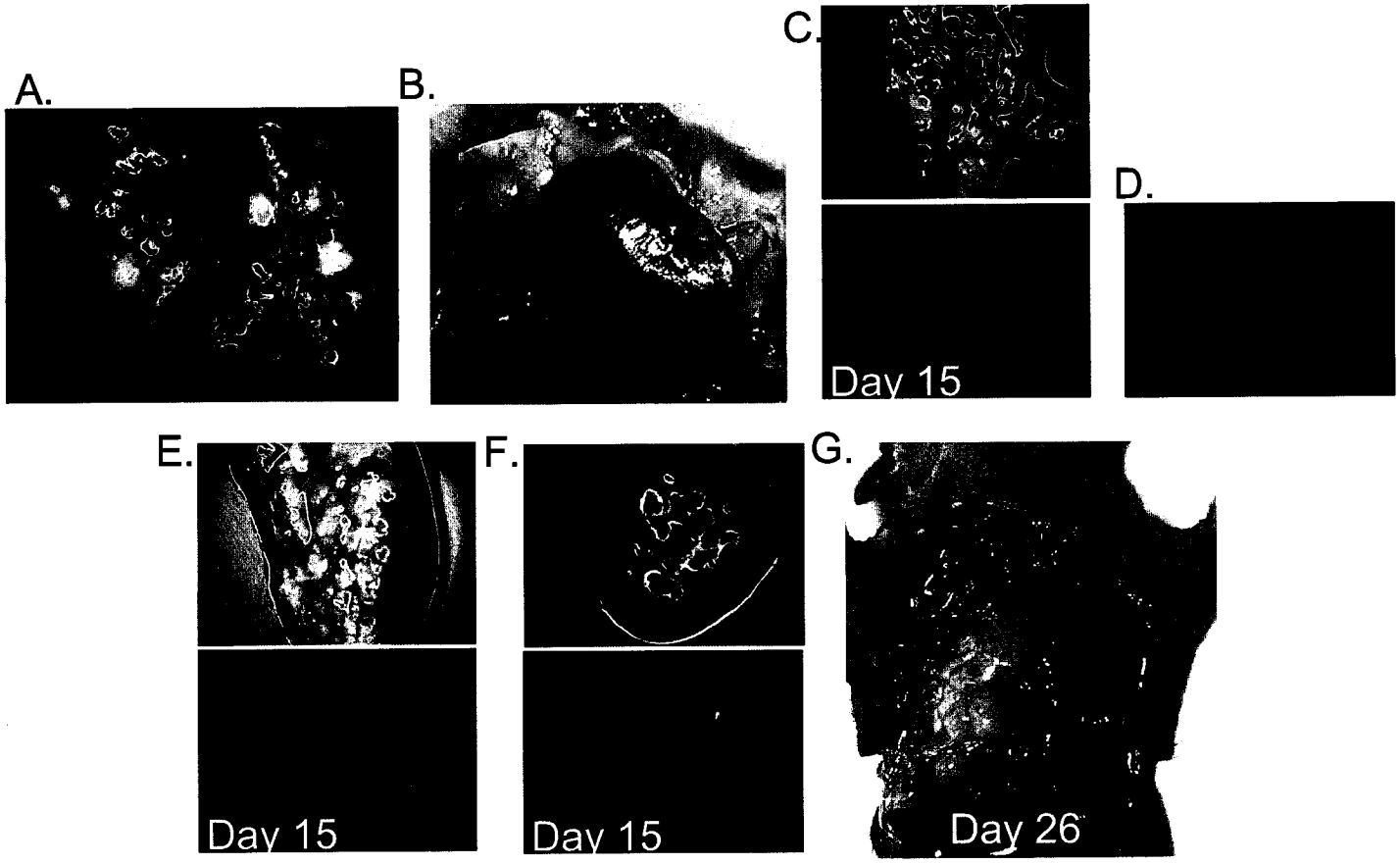
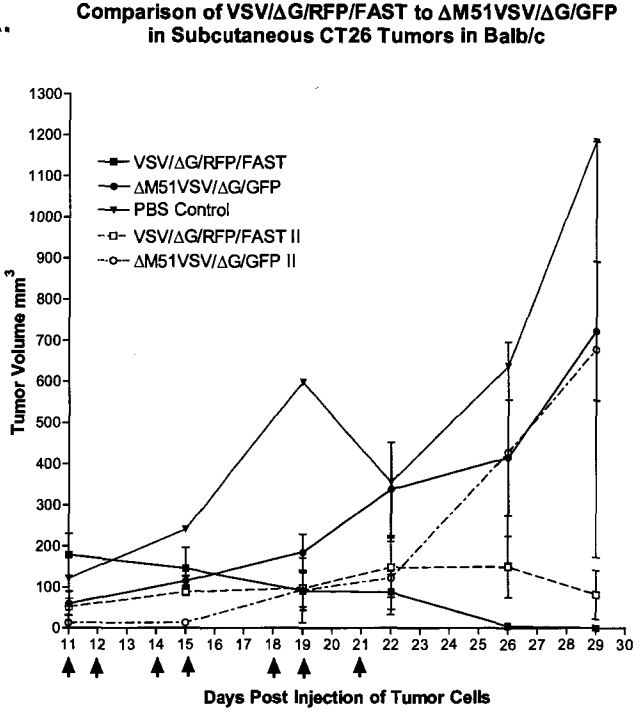

A.
B.
C.
D.
Day 15
E.
F.
G.
Day 15
Day 15
Day 26

Figure 28. Size of untreated contra-lateral tumors.
Contra-lateral CT26 tumors that were not injected but were in mice receiving either VSVΔG/mRFP1/FAST, ΔM51VSVΔG/GFP or PBS were measured and compared to the injected tumors in immune competent (N=4, except control group=2) and incompetent (N=5) mouse models. Error bars represent standard error.

A.


Comparison of VSV/ΔG/RFP/FAST to ΔM51VSV/ΔG/GFP in Subcutaneous CT26 Tumors in Balb/c
VSV/ΔG/RFP/FAST
ΔM51VSV/ΔG/GFP
PBS Control
VSV/ΔG/RFP/FAST II
ΔM51VSV/ΔG/GFP II
Tumor Volume mm3
Days Post Injection of Tumor Cells

B.

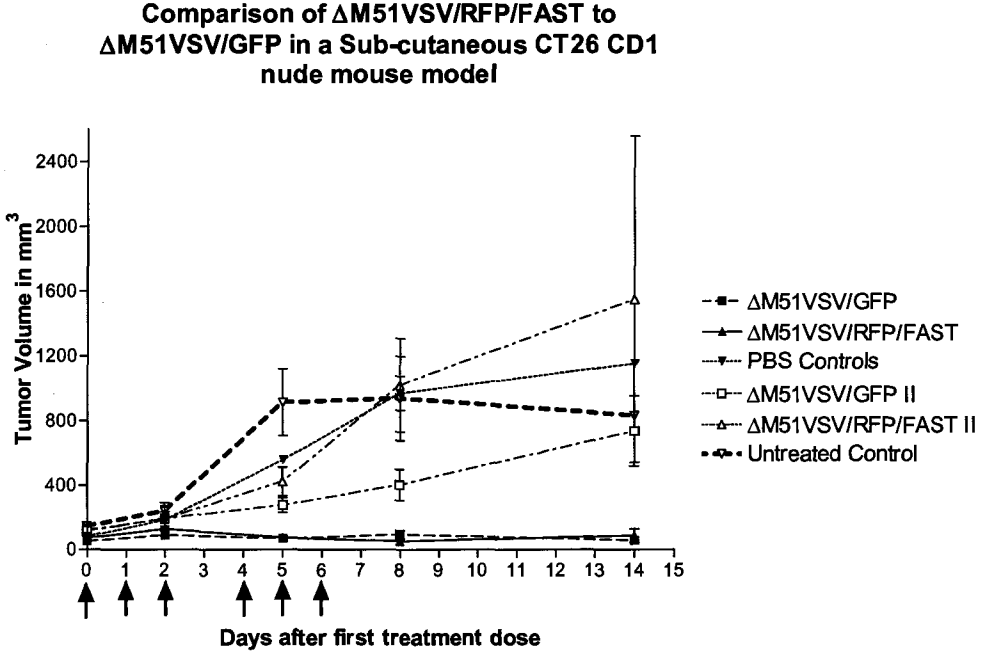

Comparison of ΔM51VSV/RFP/FAST to ΔM51VSV/GFP in a Sub-cutaneous CT26 CD1 nude mouse model
Tumor Volume in mm3
Days after first treatment dose
ΔM51VSV/GFP
ΔM51VSV/RFP/FAST
PBS Controls
ΔM51VSV/GFP II
ΔM51VSV/RFP/FAST II
Untreated Control

Table 5. FAST stimulates an anti-tumor immune response.
Splenocytes were isolated from VSVΔG/mRFP1/FAST cured mice and adoptively transferred into Balb/c mice which were subsequently challenged 24h later with 3e5 CT26 sub-sutaneously and 1e5 4T1 cells subcutaneously on the contra-lateral side. Over a 3 week period, mice were observed for tumor growth. (+) indicates tumor present; (-) indicates no tumor present.

	Control 1	Control 2	M1	M2	M3
4T1	+	+	+	+	+
CT26	+	+	+	-	-

Discussion

In this study I set out to determine whether the reoviral p14 FAST fusogenic protein can serve as a virulence factor when transferred to an unrelated virus. Furthermore, I thought to evaluate the oncolytic ability of the p14 FAST fusogenic protein, as previous reports have determined enhanced oncolysis with fusogenic proteins[23,24,349,391].

VSV expressing FAST is a functional recombinant

Various VSV recombinants were generated with the reovirus p14 FAST in either the wild type or interferon inducing ΔM51 mutant, with or without an mRFP1 reporter gene and with or without VSV G (Fig. 7). The expression of the FAST transgene was confirmed by Western blot analysis and by visual observation of the formation of large syncytia by all of the FAST recombinants in comparison to control VSV recombinant (Fig. 7 and 8). I wondered whether the rapid formation of large syncytia (Fig. 8) may lead to premature cell death and negatively impact on VSV replication, however, one-step growth curves show that VSV/FAST grows at a similar rate and to similar titers as VSV/GFP (Fig. 10). Thus the replication of VSV is sufficiently rapid to reach a plateau prior to extensive killing of infected cells by syncytium formation. In all cases the replicating FAST recombinant viruses achieved titers within a log of the parent (Table 3). The ability of the FAST/VSV recombinant to induce significant syncytium formation in monolayers of cultured cells in vitro was noted in all cell

types that are normally permissive by the parent virus (Fig. 13), demonstrating the tropism of the recombinant FAST virus was not affected. Having noted through Western blot analysis that FAST was located in the virion (Figure 7b), I tried to determine if it was a functional component of the envelope. Attempts at serially passaging the replication incompetent FAST virus were unsuccessful (Fig. 14), suggesting the FAST protein is either not membrane bound within the virion, is not functional, or is found at such low frequency within the membrane that fusion is not possible. A more likely explanation would be that the replication incompetent FAST is not budding into the supernatant.

FAST is a virulence factor

VSV is neurotoxic, generating encephalitis, leading to paralysis and death[178,179,326,392], as such the in vivo safety profile of the replication competent VSV/FAST recombinant was verified by intranasal instillation in mice. Following intranasal delivery VSV/FAST induced hindlimb paralysis at lower doses than VSV/GFP and generated high titers of virus in the brains of these mice more consistently (Table 4) (Fig. 16). I also detected enhanced entry into the CNS following IV administration of VSV/FAST (Fig. 17), especially at the 48 hour time-point and saw hindlimb paralysis within four days by this route. Interestingly, the brain titers are still increasing after 48h in contrast to all other titers and would suggest that the peak titers of VSV/FAST in the brain has not yet been reached at 96h (Fig. 17). This enhanced spread of the virus and more

efficient and rapid entry into the CNS by VSV/FAST establishes the FAST fusogenic protein as a virulence factor.

The high titers seen in brains after delivery of 5e7 VSV/GFP did not necessarily lead to hindlimb paralysis while this dose of VSV/FAST usually did (Table 4), despite similar viral load in the brain (Fig 16). In addition, although the VSV/FAST virus does appear to progress more slowly through brain tissue, it does show a greater extent of infection and very consistently demonstrates stronger staining by IHC (Fig. 18). The formation of FAST induced syncytia in the brains of mice likely leads to the greater extent of histopathology and increased incidence of hindlimb paralysis, despite yielding comparable brain tissue titers as VSV/GFP. Altogether, these findings would imply that not only does this virus enter the CNS more consistently but likely does more damage once it arrives. It is perhaps somewhat paradoxical that the VSV/FAST virus appears to progress more slowly into the brain than the VSV/GFP parent following intranasal infection when it enters more efficiently following intravenous delivery.

Although no differences in the acute T cell response to VSV/FAST were detected (Fig. 19), I cannot determine from these data whether or not the FAST protein gives this virus any immune evasive properties to explain the enlarged spread and increased virulence. The infections investigated in this study were all

of an acute nature and in some cases the mice were reaching endpoint prior to day 7 when a full-blown adaptive immune response would come to bear.

While it is not clear why enhanced tissue damage might benefit the reovirus from which the FAST protein is derived, this enhanced spread from the blood stream in the infected host could help these viruses disseminate more rapidly prior to the development of an effective immune response. Alternatively, the enhanced IHC staining I observed in VSV/FAST infected tissues may indicate that by fusing together the cytoplasm of multiple cells these viruses may have an enhanced ability to commandeer protein translation machinery to generate more viral protein leading to enhanced replication, especially for a virus like reovirus that does not need to bud.

FAST enhances the spread of VSV in tumors

Other fusogenic viruses have demonstrated enhanced oncolysis by inducing anti-tumor immunity in mouse models of cancer[23,24,126,172,226,229,265]. The possibility that FAST could enhance tumor oncolysis was suggested by the in vitro cancer cell line screen which demonstrated less viable cancer cells in the wells infected with the VSV/FAST recombinant (Fig. 13). The fact that the interferon-inducing mutant FAST recombinant was interferon sensitive (Fig. 15) and did not result in toxicity in mice in vivo (Table 4) supported the rationale to evaluate the FAST recombinant in animal tumor models.

The IHC performed on harvested tumors consistently demonstrated a greater degree of spread of the FAST recombinant compared to the parent virus in 3 independent experiments (Fig. 20), similar to the enhanced spread observed in the brain sections (Fig. 18). Interestingly, a larger area of spread is seen in subsutaneous CT26 tumors compared to VSV/GFP despite being given at 1 log lower dose, substantiating the enhanced spread. Because VSV is not normally seen within the tumor core, likely a hypoxic and low pH environment[150,256,282,299,344,346,371,372,377], I postulate that a possible explanation for the enhanced spread of VSV/FAST within tumor cores is the ability of the FAST recombinant to spread in a low pH environment (Fig. 11). In addition, the VSV/FAST recombinant lacking VSV G, the protein that dictates viral entry, which does not function at pH 6.8, is still able to spread (Fig. 12) substantiating that the FAST recombinants can spread independently of a functioning G protein. The enhanced spread was not due to an insensitivity to interferon, as determined in vitro (Fig. 15) and subsequently confirmed in vivo where mice treated with the interferon-inducing mutant FAST recombinant did not develop any neurotoxic signs (Table 4). Although enhanced spread of viruses expressing fusogenic proteins has also been attributed to the ability to evade the acquired immune response[32,280], it remains to be determined if this is a contributing factor in this study.

FAST is an oncolytic agent

The observation that the VSV/FAST recombinant lacking the VSV G gene can decrease tumor burden in different tumor models (Fig. 25-26), unlike the replication incompetent VSV, implies that FAST is an oncolytic factor. FAST could be mediating oncolysis by allowing the replication incompetent VSV to spread in the absence of the G protein and allowing the virus to effectuate its oncolytic functions. FAST could also be contributing to the oncolytic effect by inducing syncytia, a feature known to generate potent anti-tumor immunity[24,173]. In addition, the FAST induced syncytia are also known to stimulate apoptosis[327].

The ability of the VSV/FAST recombinant to diminish in vivo tumor burden appears to be as effective as VSV/GFP in the immune competent mouse model (Fig. 22). Whereas a difference in favor of VSV/FAST is appreciated in the immune compromised model only when the virus is administered systemically (IV), but not locally (IT) (Fig. 23-24). The lack of difference between the FAST recombinant and the parent VSV in both the immune-competent and nude mouse models, could be due to too much FAST within tumor cells which may be counter-productive, thereby killing the cells before the virions are assembled. Not only does FAST initiate apoptosis[327], but coupled with the toxicity of the M and G proteins[39,52,58,133,228,237,277,352,422], it is conceivable that the FAST recombinant may extinguish itself yet still be able to induce a potent innate anti-tumor immune response on par with VSV/GFP. Furthermore, the fact that less FAST recombinant results in a similar efficacy as VSV/GFP in the IV immune

competent model (Fig. 22), and that efficacy is also seen with only 3e4 PFU of VSV/ΔG/mRFP1/FAST IT treatments (Fig. 25) – are suggestive that less VSV/FAST recombinant is as good as 1e8 VSV/GFP, and possibly an advantage from a clinical safety perspective.

The discrepancy between IT replication incompetent FAST virus (Fig. 25) producing cures in comparison to the IV replication competent FAST virus (Fig. 22) in immune competent models, may be because the recombinant FAST virus is more toxic when administered systemically. Granted, the increased dosing regimen, to compensate for a lack in adaptive immune response, may also be a contributing factor. It is possible however, that tumor associated immune cells (neutrophils, APCs & lymphocytes) may not be able to effectuate a response if they come in direct contact with the infected peripheral tumor cells as they might fuse, although such hybrids have been described as potent immunogens[288]. Furthermore, peripherally located immune cells or lymph nodes may somehow be inhibited by the VSV/FAST recombinant. Although healthy cells can inhibit VSV replication, VSV can infect dendritic cells rendering them defective in antigen presentation, however, infection with the Δ51M VSV recombinant allows dendritic cell maturation[6]. VSV G has also been shown to mediated infection in dendritic cells[148,149] and VSV G pseudotyped lentivirus has been demonstrated to be able to transduce peripheral blood lymphocytes[137,350]. Perhaps in a similar fashion VSV/FAST may gain entry and be able to express FAST prior to effective activation of the anti-viral response. Although the number of FAST proteins

required to enable fusion has been established in vitro[385], the kinetics of FAST expression and the amount required to induce apoptosis is currently unknown.

It has been demonstrated that VSV is dependent on many host factors to effectively eradicate sub-cutaneous CT26 tumor cells, such as the innate immune response during vascular shutdown[43,365] as well as an adaptive anti-tumor immune response[369]. It is interesting to observe that a systemically administered VSV/FAST recombinant may enhance tumor cell death in the absence of an immune response (Fig. 23), which may prove valuable in immune compromised patients undergoing chemotherapy and radiation.

FAST mediates an anti-tumor immune response

The decreased contra-lateral tumor burden observed in immune competent mice treated with replication incompetent VSV/FAST (Fig. 28), is likely the result of an anti-tumor immune response. This is further supported when splenocytes isolated from these cured animals are able to transfer anti-tumor immunity to naïve mice (Table 5), demonstrating that FAST is also able to facilitate an anti-tumor immune response. However it should be noted that further direct evidence is required to fully substantiate this claim.

FAST does not improve the VSV platform in the Balb/c CT26 tumor model

Although our study demonstrates that VSV/FAST spread is larger and can penetrate the tumor core in CT26 tumors, this does not translate into more

obvious efficacy in vivo animal tumor models. It has previously been identified that only a fraction of an IV administered load of oncolytic virus reaches a tumor[195,280], and in the sub-cutaneous CT26 tumor model that fraction of VSV/GFP is stuck at the rim[43], possibly because of low pH inhibition of G protein function, or hypoxia mediated anti-virus factors[180,365] or because of the vascular shut down phenomenon[43]. Microsphere data suggest a difference in the vascular shut down phenomenon between VSV/FAST and VSV/GFP, whereby it is present for a shorter period of time in VSV/FAST treated tumors. Perhaps there are still remnant tumor rim cells or tumorigenic stem cells, factors which have been identified as critical for tumor expansion[112,235,319].

Models of viral spread based on laboratory and clinical samples have suggested that the innate immune response against the virus has to be abolished in order for complete control of the tumor by the virus[409]. This is further corroborated in the CT26 tumor model whereby VSV spread is enhanced in neutrophil depleted animals[43], however it has yet to be determined if neutrophils impede the spread of VSV/FAST. Another intriguing explanation for the lack of enhanced efficacy may reside in the toxicity of FAST, whereby the infected cells die before infecting other cells. Such a mechanism has been demonstrated by oncolytic tumor models to lead to failure of tumor eradication[273]. Furthermore, immune mediators such as antigen presenting cells or lymphocytes may be destroyed when coming into contact with tumor cells expressing FAST, leading to a failed anti-tumor immune response. On the contrary, like other fusogenic

proteins, FAST may enhance the anti-viral response[167], a counter-productive attribute.

FAST enables the VSVΔG platform

The ability of FAST to allow a replication incompetent VSV to spread has several advantages over the parent VSV. Given that VSV G has such a broad tropism[227,249,334], it may be beneficial in certain circumstances to tailor the tropism by pseudotyping a VSVΔG/FAST virus with a known surface protein to target infection. This approach would allow for specific targeting of the tumor cell type using the pseudotyped protein, yet FAST would indiscriminately kill the targeted cell as well surrounding tumor cells – a beneficial strategy for heterogeneous tumors. In a similar fashion, FAST could also be used to enhance the spread of other, possibly non-vesiculovirus based, oncolytic viruses. Combinatorial strategies including other oncolytic viruses or targeted therapies have yet to be explored.

A key, unexplained, advantage using the replication incompetent FAST recombinant is that despite its expression of the wild type M protein, no toxicities were noted in immune compromised mice or in immune competent mice. Animals survived up to at least 36 days after intranasal administration and up to 42 days with IT injections without any neurotoxicity, yet enhanced survival and cures were achieved. Work regarding the neurotoxicity of this recombinant is discussed in further detail in chapter 3 of this thesis.

Summary

The novel VSV/FAST recombinant has demonstrated increased spread in animal models, and is highly suggestive that FAST is not only a virulence factor but an oncolytic agent. Despite equivalent efficacy with the replication competent FAST recombinant in immune competent models, enhancements were observed in the immune compromised models. Furthermore, the FAST recombinant lacking VSV G was still able to spread and effectuate cures, possibly by stimulating an adaptive anti-tumor immune response despite being administered at a dose 4 logs lower than the parent virus. Although the data from these pilot experiments are highly suggestive, larger studies are now wanranted to substantiated these claims. It should however be noted that this is the first study to report the systemic IV administration of a replication competent oncolytic virus expressing a fusogenic transgene.

Chapter 2: Strategic shuffling of diverse envelope proteins for immune evasion in oncolytic virotherapy.

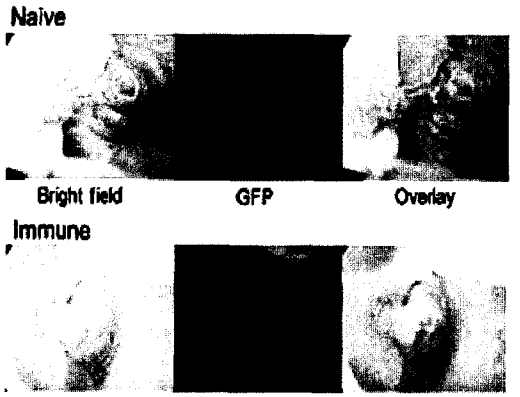
Introduction

Tumor-targeted oncolytic viruses represent a potent new class of therapeutic agents, however host immunity remains an imposing obstacle to effective application in the clinic[28]. Specifically, virus neutralization by host antibodies is a major detriment to repeated treatments[144,293,330] (Fig. 29A).

Studies have demonstrated that the G protein of the vesiculoviruses, of which there are ~1200 per virion, are the main antigenic determinants[12,290](Fig. 29B). Neutralizing antibodies to VSV can be detected as early as 4 days in treated mice[293] (Fig. 29C). The neutralizing antibodies recognize 4 epitopes on VSV G from both Indiana and New Jersey serotypes, where 200 - 500 IgG molecules per virion are required to impede the binding to cells and subsequent infection in vitro and in vivo[12,45,117,193]. Whereas non-neutralizing antibodies are unable to neutralize the virus in vitro, but do so in vivo through a complement or cell mediated mechanism[131,216]. Furthermore, cross reactive antibodies are able to neutralize VSV from more than one serogroup, and they are more likely to be formed if the oncolytic virus is administered through an intravenous route[56]. This later issue bears relevance, as the ideal oncolytic virus would likely be administered hematogenously in order to treat systemic disease. Currently, a VSV

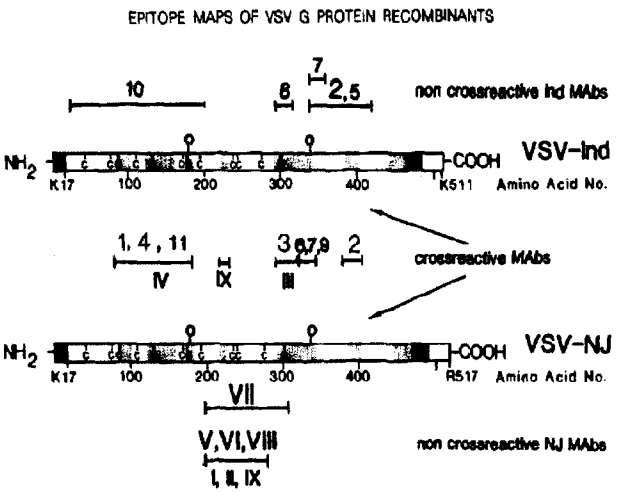
Figure 29. No virus is delivered to the tumor after immunization with VSV.
After mice are exposed to VSV intravenously (A), a second dose of VSV/GFP virus is unable to reach the tumor after 72h. (B) The VSV glycoprotein is the main antigenic determinant. Antibodies binding to 1 of at least 4 epitope regions (yellow highlight) on the VSV IND G protein which neutralizes the virion. Cross reacting and non-neutralizing antibodies also recognize other distinct epitopes on the G protein and can initiate complement mediated cascades (Image C reprinted from Keil et al. 1989 with permission to alter from Elsevier, License number 1943061125017). (C) Serum antibody titers confirm the presence of neutralization of virus as early as 4 days after immunization (Images A & C taken with permission from Power et al. 2007).

A.



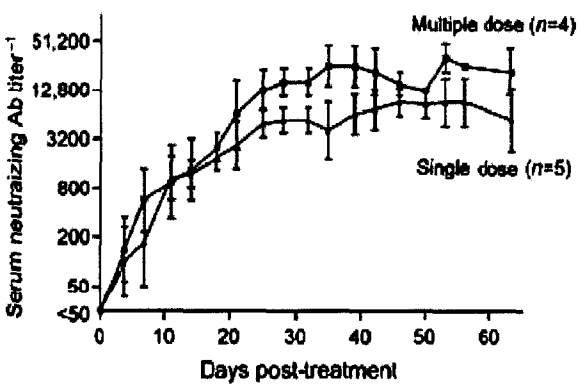
Power et al. Mol. Ther., 2007.

B.



Used with permission from Keil et al. Virology, 1989.

C.



Power et al. Mol. Ther., 2007.

based oncolytic therapy requiring multiple doses would not be able to overcome these antibodies.

Several antibody evasion strategies have been proposed for VSV. Given that the neutralizing antibody response is predominantly directed against the G protein, genetic modification of the VSV Indiana glycoprotein, as previously identified in VSV New Jersey glycoprotein escape mutants[236], could generate immunologically distinct VSV viruses[27]. Along similar lines, swapping entire glycoproteins from within the rhabdoviruses or from other virus families could also allow for evasion of pre-formed antibodies[318]. Foreign glycoproteins expressed from recombinant vesicular stomatitis viruses have efficiently been incorporated into VSV virus particles[337], such as the influenza virus hemagglutinin neuraminidase, CD4[206,309,337] and the env and gag genes from HIV[317]. Furthermore, serotype switching for cancer gene therapy has been successfully demonstrated with adenovirus[330].

Some progress has already been made with respect to increasing oncolytic VSV efficacy in the presence of neutralizing antibodies. Using a cell carrier system to smuggle virus through the circulation, virus was delivered to tumors and effectuated cures in immunized mice, thereby evading the immune system[157,292,383]. Similarly, serotype switching of the VSV glycoprotein with other members of the rhabdovirus family has demonstrated preliminary evidence, in animal models, of immune evasion in order to enhance boosting in vaccine

strategies[318]. To circumvent neutralizing antibodies and create vectors for boosting, recombinant VSV glycoprotein exchange vectors encode the N, P, M, and L genes of the VSV IND serotype, but have the IND G gene serotype substituted with either the gene expressing the VSV NJ serotype G protein or the gene for the G protein of the related Chandipura virus. It is claimed that these glycoprotein exchange vectors are able to circumvent the neutralizing antibody response to the VSV IND G protein because they boost the immunity against an unrelated HIV protein[318]. It has yet to be determined if such a serotype switching strategy could be used for oncolytic purposes. It has been proposed that that such glycoprotein exchange vectors could be adapted for use as an oncolytic[27], with the main difference being the dose, the number of doses and route of administration. For vaccination purposes in mice, 25ul of 1e5 PFU IN is given and boosted with 6.25e6 PFU IP[318], and in non-human primates a total of 400ul of 5e6 PFU IN followed by a single boost results in effective HIV vaccine[301]. In comparison, the oncolytic therapeutic doses of 100ul of 1-5e8 PFU IV/dose for a total of at least 6 doses is required to effectuate cures in a mouse tumor model[369].

In this study I sought to develop recombinant VSV virus bearing G's from different vesiculoviruses: VSV Indiana, VSV New Jersey, Chandipura, Isfahan, and the previously un-sequenced member Maraba. As well, a non-rhabdoviridae envelope protein from a beta-retrovirus was used as an immune evasion recombinant. JSRV is a beta-retrovirus whose natural host is sheep, where the JSRVenv is responsible for inducing pulmonary adenocarcinoma. Recently it was

confirmed that the cytoplasmic tail of JSRVenv is responsible for inducing the cellular transformation. JSRVenv binds to the Hyal-2 receptor found in various organs but not human adult brain[220,272,370], and is also over expressed in various cancers[31,107,221].

Herein, I have investigated the hypothesis of envelope protein shuffling as a strategy for immune evasion, using a model oncolytic agent based on the vesicular stomatitis virus. A panel of novel recombinant viruses expressing engineered or evolutionarily divergent envelope proteins was tested for their ability to escape neutralizing antibody and effect oncolytic activity in vitro and in vivo. The findings indicate that glycoprotein shuffling can be an effective strategy for immune evasion for enveloped oncolytic viruses, however serotype distinctions based on *in vitro* neutralization assays are poor predictors of *in vivo* clinical efficacy.

Material and Methods

Viruses and Cells:

BHK-T7 were a gift from Karl-Klaus Conzelmann, and Vero, CT26, SW620 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA 20108 USA). Cells were propagated in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal calf serum (Cansera, Etobicoke, Ontario, Canada). BHK-T7 were gown in the presence of G418 (Invitrogen) at a final concentration of 0.2mg/ml and used for virus rescues.

For production of virus used in animal experiments Vero cells were infected at an MOI of 0.01. Culture supernatants were collected 24 or 48 hours later and cleared by centrifugation and filtration through a 0.2 μm filter. Stocks were titered on Vero cells. A one step growth curve was performed in a 6 well dish that was infected with virus at an MOI of 5 and after 45min of adsorption, the wells were washed 4 times with 3ml of PBS followed by application of 3ml of media containing 10% FBS and incubated at 37°C. A 100ul aliquot of supernatant was obtained from 3 independent wells at time points 0, 2, 4, 6, 8, 12, 24, 30, 48 and 72h and stored at -80°C and only underwent 1 freeze thaw prior to titering on Vero cells. Error bars represent standard deviations of three replicates per time point.

Maraba virus cDNA Library Synthesis and Isolation of the Maraba G gene:

Maraba virus RNA was isolated from pure stock (Gift from Robert Tesh, University Texas Medical Branch, TX, USA) as described in the viral RNA isolation section. A cDNA library was generated using the SuperScript Choice System for cDNA Synthesis (Invitrogen, 18090-019). Fragments of cDNA were blunt end ligated into pT7 Blue3 vector (Novagen, 70182-3) and transformed into competent cells (NovaBlue Singles Competent Cells, Novagen, 70181-3). Individual colonies were isolated and plasmid DNA was purified using Qiaprep Spin Miniprep Kit (Qiagen, 27106). All sequencing was performed using an Applied Biosystems 3730 DNA Analyzer (StemCore Laboratories, Ottawa Health Research Institute, Ottawa, Canada).

Generation of Recombinants:

Vesiculoviruses, Isfahan, Chandipura and Maraba were a gift from Robert Tesh, University Texas Medical Branch, TX, USA. Viral RNA was isolated from pure virus stock. RT-PCR was performed as described to generate cDNA's which were then PCR amplified, see *RT – PCR and Sequencing of Rescued Recombinants* for primer sequences. The PCR products were gel purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, 28-9034-70) and blunt end ligated into the pT7Blue3 EcoRI site (Novagen, 70182-3). The plasmids containing sequences for the VSV IND G, Maraba G, and Isfahan G were subsequently digested with MluI/XhoI whereas the plasmid containing Chandipura G sequence was digested with MluI/HindIII. The fragments were then

subcloned into their respective MluI/XhoI or MluI/HindIII sites within a vector containing the 3' stop-start sequence, GGTATGAAAAAAACTAACAGATATCACG flanked by XhoI or HindIII and NheI sites. Upon identification of positive plasmid clones, all inserts were digested with MluI/NheI and subcloned into the genome vector containing the firefly luciferase gene (FL), pVSV-XN FL and the pVSV-XNΔM51 FL. Additionaly, the Isfahan G sequence was also cloned into the pVSV-XNΔM51 RL containing the Renilla luciferase gene. Similarly, the JSRVenv cDNA (gift from Shan-Lu Liu, McGill University, Montreal, Canada) was amplified between nucleotides 1 and 1660 and the transmembrane and cytoplasmic tail containing region of VSV IND G followed by the stop-start sequence was incorporated using the overlapping primer based PCR insertion Quick change II XL kit (Stratagene, 2005215), thereby generating a JSRVenv-VSV G chimera. Upon identification of a positive clone, the plasmid was digested with MluI/NheI and subcloned into the genome vector containing the firefly luciferase gene (FL), pVSV-XN FL.

The genome plasmids containing VSV IND G, Isfahan G, Chandipura G and Maraba G were used to rescue infectious virus using a similar technique to that previously described[214]. Briefly, BHK cells expressing the T7 RNA polymerase (BHK-T7) were grown to approximately 85% confluency in sterile polystyrene 24-well microtiter plates (Costar 3526, Corning, Corning, NY, USA). BHK-T7 cells in 400ul of serum free media per well were transfected with each well receiving an additional 100ul of serum free medium containing 2ul of

Lipofectamine 2000 (Invitrogen), 0.5ug of the genome plasmid and 0.5ug of the support plasmids containing the elements of the VSV ribonucleoprotein (125ng pBS-VSV N, 313ng pBS-VSV P, 63ng pBS-VSV L). After 5 hours of tranfection, the serum free medium was replaced with 500ul of medium containing 10% FBS. Wells in which cells were observed for CPE 72 – 96h after transfection were passaged, amplified and filtered through a 0.2μm filter. Rescue efficiency with this method is <1.5%.

Rescue of the JSRVenv-VSV G chimera recombinant was performed using 90% confluent BHK-T7 cells in 6 well plates (Costar 6 Well Microplates, Corning, NY, USA) which were incubated with Vaccinia-T7 at an MOI of 1 and after 1h of adsorption time, the inoculum was removed and 1.5ml of serum free medium was added to the wells. To each well, the cells were transfected with an additional 500ul of serum free medium containing 5ul of Lipofectamine 2000 (Invitrogen), 2ug of the genome plasmid and 2.5ug of the support plasmids containing the elements of the Maraba ribonucleoprotein (1ug pCI-Neo-Maraba N, 1.25ug pCI-Neo-Maraba P, 0.25ug pCI-Neo-Maraba L). After 5 hours of transfection, the serum free medium was replaced with 2ml of medium containing 10% FBS. Wells in which cells were observed for CPE as early as 24h after transfection were filtered, passaged, filtered amplified and filtered again through 0.2μm filters. Rescue efficiency with this protocol is 100%.

All rescued recombinants were plaque purified 3 times, amplified, filtered through 0.2μm filters and titrated on Vero cells. VSV NJ was a gift from C.Y. Kang, McMaster University, Ontario, Canada. Anthony T. Power produced the VSV IND N-Gly mutant and was responsible for its rescue, sequencing, and confirmed expression the expression of the mutant by Western blotting.

Calculation of degree of protein sequence similarity:

The MegAlign software version 4.03 (DNASTAR Inc. Madison, WI USA) was utilized with the Jotun Hein alignment method to perform multiple sequence alignments. Subsequently, sequence pair distances of the envelope/glycoproteins were determined using the clustal method with PAM250 residue weight table and reported percent identity relative to VSV IND. The degree of similarity was corroborated using the pubmed web based comparison tool Blast 2 Sequences, National Center for Biotechnology Information (NCBI)[378]. Accession numbers for the envelope proteins used to calculate degree similarity are: Isfahan CAH17547.1; VSV IND NP_041715; VSV NJ AAG00865; Chandipura AAA42916; JSRVenv CAA77115.1.

Viral RNA isolation, RT – PCR and Sequencing of Rescued Recombinants:

From 500ul of pure virus stock (PFU>1e9/ml), RNA was extracted using 200ul of Trizol (Invitrogen). After 5 min incubation at room temperature, 200ul of chloroform was added and after 5 min it was followed by a 15min spin at 14000 rpm. The chloroform extraction was then repeated on the upper aqueous phase. To

the aqueous phase, $1/10^{th}$ volume of 3M sodium acetate and 3 volumes of 100% ethanol was added. After 30min at -80°C, the sample was then centrifuged for 30 min at 14000 rpm at 4°C. The precipitated pellet was washed with 70% ethanol followed by a 5 min spin at 14000 rpm at 4°C, and then air dried in a speedvac. The dried pellet was resuspended in 20ul of RNAse free water.

To 10ul of the RNA sample was added 1ul of random hexamer primers (50-250ng from Invitrogen) and 10 mM dNTP's (Invitrogen), followed by a 5 min incubation at 65°C and a quick chill on ice. After adding 2ul of 0.1M DTT (dithiothreitol, Invitrogen), 1ul of RNAse inhibitor (Invitrogen) and 4ul of first strand buffer (Invitrogen), the sample was incubated at 42°C for 2 min before adding 1ul (200 units) of superscript II (Invitrogen). The sample was then incubated for 50 min at 42°C followed by inactivation for 15 min at 70°C and the addition of 1ul (2 units) of RNAse H (Invitrogen).

The PCR reaction was performed on 2ul of the RT-product, using PFU turbo DNA polymerase (Stratagene). A 35 cycle PCR protocol with a 4 min extension time was used for all PCR reactions. Annealing times were optimized based on primer sets: VSV IND G: FWD CCG AAT TCC GAT CGA TCT GTT TCC TTG ACA and REV CCC TCG AGA ATT GAG GCC TCT TTG AGC A at 55°C; Maraba G: FWD GGA CGC GTG AGA TGT TGA GAC TTT TTC and REV CCC CTC GAG CTG TTA TTT ATT TCC C at 55°C; Isfahan G: FWD CCG AAT TCG CCA CCA TGA CTT CAG TCT TAT TC and REV CCC TCG

AGT CAT CTG TGA AAA GCT TGT CT at 58°C; Chandipura G: FWD AAC AGA ATT CCT ACG AAT TCA GAA ACA TGA and REV CTC TGC AGC ATC ATC CAC CGG GTT GAG ATC C at 58°C; JSRVenv: FWD GGA CGC GTT TTC GCC ACC ATG CCG AAG and REV CCG CTA GCC GTG ATA TCT GTT AGT TT at 55°C.

PCR products were gel purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, 28-9034-70), and the resulting product was then blunt end ligated into pT7Blue3 vector (Novagen, 70182-3) and transformed into competent cells (NovaBlue Singles Competent Cells, Novagen, 70181-3). Individual colonies were isolated and plasmid DNA was purified using Qiaprep Spin Miniprep Kit (Qiagen, 27106). All sequencing was performed using the Applied Biosystems 3730 DNA Analyzer (StemCore Laboratories, Ottawa Health Research Institute, Ottawa, Ontario).

Serum-neutralizing antibody assay:

Two-fold serial dilutions of mouse serum were heat inactivated at 56°C for 30 minutes, and then incubated with 1000 – 2000 PFU of VSV or VSV envelope protein recombinant in a total volume of 100ul for 45 min at 37°C and then applied to a monolayer of Vero cells in a 96-well plate. Wells were examined for cytopathic effects 48h post-inoculation as well as visualized for GFP or luciferase activity to confirm infection. Neutralizing titer was taken as the highest dilution factor of serum that prevented the appearance of infection as determined

using the fluorescent microscope or by IVIS, or by observing cytopathic effects visually.

Mouse Experiments:

Female, 8 to 10 week-old Balb/c and Balb/c nude mice were obtained from Charles River Laboratories (Wilmington, MA). All experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Services. Intravenous administration of 100ul of virus was performed through the tail vein at indicated the PFU. Immune competent mice were immunized with 1e7 VSV/mRFP1 IV a minimum of 4 weeks prior to challenge. Nude mice were passively immunized with 100ul of immune serum, injected IP 24h prior to challenge. Immune serum was obtained from mice that were primed intravenously with 1e7 ΔM51VSV/GFP, and serum was collected no earlier than 4 weeks after priming and the presence of neutralizing antibody titers was confirmed prior to passive transfer. The mouse tumor model consisted of subcutaneously injected 1e6 CT26 cells to establish hind flank tumors. Challenges with virus began when subcutaneous tumors were visible and measure at least $13mm^3$ or greater. Mice were monitored daily and euthanized at indicated time points or upon signs of morbidity by carbon dioxide narcosis. To assess tumor viral titers, tumors were harvested and immediately placed at minus 80 degrees Celsius. Viral content of homogenized tissues was determined by plaque assay on Vero cells. All tissues had undergone only one freeze thaw cycle and tissue weight was measured after homogenization.

In vitro & vivo IVIS imaging:

In vitro tissue culture 96 well plates were incubated with 2ul of d-luciferin (10mg/ml in PBS) (Molecular Imaging Products Company, Ann Arbor, MI) for Firefly luciferease imaging where applicable. Mice were injected with 200 ul (10 mg/ml in PBS) of d-luciferin intraperitoneally for Firefly luciferase imaging. Mice were anesthesized under 3% isofluorane (Baxter Corp., Deerfield,IL) and imaged with the in vivo imaging system 200 Series Imaging System (Xenogen Corporation, Hopkinton, MA). Data acquisition and analysis was performed using Living Image v2.5 software. For each experiment, where appropriate, images were captured under identical exposure, aperture and pixel binning settings, and bioluminescence was plotted on identical color scales.

Results

Generating recombinants with distinct envelope proteins

cDNAs encoding envelope glycoproteins were obtained by RT-PCR from members of the Rhabdoviridae family, vesiculovirus genus: VSV IND, VSV NJ, Maraba, Isfahan and Chandipura. The JSRV envelope glycoprotein cDNA was a kind gift from Shan-Lu Liu (McGill University, Montreal, Canada). The JSRVenv protein was truncated at the nucleotide level to remove the transforming carboxy terminus and the transmembrane domain. The remaining amino terminus consisted of the ectodomain of the JSRVenv protein and was fused in frame to the carboxy terminus of VSV G containing the transmembrane and cytoplasmic domains followed by a stop-start sequence at the 3' end (Fig. 30).

The diversity of the protein sequences compared to VSV IND encompassed a wide range and varied from Maraba 77%, VSV NJ 48%, Isfahan 39%, Chandipura 36%, to the JSRV chimera 19% (Table 6) as determined using MegAlign software (DNASTAR) and NCBI web based Blast 2 Sequences (Fig.31).

The JSRVenv-VSV G gene with a 3' stop-start signal was subsequently subcloned into the VSV IND genome pVSV-XN/FL vector (Fig. 32). The vesiculovirus G protein cDNAs were subcloned into a transfer vector to obtain a stop-start signal at the 3' end. Vesiculovirus cDNAs were then subcloned into

Figure 30. Sequence of the JSRVenv recombinant chimera.
The first 1660 nucleotides of JSRVenv containing the ectodomain components of JSRVenv were fused to the transmembrane domain and cytoplasmic tail of VSV Indiana G, resulting in a chimeric gene encoding for the JSRVenv-VSV G envelope protein depicted. Plain letters represent the amino acid sequence from JSRVenv, underlined letters are the amino acid sequence from the VSV IND G transmembrane domain and cytoplasmic tail, the boxed sequence is the intergenic nucleotide stop-start sequence.

MPKRRAGFRKGWYARQRNSLTHQMQRMTLSEPTSELPTQRQIEA
LMRYAWNEAHVQPPVTPTNILIMLLLLLQRVQNGAAAAFWAYIPDPPMIQSLGWDREI
VPVYVNDTSLLGGKSDIHISPQQANISFYGLTTQYPMCFSYQSQHPHCIQVSADISYP
RVTISGIDEKTGKKSYGNGSGPLDIPFCDKHLSIGIGIDTPWTLCRARVASVYNINNA
NATFLWDWAPGGTPDFPEYRGQHPPIFSVNTAPIYQTELWKLLAAFGHGNSLYLQPNI
SGSKYGDVGVTGFLYPRACVPYPFMLIQGHMEITLSLNIYHLNCSNCILTNCIRGVAK
GEQVIIVKQPAFVMLPVEIAEAWYDETALELLQRINTALSRPKRGLSLIILGIVSLIT
LIATAVTASVSLAQSIQAAHTVDSLSYNVTKVMGTQEDIDKKIEDRLSALYDVVRVLG
EQVQSINFRMKIQCHANYKWICVTKKPYNTSDFPWDKVKKHLQGIWFNTNLSLDLLQL
HNEILDIENSPKATLNIADTVDNFLQNLFSNFPSLHSLWK**SSIASFFFTIGLIIGLFL**
VLRVGIYLCIKLKHTKKRQIYTDIEMNRLGTGGTATGAAAAAAACTAACAGATATCACG

VSV Indiana G amino acid sequence of transmembrane domain and cytoplasmic tail used to replace the membrane and cytoplasmic portion of JSRV env **SSIASFFFTIGLIIGLFLVLRVGIYLCIKLKHTKKRQIYTDIEMNRLGT**

VSV intergenic stop-start nucleotide sequence GGTATGAAAAAAACTAACAGATATCACG

Table 6. Envelope protein sequence comparison.
Both MegAlign and Blast 2 Sequences were utilized to determine the percent identity between the various envelope proteins to the VSV IND G protein.

Envelope Protein	% Similarity to VSV Indiana Using pubmed web based Blast 2 Sequences comparison tool	% Similarity to VSV Indiana Using MegAlign DNASTAR software
VSV Indiana glycoprotein	100%	100%
VSV New Jersey glycoprotein	51%	48.1%
VSV Indiana N-Gly mutant	99%	Not Available
Maraba glycoprotein	81%	77.1%
Isfahan glycoprotein	41%	38.9%
Chandipura glycoprotein	41%	36.4%
JSRV envelope protein	No significant homology	11.4%
JSRVenv–VSV G chimera	No significant homology	19.4%

Figure 31. NCBI Glycoprotein sequence alignment.
Alignment of protein sequences between the various envelope proteins and VSV IND G using the web based Blast 2 sequences.

Score = 728 bits (1879), Expect = 0.0
Identities = 332/405 (81%), Positives = 368/405 (90%), Gaps = 0/405 (0%)

```
          MCHAAKWVTTCDFRWYGPKYITHSIHSMSPTLEQCKTSIEQTKQGVWINPGFPPQSCGYA  60
          MCHA+KWVTTCDFRWYGPKYIT SI S +P++EQCK SIEQTKQG W+NPGFPPQSCGYA
          MCHASKWVTTCDFRWYGPKYITQSIRSFTPSVEQCKESIEQTKQGTWLNPGFPPQSCGYA  133

Query  61   TVTDAEVVVVQATPHHVLVDEYTGEWIDSQLVGGKCSKEVCQTVHNSTVWHADYKITGLC  120
            TVTDAE V+VQ TPHHVLVDEYTGEW+DSQ + GKCS  +C TVHNST WH+DYK+ GLC
Sbjct  134  TVTDAEAVIVQVTPHHVLVDEYTGEWVDSQFINGKCSNYICPTVHNSTTWHSDYKVKGLC  193

Query  121  ESNLASVDITFFSEDGQKTSLGKPNTGFRSNYFAYESGEKACRMQYCTQWGIRLPSGVWF  180
            +SNL S+DITFFSEDG+ +SLGK  TGFRSNYFAYE+G KAC+MQYC  WG+RLPSGVWF
Sbjct  194  DSNLISMDITFFSEDGELSSLGKEGTGFRSNYFAYETGGKACKMQYCKHWGVRLPSGVWF  253

Query  181  ELVDKDLFQAAKLPECPRGSSISAPSQTSVDVSLIQDVERILDYSLCQETWSKIRAKLPV  240
            E+ DKDLF AA+ PECP GSSISAPSQTSVDVSLIQDVERILDYSLCQETWSKIRA LP+
Sbjct  254  EMADKDLFAAARFPECPEGSSISAPSQTSVDVSLIQDVERILDYSLCQETWSKIRAGLPI  313

Query  241  SPVDLSYLAPKNPGSGPAFTIINGTLKYFETRYIRVDISNPIIPHMVGTMSGTTTERELW  300
            SPVDLSYLAPKNPG+GPAFTIINGTLKYFETRYIRVDI+ PI+  MVG +SGTTTERELW
Sbjct  314  SPVDLSYLAPKNPGTGPAFTIINGTLKYFETRYIRVDIAAPILSRMVGMISGTTTERELW  373

Query  301  NDWYPYEDVEIGPNGVLKTPTGFKFPLYMIGHGMLDSDLHKSSQAQVFEHPHAKDAASQL  360
            +DW PYEDVEIGPNGVL+T +G+KFPLYMIGHGMLDSDLH SS+AQVFEHPH +DAASQL
Sbjct  374  DDWAPYEDVEIGPNGVLRTSSGYKFPLYMIGHGMLDSDLHLSSKAQVFEHPHIQDAASQL  433

Query  361  PDDETLFFGDTGLSKNPVELVEGWFSSWKSTLASFFLIIGLGVAL  405
            PDDE+LFFGDTGLSKNP+ELVEGWFSSWKS++ASFF IIGL + L
Sbjct  434  PDDESLFFGDTGLSKNPIELVEGWFSSWKSSIASFFFIIGLIIGL  478
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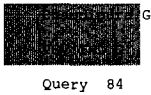
Score = 403 bits (1036), Expect = 3e-110
Identities = 191/456 (41%), Positives = 275/456 (60%), Gaps = 10/456 (2%)



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            +I FP N K +WK V  N  YCP   +      L   +      P      ++DG++CHA+
            TIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHNDLIGTAIQVKMPKSHKAIQADGWMCHAS  78

Query  84   KWVTTCDFRWYGPKYITHSIHNIKPTRSDCDTALASYKSGTLVSLGFPPESCGYASVTDS  143
            KWVTTCDFRWYGPKYIT SI +  P+   C  ++   K GT ++ GFPP+SCGYA+VTD+
Sbjct  79   KWVTTCDFRWYGPKYITQSIRSFTPSVEQCKESIEQTKQGTWLNPGFPPQSCGYATVTDA  138

Query  144  EFLVIMITPHHVGVDDYRGHWVDPLFVGGECDQSYCDTIHNSSVWIPADQTKKNICGQSF  203
            E +++ +TPHHV VD+Y G WVD  F+ G+C    C T+HNS+ W  +D   K +C  +
Sbjct  139  EAVIVQVTPHHVLVDEYTGEWVDSQFINGKCSNYICPTVHNSTTW-HSDYKVKGLCDSNL  197

Query  204  TPLTVTVAYD--KTKEIAAGGIVFKSKYHSHMEGARTCRLSYCGRNGIKFPNGEWVSLML  261
              + +T   +  +   +  G  F+S Y ++  G + C++ YC   G++ P+G W  +
Sbjct  198  ISMDITFFSEDGELSSLGKEGTGFRSNYFAYETGGKACKMQYCKHWGVRLPSGVWFEM--  255

Query  262  KLRSKRNLYFPC-LKMCPTGIRGEIYPSIRWAQVLTSEIQRILDYSLCQNTWDKVERKEP  320
              + ++L+       CP G              L  +++RILDYSLCQ TW K+    P
Sbjct  256  ---ADKDLFAAARFPECPEGSSISAPSQTSVDVSLIQDVERILDYSLCQETWSKIRAGLP  312

Query  321  LSPLDLSYLASKSPGKGLAYTVINGTLSFAHTRYVRMWIDGPVLKEPKGKRESPSGISSD  380
            +SP+DLSYLA K+PG G A+T+INGTL +  TRY+R+ I  P+L    G   S +    +
Sbjct  313  ISPVDLSYLAPKNPGTGPAFTIINGTLKYFETRYIRVDIAAPILSRMVG-MISGTTTERE  371

Query  381  IWTQWFKYGDMEIGPNGLLKTAGGYKFPWHLIGMGIVDNELHELSEANPLDHPQLPHAQS  440
            +W  W  Y D+EIGPNG+L+T+ GYKFP ++IG G++D++LH  S+A   +HP +  A S
Sbjct  372  LWDDWAPYEDVEIGPNGVLRTSSGYKFPLYMIGHGMLDSDLHLSSKAQVFEHPHIQDAAS  431

Query  441  IADDSEEIFFGDTGVSKNPVELVTGWFTSWKESLAA  476
              D E +FFGDTG+SKNP+ELV GWF+SWK S+A+
Sbjct  432  QLPDDESLFFGDTGLSKNPIELVEGWFSSWKSSIAS  467
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Score = 416 bits (1070), Expect = 3e-114
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          M  +L++  + +G     +C   IVFP   K  WK V      YCP S++LN   DL   A
          MKCLLYLAFLFIGV----NCKFTIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHNDLIGTA  56

Query  61   FDSKVPIGITPSNSDGYLCHAAKWVTTCDFRWYGPKYITHSVHSLRPTVSDCKAAVEAYN  120
               K+P        +DG++CHA+KWVTTCDFRWYGPKYIT S+ S  P+V  CK ++E
Sbjct  57   IQVKMPKSHKAIQADGWMCHASKWVTTCDFRWYGPKYITQSIRSFTPSVEQCKESIEQTK  116

Query  121  AGTLMYPGFPPESCGYASITDSEFYVMLVTPHPVGVDDYRGHWVDPLFPTSECNSNFCET  180
             GT + PGFPP+SCGYA++TD+E  ++ VTPH V VD+Y G WVD  F   +C++  C T
Sbjct  117  QGTWLNPGFPPQSCGYATVTDAEAVIVQVTPHHVLVDEYTGEWVDSQFINGKCSNYICPT  176

Query  181  VHNATMWIPKDLKTHDVCSQDFQTIRVSVMYPQTK-PTKGADLT-LKSKFHAHMKGDRVC  238
            VHN+T W   D K   +C  +  ++ ++ ++      +  + G + T  +S + A+  G + C
Sbjct  177  VHNSTTW-HSDYKVKGLCDSNLISMDITFFSEDGELSSLGKEGTGFRSNYFAYETGGKAC  235

Query  239  KMKFCNKNGLRLGNGEWIEVGDEVMLDNSKLLSLFPDCLVGSVVKSTLLSEGVQTALWET  298
            KM++C   G+RL +G W E+ D+ +   ++    FP+C  GS + +   +  +  + E +
Sbjct  236  KMQYCKHWGVRLPSGVWFEMADKDLFAAAR----FPECPEGSSISAPSQTSVDVSLIQDV  291

Query  299  DRLLDYSLCQNTWEKIDRKEPLSAVDLSYLAPRSPGKGMAYIVANGSLMSAPARYIRVWI  358
            +R+LDYSLCQ TW KI    P+S VDLSYLAP++PG G A+ + NG+L      RYIRV I
Sbjct  292  ERILDYSLCQETWSKIRAGLPISPVDLSYLAPKNPGTGPAFTIINGTLKYFETRYIRVDI  351

Query  359  DSPILKEIKGKKESASGIDTVLWEQWLPFNGMELGPNGLIKTKSGYKFPLYLLGMGIVDQ  418
             +PIL  + G   S +  +  LW+ W P+  +E+GPNG+++T SGYKFPLY++G G++D
Sbjct  352  AAPILSRMVG-MISGTTTERELWDDWAPYEDVEIGPNGVLRTSSGYKFPLYMIGHGMLDS  410

Query  419  DLQELSSVNPVDHPHVPIAQAFVSEGEEVFFGDTGVSKNPIELISGWFSDWKETAAALGF  478
            DL   S      +HPH+  A + + + E +FFGDTG+SKNPIEL+ GWFS WK + A+  F
Sbjct  411  DLHLSSKAQVFEHPHIQDAASQLPDDESLFFGDTGLSKNPIELVEGWFSSWKSSIASFFF  470

Query  479  AAISVILIIGLMRLLPLLCRRRK--QKKVIYKDVELN  513
                +I +  ++R+   LC + K  +K+ IY D+E+N
Sbjct  471  IIGLIIGLFLVLRVGIHLCIKLKHTKKRQIYTDIEMN  507
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Score = 523 bits (1347), Expect = 2e-146
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          M   L LA +   +  K  IVFP +  G+WK VP  Y+YCP+S+D N H    G  +++
          MKCLLYLAFLFIGVNCKFTIVFPHNQKGNWKNVPSNYHYCPSSSDLNWHNDLIGTAIQVK  60

Query  61   MPKGLTTHQVDGFMCHSALWMTTCDFRWYGPKYITHSIHNEEPTDYQCLEAIKAYKDGVG  120
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Sbjct  61   MPKSHKAIQADGWMCHASKWVTTCDFRWYGPKYITQSIRSFTPSVEQCKESIEQTKQGTW  120

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             NPGFPPQSCGY TVTDAEA I+ VTPH V VDEYTGEW+D  FI G+C    IC TVHNS
Sbjct  121  LNPGFPPQSCGYATVTDAEAVIVQVTPHHVLVDEYTGEWVDSQFINGKCSNYICPTVHNS  180

Query  181  TKWFTSSDGESVCSQLFTLVGGTFFSDSEEITSMGLPETGIRSNYFPYISTEGICKMPFC  240
            T W +    + +C      + TFFS+  E++S+G   TG RSNYF Y +    CKM +C
Sbjct  181  TTWHSDYKVKGLCDSNLISMDITFFSEDGELSSLGKEGTGFRSNYFAYETGGKACKMQYC  240

Query  241  RKPGYKLKNDLWFQITDPDLDKTVRDLPHIKDCDLSSSIITPGEHATDISLISDVERILD  300
            + G +L + +WF++ D DL    R      +C   SSI  P + + D+SLI DVERILD
Sbjct  241  KHWGVRLPSGVWFEMADKDLFAAAR----FPECPEGSSISAPSQTSVDVSLIQDVERILD  296

Query  301  YALCQNTWGKIEAGEPITPVDLSYLGPKNPGVGPVFTIINSSLHYFTSKYLRVELESPVI  360
            Y+LCQ TW KI AG PI+PVDLSYL PKNPG GP FTIIN +L YF ++Y+RV++ +P++
Sbjct  297  YSLCQETWSKIRAGLPISPVDLSYLAPKNPGTGPAFTIINGTLKYFETRYIRVDIAAPIL  356

Query  361  PRMEGRVAGTRIVRQLWDQWFPFGEAEIGPNGVLKTKQGYKFPLHIIGTGEVDSDIKMER  420
             RM G ++GT    R+LWD W P+ + EIGPNGVL+T  GYKFPL++IG G +DSD+ +
Sbjct  357  SRMVGMISGTTTERELWDDWAPYEDVEIGPNGVLRTSSGYKFPLYMIGHGMLDSDLHLSS  416

Query  421  IVKHWEHPHIEAAQTYLKKDDTEEVIYYGDTGISKNPVELVEGWFSGWRSS  471
              + +EHPHI+ A + L  D   E +++GDTG+SKNP+ELVEGWFS W+SS
Sbjct  417  KAQVFEHPHIQDAASQLPDD---ESLFFGDTGLSKNPIELVEGWFSSWKSS  464
```
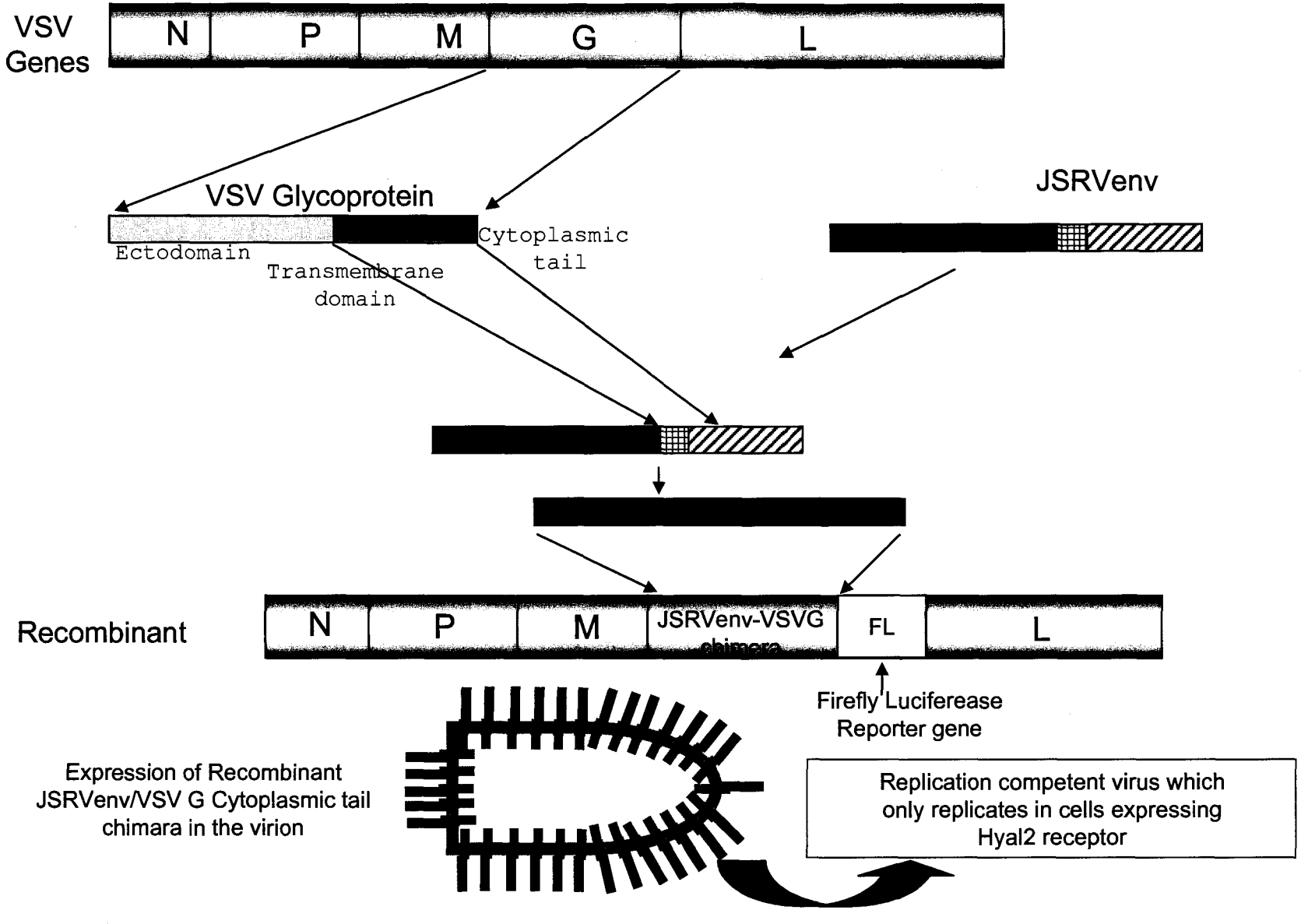

Figure 32. Cloning strategy for the JSRVenv - VSV G recombinant.
The ectodomain from JSRVenv was fused to the transmembrane and cytoplasmic domains of VSV IND G containing a 3' stop-start signal. The chimeric gene was subsequently subcloned into the VSV IND genome pVSV-XN/FL vector, thereby replacing the endogenous VSV IND G. Note that the genome vector encodes the firefly luciferase gene (FL). The virion depicts the position of the chimeric protein within the viral membrane.


VSV Genes
N
P
M
G
L
VSV Glycoprotein
Ectodomain
Transmembrane domain
Cytoplasmic tail
JSRVenv
Recombinant
N
P
M
JSRVenv-VSVG chimera
FL
L
Firefly Luciferease Reporter gene
Expression of Recombinant JSRVenv/VSV G Cytoplasmic tail chimara in the virion
Replication competent virus which only replicates in cells expressing Hyal2 receptor

VSV genome vectors, pVSV-XN/FL and the attenuated M protein mutant pΔM51VSV-XN/FL, replacing the VSV G gene (Fig. 33A). Recombinants were rescued, purified and the envelope glycoprotein cDNA was re-isolated by RT-PCR and sequenced to confirm the identity of the recombinant.

Distinct vesiculovirus G proteins do not alter/impair VSV replication in vitro

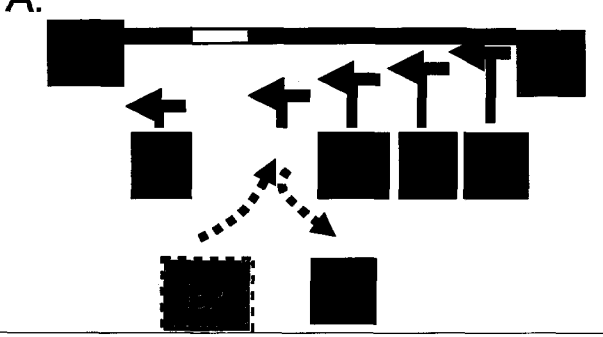
One step growth curves performed with the vesiculovirus recombinants revealed similar achievable maximum titers at 24h in comparison to the parent viruses (Fig. 33B & C), suggesting that the replication kinetics of the recombinant viruses were not grossly impeded.

JSRVenv-VSV G chimeric recombinant virus growth and tropism is restricted

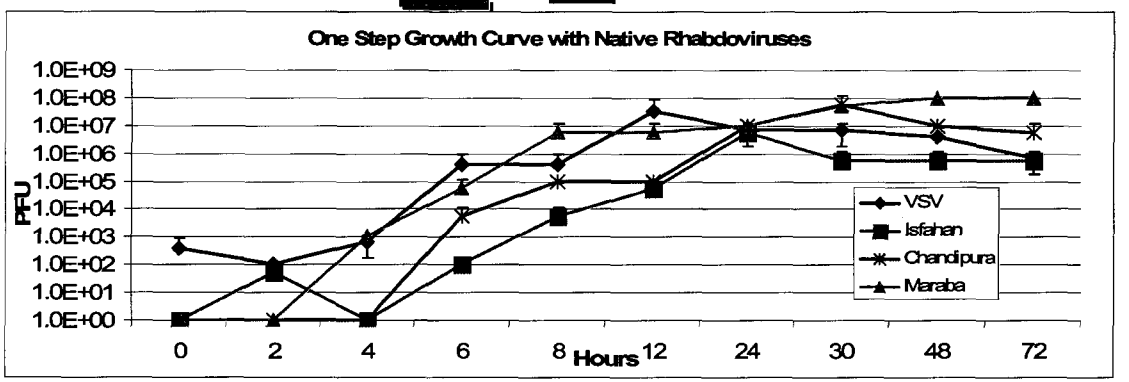
The maximum achievable titer with the chimeric recombinant after 48 hours of infection on Vero cells is 1e5 PFU/ml which is increased to 1e7 PFU/ml following centrifugation. Furthermore, unlike VSV IND G, the chimeric recombinant does not induce much CPE and infects a restricted number of cell types (Fig. 34 A-D) in comparison to the parent VSV/FL (Fig. 34 E). Of the 91304 Ewing sarcoma, CT26, HEK 293, 4T1, U2OS, Sn12c, M14, SF268, SNB19, SW620 and Vero cells infected with an MOI of 1, only the U2OS, SNB19, Vero and SW620 cells demonstrated any luminescence (Fig. 34 A-D wells with yellow circles) and of those only the SW620 cells appeared to

Figure 33. Cloning strategy and growth curve of the G recombinants.
(A) Vesiculovirus cDNA's were subcloned into VSV genome vectors, pVSV-XN/FL and the attenuated M protein mutant pΔM51VSV-XN/FL, replacing the VSV IND G gene. In comparison to the parent vesiculovirus (B), the rescued vesiculovirus G recombinants achieved titers within at least a log of the parent ΔM51VSV/FL virus at the 24h time point (C). Similar titers were achieved for the VSV/FL recombinants. Error bars represent standard deviations. One step growth curves using an MOI of 5 were repeated in triplicate for each time point and this is representative of 2 independent experiments. Note that titers are representative of concentration from supernatant and not a centrifuged stock. T=0 represent titer after 45 minute adsorption and 4 washes with 500ul of PBS.

A.



B.


One Step Growth Curve with Native Rhabdoviruses
PFU
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
2
4
6
8
Hours
12
24
30
48
72
VSV
Isfahan
Chandipura
Maraba

C.

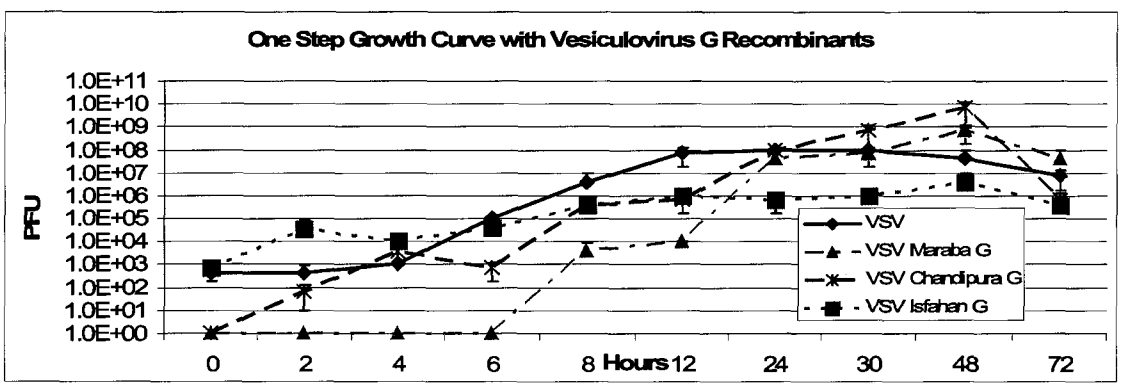
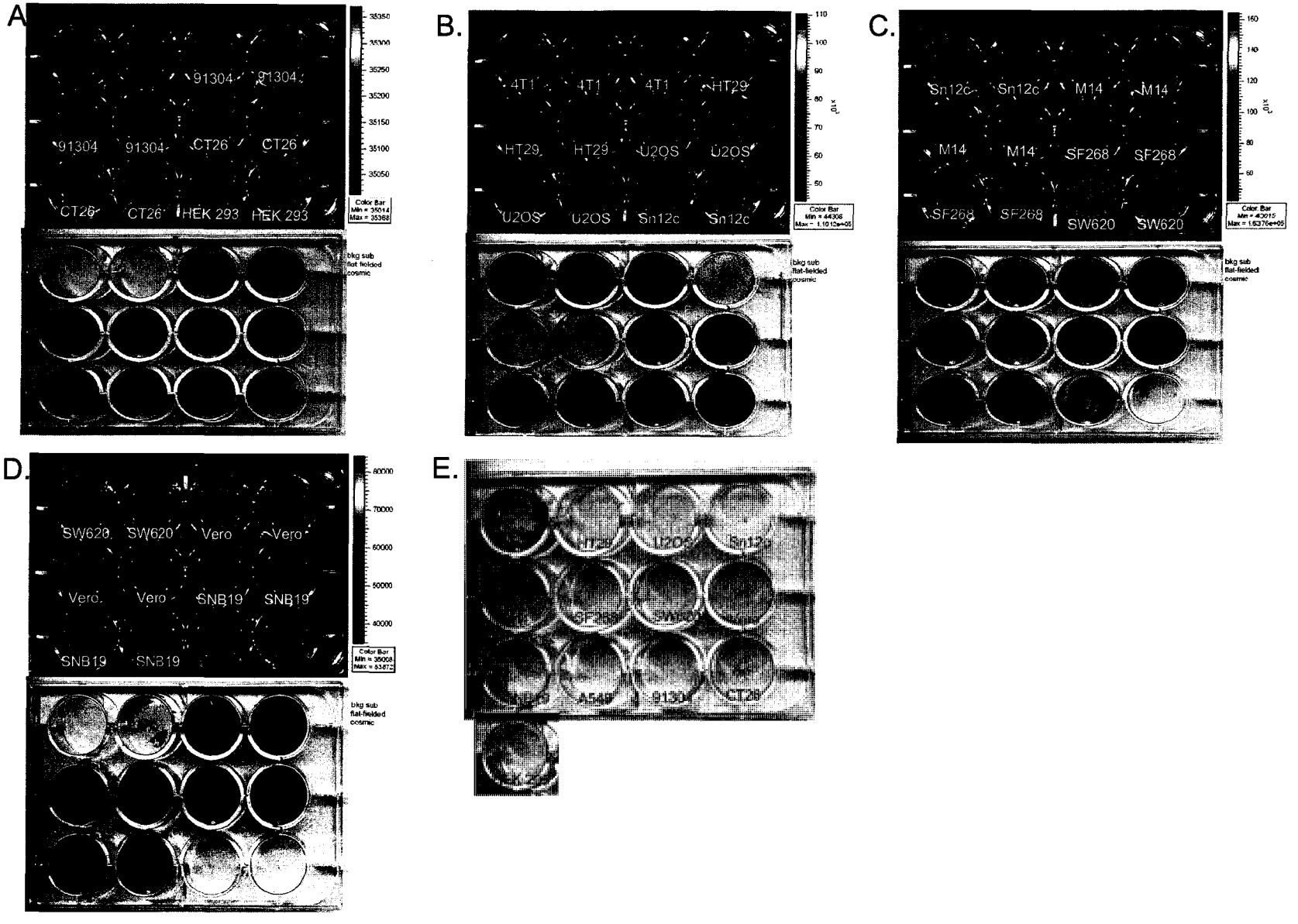

One Step Growth Curve with Vesiculovirus G Recombinants
PFU
1.0E+11
1.0E+10
1.0E+09
1.0E+08
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
1.0E+00
0
2
4
6
8
Hours
12
24
30
48
72
VSV
- VSV Maraba G
VSV Chandipura G
- VSV Isfahan G

Figure 34. Restricted tropism of the JSRVenv-VSV G chimeric recombinant.
A panel of cell lines was infected with the chimeric recombinant at an MOI of 1. (A-D) Wells were imaged with IVIS 60h post infection. In comparison to VSV/FL (E), the VSV/JSRVenv-VSVG/FL chimera only infected the U2OS, SNB19, Vero and SW620 cells types and only the SW620 demonstrated visible CPE upon crystal violet stain (C & D). Note IVIS images of wells are placed above the corresponding crystal violet stained plate. Yellow rings indicate the presence of luminescing cells. Only HT29 cells were poorly confluent at the time of infection. Samples infected with VSV/JSRVenv-VSVG/FL were done in quadruplicate.


A
91304
91304
91304
91304
CT26
CT26
CT26
CT26
HEK 293
HEK 293
B.
4T1
4T1
4T1
HT29
HT29
HT29
U2OS
U2OS
U2OS
U2OS
Sn12c
Sn12c
C.
Sn12c
Sn12c
M14
M14
M14
M14
SF268
SF268
SF268
SF268
SW620
SW620
D.
SW620
SW620
Vero
Vero
Vero
Vero
SNB19
SNB19
SNB19
SNB19
E.
CT26

demonstrate CPE (Fig, 34 D). In comparison, only the 4T1 cells demonstrated resistance to VSV/FL infection, whereas the rest of the cell types show significant CPE (Fig. 34 E).

JSRVenv – VSV G chimeric recombinant in a low pH environment

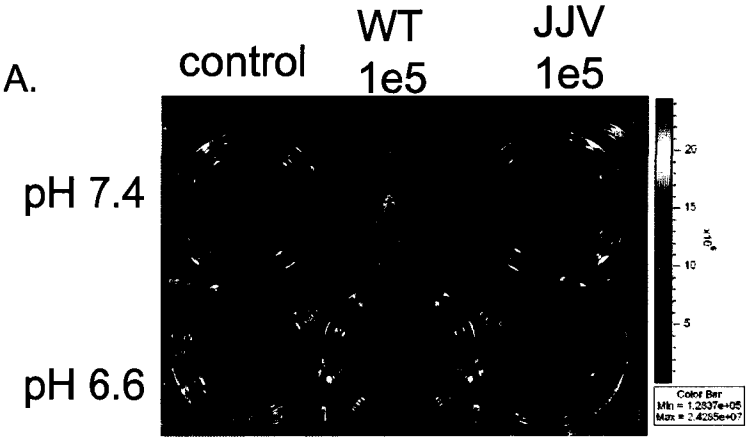
Previous studies have demonstrated that the JSRVenv is able to function in a low pH environment[75]. This was investigated with the JSRVenv-VSVG/FL chimeric recombinant by infecting a confluent monolayer of Vero cells at pH 7.4 and 6.4 in comparison to VSV/FL (Fig. 35). Wells were imaged with the IVIS at 48h after infection and demonstrated that unlike VSV/FL, the chimeric recombinant can infect and spread in Vero cells in a low pH environment (Fig. 35 A). Furthermore, crystal violet staining 60h post infection suggests the chimeric recombinant is better suited to induce CPE in this pH range, unlike VSV (Fig. 35 B).

Engineering a distinct glycoprotein recombinant

It has been described that a major neutralizing epitope within the VSV IND G glycoprotein is located between aa 257 and 271[393]. To investigate this further, Anthony Power generated a mutant glycoprotein whereby a novel glycosylation site with an aa sequence NRT, was introduced into the VSV IND G between aa 258 and 262 thereby potentially masking the main neutralizing epitope (Fig 36A). This was confirmed by sequencing (Fig. 36B) and Western

Figure 35. The JSRVenv-VSV G chimera in a low pH environment.
(A) Unlike VSV G which is sensitive to pH changes, whereby the virion is unable to spread between pH 6.4 – 6.6, the JSRVenv-VSV G chimeric recombinant can infect and spread in Vero cells in these low pH environment. (B) Furthermore, crystal violet staining 60h post infection suggests the chimeric recombinant is better suited to induce CPE in this pH range, unlike VSV. This is representative of 2 independent experiments.

A.


control
WT
1e5
JJV
1e5
pH 7.4
pH 6.6
20
15
10
5
Color Bar
Min = 1.2837e+05
Max = 2.4255e+07

B. 60 h

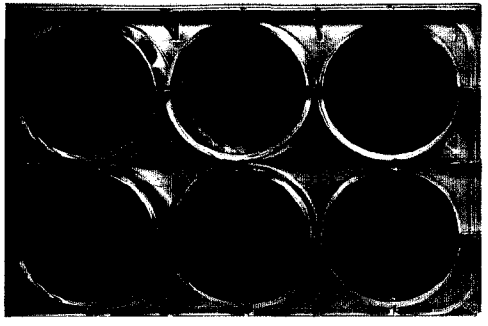
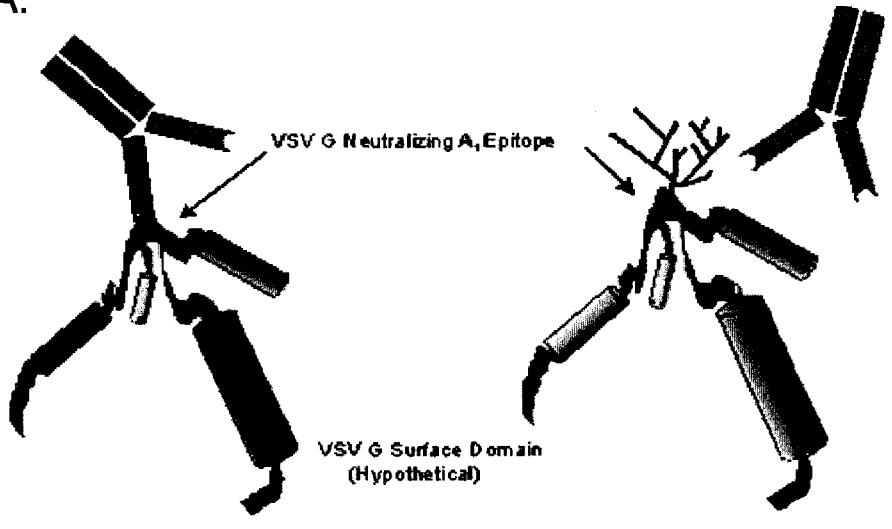


Figure 36. Engineering a VSV IND G glycosylated mutant.
This work was performed by A.T. Power. A novel glycosylation site (NRT) was inserted into the main neutralizing epitope region on VSV IND G (A) at amino acid position 259-261(B) as confirmed by sequencing. (C) Western blot confirming the expression of the glycosylated mutant G protein in lane b in comparison to the parent VSV G in lane a.

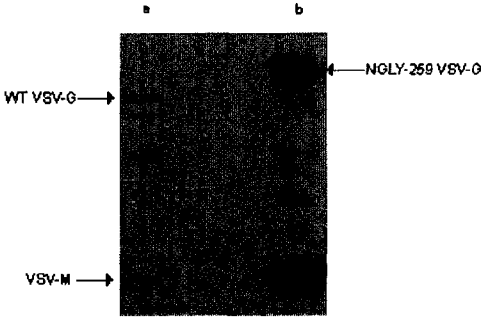
A.



B.

	252	253	254	255	256	257	258	259	260	261	262	263	264	265
VSV-Indiana	W	F	E	M	A	D	K	D	L	F	A	A	A	R
VSV-Ngly-259	W	F	E	M	A	D	K	N	R	T	A	A	A	R

C.



blot (Fig. 36C). Maximum achievable titer with this recombinant was 1e11 after concentration, suggesting that the mutation did not alter the kinetics of the virus.

In vitro neutralizing antibody assay

To determine if the recombinant viruses bearing distinct envelope glycoproteins were able to evade neutralization by anti-VSV IND serum, an in vitro neutralizing antibody assay was performed whereby a two fold serial dilution of serum containing VSV neutralizing antibodies was pre-incubated with 1-2e3 virions from each type of recombinant and then applied to a monolayer of Vero cells. The VSV IND G bearing recombinant is completely neutralized at a serum dilution of 1/1600 of anti-VSV IND serum (Fig. 37A and G) in comparison to non-immunized serum (Fig. 37E and G). In contrast, the VSV IND recombinant bearing the Isfahan G (Fig. 37B, B'), the JSRVenv-VSV G chimera (Fig. 37 E, E') and VSV NJ (Table 7) were able to completely evade neutralization. Whereas the VSV IND Maraba G, VSV IND Chandipura G recombinants (Fig. 37 C, C' and D, D') and VSV IND G N-Gly mutant recombinant (Table 7) were only partially neutralized. Neutralization titers were tabularized and included the data of the visual CPE noted from the VSV NJ and VSV IND G N-Gly mutant recombinants (Table 7).

In vivo immune evasion with vesiculovirus recombinants

To validate the findings of the in vitro antibody neutralization assay, I challenged mice immunized with VSV IND, bearing bilateral sub-cutaneous

Figure 37. In vitro antibody neutralization assay.
A total of 1e3 – 2e3 virions from each individual recombinant was pre-incubated with anti-VSV IND neutralizing serum at various dilutions, and then added to confluent Vero cells in a 96 well plate. Wells were observed 48h later for signs of infection as determined by the detection of luminescence using IVIS (A-D and B'-E') or fluorescence using GFP (G) or by visually confirming CPE (F). (A-D) Various envelope recombinants incubated with anti-VSV IND neutralizing serum. (E) 50ul of 2e3 virions of JSRVenv-VSV G FL chimeric recombinant or VSV/FL incubated on Vero cells alone, after pre-incubation with 2ul of non-neutralizing serum or after pre-incubation with 2ul of anti-VSV IND neutralizing serum. (B'-E' and G) Corresponding repeat experiments. (F-G) Confirming the neutralizing effect of the anti-VSV IND neutralizing serum in comparison to non-neutralizing serum using 2e3 virions of VSV/GFP. (F) Crystal violet stain performed 72h after wells of confluent Vero cells were infected with VSV/GFP alone, or after pre-incubation with non-neutralizing or neutralizing serum. (G) Anti-VSV IND serum neutralizes VSV/GFP at a serum dilution of 1:1600 in comparison to no neutralization by non-immunized serum. This serum was used for immune evasion experiments. Note luminescence / fluorescence scale adjusted for maximum sensitivity in all cases. Note the VSV NJ and VSV IND G N-Gly mutant recombinant do not express GFP or FL and are therefore not shown. (-) indicates well with cells and antibody only; (+) cells and virus only; Gradient = antibody gradient using a 2 fold dilution. Same anti-VSV IND serum is utilized for all recombinants per experiment.

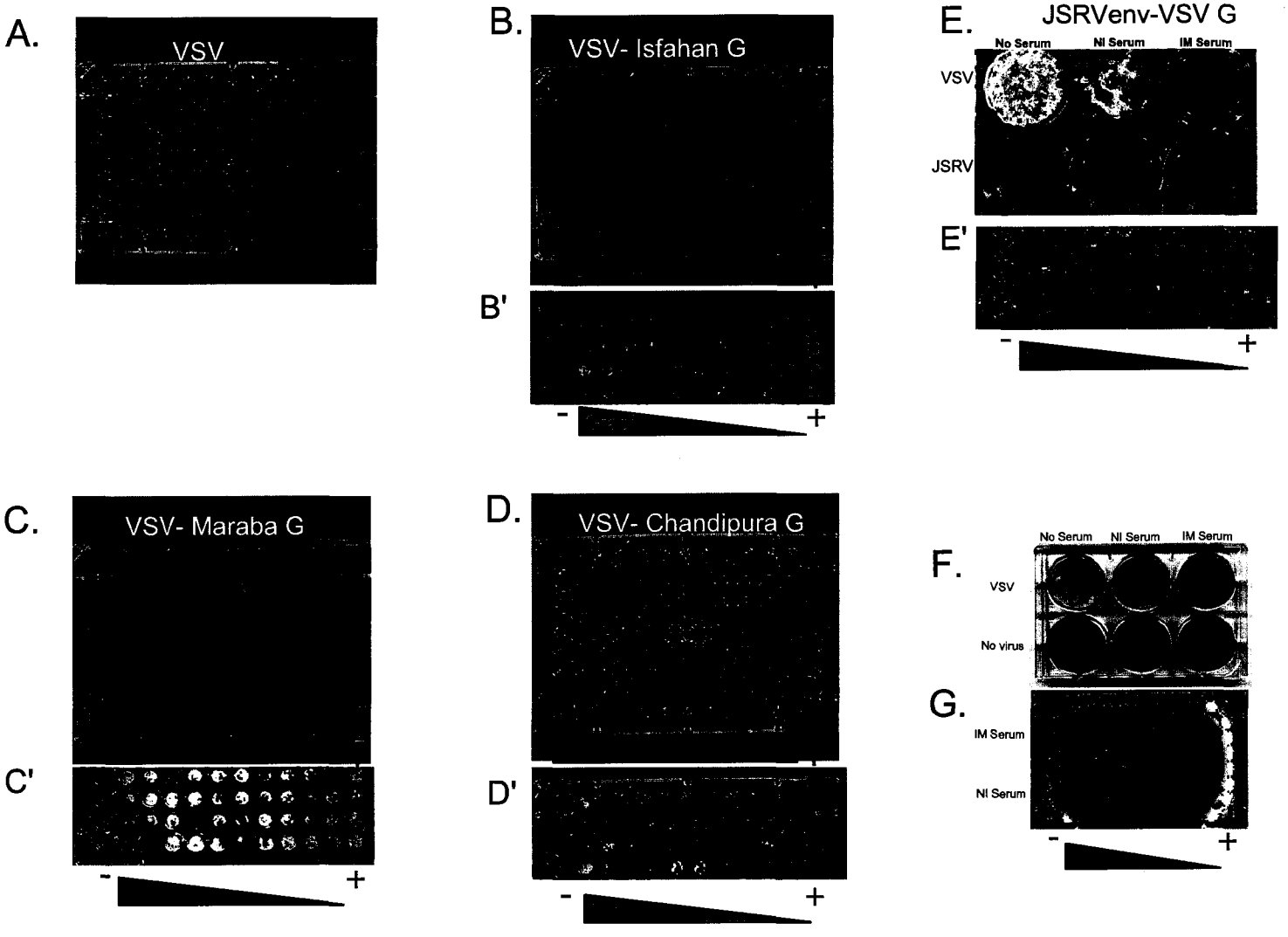

A.
VSV
B.
VSV- Isfahan G
B'
-
+
E.
JSRVenv-VSV G
No Serum
NI Serum
IM Serum
VSV
JSRV
E'
-
+
C.
VSV- Maraba G
C'
-
+
D.
VSV- Chandipura G
D'
-
+
F.
No Serum
NI Serum
IM Serum
VSV
No virus
G.
IM Serum
NI Serum
-
+

Table 7. In vitro antibody neutralization assay.
Data from an independent assay is tabularized based on the neutralizing titer determined as the highest dilution of serum that demonstrated infection in at least 2 or more of 4 wells (yellow highlight). Line indicates the neutralizing titer of VSV IND with that particular anti-VSV IND serum.

Antibody Dilution	VSV Ind G	VSV NJ G	VSV Ind N-Gly G	Maraba G	Isfahan G	Chandipura G	JSRVenv
2.00E-02	0	1	0	0.75	1	1	1
1.00E-02	0	1	1	1	1	0.5	1
5.00E-03	0	1	0.75	0.75	1	1	1
2.50E-03	0	1	1	1	1	0.75	1
1.25E-03	0	1	1	1	1	0.75	1
6.25E-04	0.25	1	1	1	1	1	1
3.13E-04	0.75	1	1	1	1	1	1
1.56E-04	0.75	1	1	1	1	0.75	1
7.81E-05	0.75	1	1	1	1	1	1
3.91E-05	1	1	1	1	1	1	1

CT26 tumors, with the recombinants bearing the distinct vesiculovirus glycoproteins (Fig. 38 E-H, J). In comparison to non-immunized mice (Fig. 38 A-D) Mice challenged with VSV IND G, Maraba G and the Chandipura G recombinants (Fig. 38 F and J respectfully) did not result in immune evasion as no signal was visualized with IVIS imaging and no virus was detected after tumor titration. Mice immunized with VSV IND and challenged with the Isfahan G recombinant resulted in the detection of firefly luminescence by IVIS (Fig. 38 J), albeit at a lower signal intensity than in non-immunized mice (Fig. 38C). None the less, in a repeat experiment the VSV Isfahan G recombinant did produce a visible signal in 5/5 mice as well as in 2/5 tumors in a repeat experiment (Fig. 38 G), and in 1/5 tumors in a third repeat experiment (Fig. 38 H). The ability of the VSV Isfahan G FL recombinant virus to evade was confirmed by verifying the anti-VSV neutralizing titer of the mice prior to challenge (ranged from 1/2400 to 1/4800) and by tumor titering (Fig. 38 H and Fig. 39). The converse experiment where the mice were immunized with Isfahan G recombinant and subsequently challenged with VSV IND also demonstrated the ability to evade pre-existing immunity as both mice had visible IVIS signal and the left tumor of M5 was also found to have virus within the tumor upon titering(Fig. 38 I). Again, to ensure that this was not a result of a low antibody titer in the immunized mice, serum from both immunized and non-immunized mice were collected the day prior to re-challenge and confirmed to be able to completely neutralize VSV IND in vitro at

Figure 38. Immune evasion in immune competent mice.
Non-immunized immune competent balb/c mice bearing bilateral sub-cutaneous CT26 tumors were treated with virus as depicted (A). Mice were imaged using IVIS at 24, 48 and 72h post IV administration of virus. After 72h all mice receiving VSV/FL demonstrate detectable luminescence from both tumors (time point shown in figure) which was subsequently confirmed by tumor titering (B). A similar experiment was repeated with the VSV Isfahan G recombinant at the indicated PFU (C and D). Mice immunized with 1e7 PFU VSV/GFP IV were challenged as depicted (E). No detectable signal was measured when challenged with VSV/FL (F). However, when immunized mice were challenged with the VSV Isfahan G recombinant a signal is detected in 5 of 5 mice and is confirmed visibly to be in 2 of 5 tumors (G) (doted circles indicate where tumors are located). This experiment was repeated (H) but mice M1, 2, 4 & 5 died 6-24h after challenge; however the surviving mouse which did have anti-VSV neutralizing serum, also had a signal in the left tumor which was confirmed to be the VSV Isfahan G recombinant virus upon titering. The converse experiment was performed where mice were immunized with VSV Isfahan G Renilla Luciferase and then challenged with VSV/FL (I), again M1 & M3 died 24h after challenge (M4 did not develop tumors), but both surviving mice had evidence of virus infection and the left tumor of M5 was positive for virus upon titering even though that mouse had VSV Isfhan G recombinant. As a pilot experiment, the remaining recombinants were tried in non-immunized and VSV/GFP immunized mice (J). Note that the photon scale bar on the right of figure is applicable to all images.

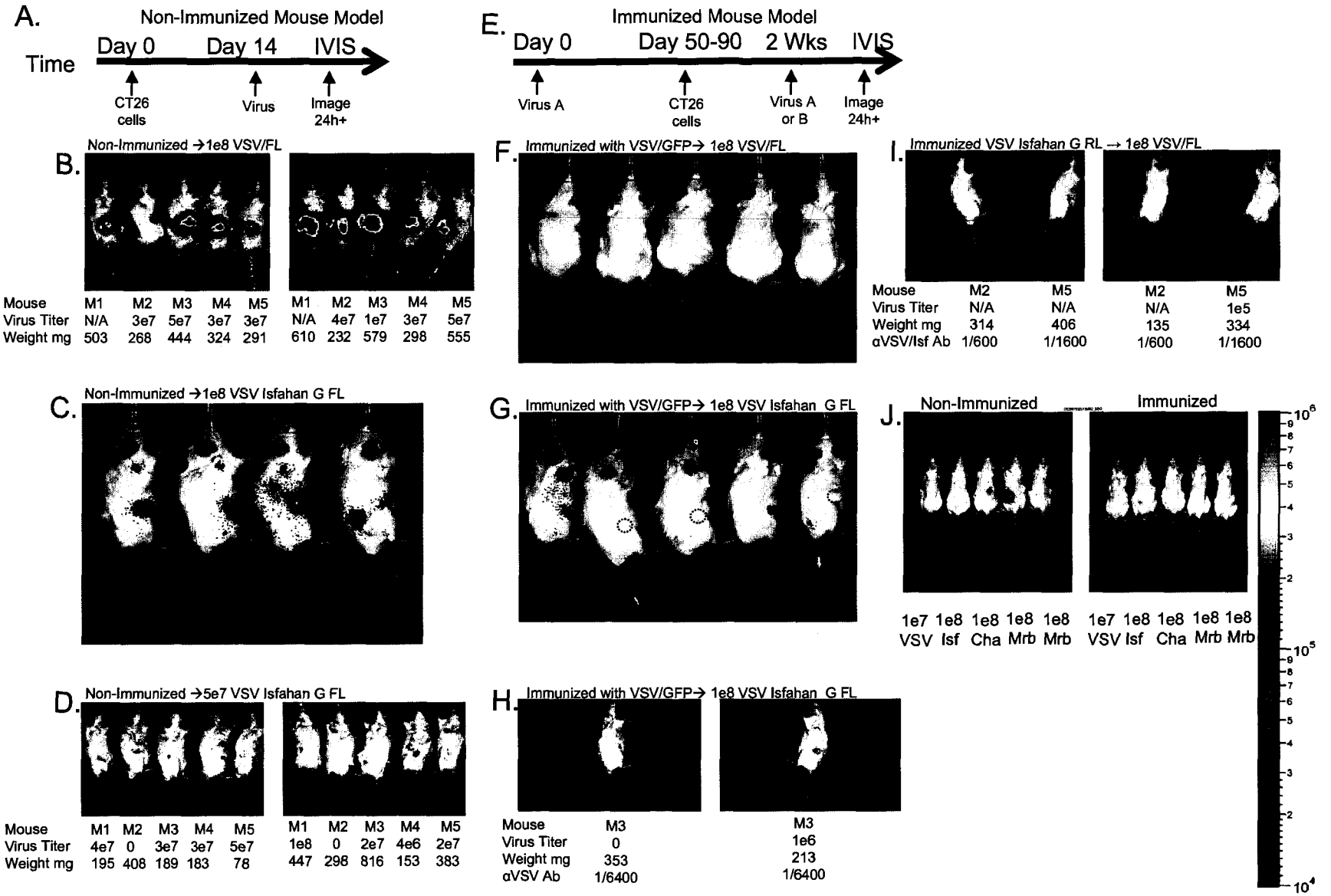
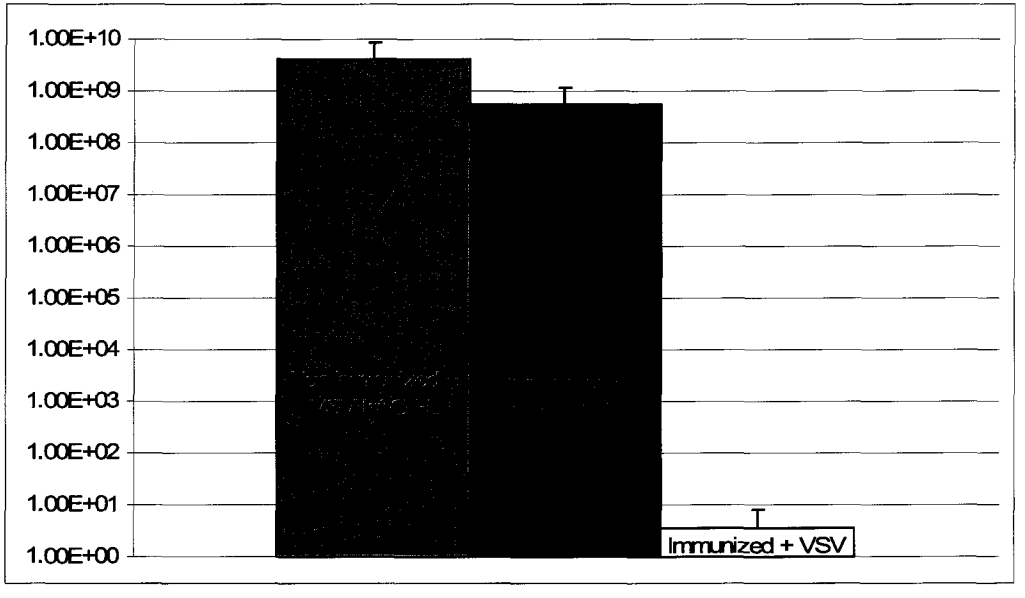

A.
Non-Immunized Mouse Model
Time
Day 0
Day 14
IVIS
CT26 cells
Virus
Image 24h+
B.
Non-Immunized →1e8 VSV/FL
Mouse M1 M2 M3 M4 M5
Virus Titer N/A 3e7 5e7 3e7 3e7
Weight mg 503 268 444 324 291
M1 M2 M3 M4 M5
N/A 4e7 1e7 3e7 5e7
610 232 579 298 555
C.
Non-Immunized →1e8 VSV Isfahan G FL
D.
Non-Immunized →5e7 VSV Isfahan G FL
Mouse M1 M2 M3 M4 M5
Virus Titer 4e7 0 3e7 3e7 5e7
Weight mg 195 408 189 183 78
M1 M2 M3 M4 M5
1e8 0 2e7 4e6 2e7
447 298 816 153 383
E.
Immunized Mouse Model
Day 0
Day 50-90
2 Wks
IVIS
Virus A
CT26 cells
Virus A or B
Image 24h+
F.
Immunized with VSV/GFP→ 1e8 VSV/FL
G.
Immunized with VSV/GFP→ 1e8 VSV Isfahan G FL
H.
Immunized with VSV/GFP→ 1e8 VSV Isfahan G FL
Mouse M3
Virus Titer 0
Weight mg 353
αVSV Ab 1/6400
M3
1e6
213
1/6400
I.
Immunized VSV Isfahan G RL → 1e8 VSV/FL
Mouse M2 M5 M2 M5
Virus Titer N/A N/A N/A 1e5
Weight mg 314 406 135 334
αVSV/Isf Ab 1/600 1/1600 1/600 1/1600
J.
Non-Immunized
Immunized
1e7 VSV
1e8 Isf
1e8 Cha
1e8 Mrb
1e8 Mrb
1e7 VSV
1e8 Isf
1e8 Cha
1e8 Mrb
1e8 Mrb
10^6
10^5
10^4

Figure 39. Titers in immune evaded tumors in immune competent mice.
Tumors from VSV/GFP immunized mice challenged IV with 1e8 PFU of VSV Isfahan G FL recombinant achieved titers within a log of tumors from non-immunized mice given VSV Isfahan G FL recombinant. In comparison, VSV/GFP immunized mice challenged with VSV/FL did not have any significant viral titers. Error bars represent standard deviation, 3 mice per group.


1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1.00E+04
1.00E+03
1.00E+02
1.00E+01
1.00E+00
Immunized + VSV

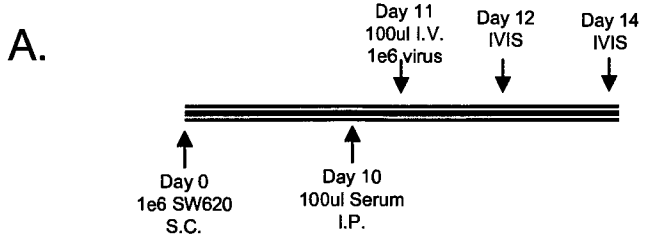
a titer of 1/600 and 1/1600 compared to non-immunized serum (Fig. 38 I). It should be noted that mice that were re-challenged with virus (Fig. 38 G, H and I) were less active, hunched with piloerection and in some cases animals had to be euthanized. In all cases there were at least 5 mice per group except for the preliminary pilot screen (Fig. 38 J).

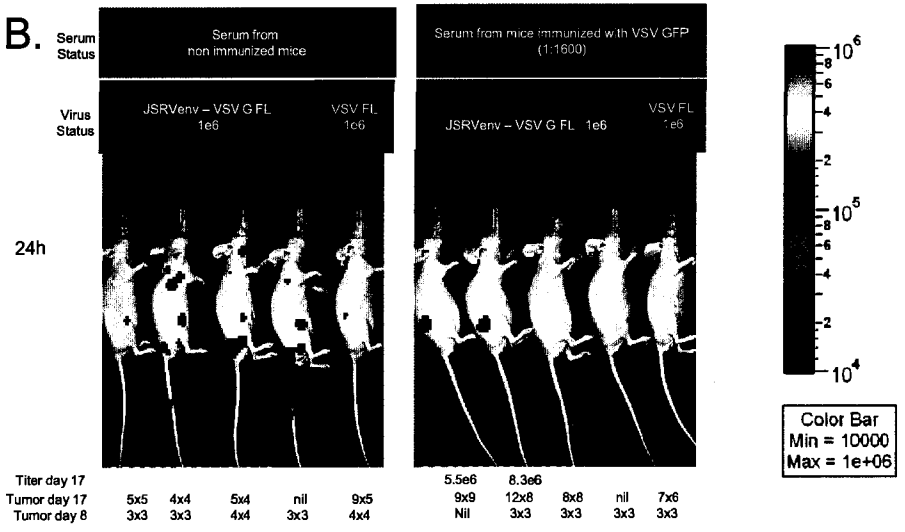
In vivo immune evasion with the VSV-JSRVenv recombinant

The 30% success rate in evading the immune system and obtaining virus at the tumor site with the VSV Isfahan G recombinant, suggested only a partial evasion, possibly by cross reacting antibodies. To minimize any chance of cross neutralization, I proceeded to use the JSRVenv-VSV G FL chimera which had an envelope protein with little similarity to that of the vesiculoviruses, yet was still functional as an oncolytic virus. Nude Balb/c mice bearing unilateral sub-cutaneous SW620 tumors were passively immunized IP with 100ul of serum from immune competent Balb/c mice immunized IV with VSV/GFP (Fig. 40 A). The anti- VSV IND neutralizing serum effects were confirmed in vitro and in vivo prior to IP injection into these nude mice (Fig. 37 F,G and 38 F). The passively immunized nude mice were then challenged with either 1e6 PFU of JSRVenv-VSV G FL recombinant or 1e6 PFU of VSV IND FL (Fig. 40). Mice were imaged at 24, 48 and 72h with IVIS. Compared to control mice, immunized mice challenged with the JSRVenv-recombinant demonstrated virus at the tumor in 62.5% of cases[231] of challenged mice in comparison to 0% of the mice challenged with VSV IND (Fig. 40 B & C). To confirm presence of virus, tumors were

harvested and titered. Although tumors were measured over a three week period, they failed to demonstrate any significant decrease in size in comparison to controls after 1 treatment.

Figure 40. Immune evasion in nude mice.
Balb/c nude mice bearing unilateral sub-cutaneous SW620 tumors were administered IV either 1e6 PFU of VSV/FL or JSRVenv-VSV G FL alone or 24h after IP injection of pre-immunized serum or non-immunized serum (A). Mice were imaged at indicated time points. B and C are independent experiments. Tumor sizes were followed for a period of three weeks whereupon the tumors titers were obtained. No adverse effects were noted.


A.
Day 11
100ul I.V.
1e6 virus
Day 12
IVIS
Day 14
IVIS
Day 0
1e6 SW620
S.C.
Day 10
100ul Serum
I.P.


B.
Serum Status
Serum from non immunized mice
Serum from mice immunized with VSV GFP (1:1600)
Virus Status
JSRVenv – VSV G FL 1e6
VSV FL 1e6
JSRVenv – VSV G FL 1e6
VSV FL 1e6
24h
Titer day 17
5.5e6
8.3e6
Tumor day 17
5x5
4x4
5x4
nil
9x5
9x9
12x8
8x8
nil
7x6
Tumor day 8
3x3
3x3
4x4
3x3
4x4
Nil
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3x3
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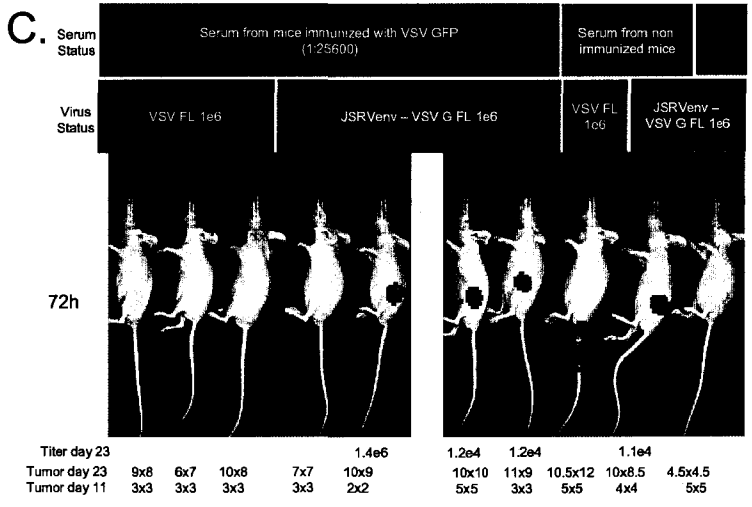
10^4
Color Bar
Min = 10000
Max = 1e+06


C.
Serum Status
Serum from mice immunized with VSV GFP (1:25600)
Serum from non immunized mice
Virus Status
VSV FL 1e6
JSRVenv – VSV G FL 1e6
VSV FL 1e6
JSRVenv – VSV G FL 1e6
72h
Titer day 23
1.4e6
1.2e4
1.2e4
1.1e4
Tumor day 23
9x8
6x7
10x8
7x7
10x9
10x10
11x9
10.5x12
10x8.5
4.5x4.5
Tumor day 11
3x3
3x3
3x3
3x3
2x2
5x5
3x3
5x5
4x4
5x5

Discussion

Tumor-targeted oncolytic viruses represent a potent new class of therapeutic agents; however host immunity remains an imposing obstacle to effective application in the clinic[293]. Antibodies in particular have been shown to be the component in VSV antiserum which neutralize virus[45,144,193,217,218,293] and impair systemic delivery to the tumor[293]. Additional studies have identified antigenic regions on the VSV G protein which are responsible for the binding of neutralizing as well as non-neutralizing antibodies[45,193,217,218]. Although others have successfully generated VSV recombinants bearing distinct glycoprotein serotypes[318] they have not been used in the context of overcoming pre-existing neutralizing antibodies to generate tumor oncolysis. As such, I have investigated the potential of envelope protein shuffling as a strategy for immune evasion from the VSV IND antiserum.

Functional recombinants bearing distinct envelope proteins

Using a model oncolytic agent based on vesicular stomatitis virus, a panel of novel recombinant viruses expressing engineered or evolutionarily divergent envelope proteins was tested for their ability to escape VSV IND neutralizing antibody. The similarity of their envelope proteins ranged from 99% to 19% with as compared to VSV IND G (Table 6). I have confirmed that the VSV recombinants bearing various vesiculovirus glycoproteins are functional, as they can achieve similar titers as the parent virus suggesting similar viral replication

kinetics (Fig. 33). In addition, the introduction of a non-rhabdovirus related envelope protein, the JSRVenv from a beta retrovirus, also yielded a replication competent recombinant virus. Granted, the chimeric JSRVenv recombinant had significantly lower titers (1e7 PFU/ml), and a restricted tropism in comparison to the parent VSV (Fig. 34). The fact that the Hyal2 receptor for JSRVenv is not ubiquitously expressed by all tumor cell lines[31,220,370] would explain the restricted tropism of the virus, coupled with the fact that the recombinant was amplified at pH 7.4 may explain the low titers since it has been reported that this env protein functions better in a low pH environment[75]. However, despite the low titer and restricted tropism, the chimeric recombinant still demonstrated oncolytic activity in SW620 and Vero cell lines. Even under low pH conditions (Fig. 34 and 35) the JSRVenv chimeric recombinant could infect and spread, a feature not characteristic of VSV G, thereby confirming a functional envelope protein. Furthermore, the ability to function in a low pH environment is certainly a desired quality for an oncolytic virus in order to allow it to penetrate low pH tumor cores.

Evading VSV IND antiserum in vitro

As expected, switching the glycoprotein with those from distinct serogroups of VSV and modifying the immunodominant neutralizing epitope or using the unrelated JSRVenv protein was sufficient to achieve immune evasion *in vitro* compared to the parent VSV IND (Fig. 37). Although the N-Gly mutant recombinant was neutralized at 32 fold more antibody titer than the parent virus, it still indicated that the virus was being neutralized. This suggests that a higher

concentration of an antibody with low avidity was required to neutralize the virus[12]. In addition the Maraba G and Chandipura G recombinants were partially neutralized at the lower dilutions, and their neutralization patterns were not linear, possibly due to conformational changes of G proteins although I did not explore this further. However, both the Isfahan G and JSRVenv-VSV G chimera were able to evade undiluted anti-VSV serum (Fig. 37 and Table 7), making them ideal candidates for an in vivo immune evasion experiment. The ability to evade neutralization in comparison to the parent virus also provides indirect evidence that the recombinants are expressing their repective membrane proteins.

Evading VSV IND antiserum in vivo

An in vivo immune evasion experiment was performed in immune competent mice (Fig. 38). A preliminary screen suggested that the Isfahan G recombinant was able to evade neutralizing serum in mice immunized with VSV/GFP (Fig. 38J) unlike the immunized mice challenged with VSV/FL where no signal is ever detected (Fig. 38F). This supported the possibility that the Isfahan G recombinant was an evasion candidate. In contrast, the remaining vesiculovirus G recombinants were unable to achieve immune evasion in tumor-bearing animals (Fig. 38 J), likely due to recognition by broadly cross-reactive antibodies. Furthermore, immune evasion and tumor infection was achieved with the Isfahan G recombinant in repeat experiments (Fig. 38 G, H), however the 30% incidence of obtaining virus at the tumor site suggested the possibility of a partial evasion. Coupled with the fact that anti-VSV Isfahan G RL recombinant serum

appears to impede VSV FL also supports the notion of cross reacting antibodies(Fig. 38I). Despite partial escape, VSV Isfahan G tumor titers were within a log of that of the parent virus (Fig. 39).

Like the Isfahan recombinant the JSRVenv-VSV G chimeric recombinant virus was able to evade neutralization in vitro (Fig. 37E, E'). However, in order to test its ability to evade neutralization in vivo, nude mice bearing SW620 subcutaneous xenografts were passively immunized with serum 24h prior to IV challenge with the chimeric recombinant (Fig. 40). Immune evasion was achieved with the recombinant in 50% of mice and up to 75% in a repeat experiment and was confirmed by tumor tittering, whereas no signal is detected by IVIS or by tumor titering in immunized mice challenged with VSV/FL. It should be noted that due to the limited titer achieved with the chimeric recombinant only 1e6 PFU/100ul was utilized per challenge which is 2 logs lower than what has previously been used with VSV, possibly explaining the weak signal obtained in non-immunized mice receiving VSV/FL (Fig. 40B &C). Despite in vitro data demonstrating that both VSV/FL and JSRVenv-VSV G FL recombinant generate substantial CPE in SW620 cells (Fig. 34 C-E), in vivo only the chimeric recombinant appeared to infect the tumor. A possible explanation for the shortlived VSV/FL signal in comparison to the chimeric recombinant in the in vivo SW620 tumors may be due to the pH of the tumor microenvironment (Fig. 35).

Although immune evasion with the JSRVenv-VSV G FL chimera appears to be more consistent than for the VSV Isfahan G recombinant, the fact that these mice were passively immunized as well as immune incompetent, must not be overlooked. Previous work has validated the use of anti-VSV IND neutralizing serum in passive immunizations of SCID mice, whereby IP administration protected them from VSV encephalitis[12,13]. The controls used in this study are in keeping with what others have reported using this technique and dose range[13,57,292,330], further legitimizing our nude animal model. Although studies with single chain antibodies have demonstrated that Fc is not required to protect against a lethal dose of VSV[12,192], non-neutralizing antibodies are thought to function through an Fc mediated mechanism and Balb/c nude mice, unlike SCIDs, are able to fix complement (Charles River Laboratories, Wilmington, MA), further validating the use of our animal model. However, other than confirming in vitro anti-VSV serum neutralization titers, I did not determine the amount and avidity of the antibodies in the serum, a shortcoming of this study.

The in vitro to in vivo discrepancy

The disparity between the in vitro antibody neutralization and in vivo evasion experiment may lie in the in vivo setting. Non-neutralizing antibodies have been identified as able to bind epitopes on the G proteins (Fig. 29B)[193], and are thereby though to mediate their effects through an antibody mediated complement dependent lysis of virions or infected cells, or even through antibody dependent cell cytotoxicity[131,216]. This study suggests, indirectly that non-

neutralizing antibodies are contributing to in vivo virus neutralization in both immune competent and nude mice.

From evasion to efficacy

The fact that tumor infection with VSV IND is never observed in the presence of VSV IND anti-serum, yet is achieved with recombinants bearing Isfahan and JSRV envelope protein, supports our hypothesis. However, the dose that managed to escape immune detection was insufficient to impede or decrease tumor burden (Fig. 40), implying that significant improvements remain to be achieved.

A multiple dosing regimen with the immune evader may enhance tumor oncolysis, however it may also provoke a specific immune response against the evader. It may be necessary to engineering a repertoire of chimeric or pseudotyped viruses not only to allow evasion and further enhance tumor oncolysis or even gene therapy, but to engineer them such as to minimize the anti-viral immune response. Implementing other types of immune evasion features used by other oncolytic viruses such as Vaccinia and NDV may also be useful[32,231,280]. Alternate dosing regimens may also permit skirting the immune system, as it has been observed in animals that VSV immunity can be broken down by repeated inoculations[76] and it is interesting to note a similar desensitization strategy has been demonstrated in human clinical trials with

NDV[212,232]. Although I have not observed these benefits in our animal models, these tactics warrant future experimentation.

Significant advances in understanding the in vitro interactions between the envelope proteins and the anti-VSV response have been achieved. However, there are still unexplained in vivo observations which need to be addressed, such as the inexplicable toxicities which occurred in the immune competent VSV immunized mice upon challenge with the VSV-Isfahan G recombinant. Similar toxicities have been observed by others which suggests a possible over-sensitized system or anaphylactic like mechanisms[399]. Similarly, immune exhaustion, the concept whereby overdosing with virus may deplete T-cells and interferons may also decrease the viral toxicity threshold [9,204]. In addition, the decreased intensity of infection as observed by IVIS with the various envelope protein recombinants in comparison to the parent VSV/FL has not been explained, although envelope proteins are known to affect the stability of VSV[337]. Others have reported that non-immune serum can neutralize virus in mice and humans[144,255], however this has not been observed in our study. Altogether, these points emphasize the need for continued investigation of the interaction between the viral envelope proteins and the hosts immune response in order to develop a better oncolytic virus.

Summary

To demonstrate that envelope protein switching could be used to deliver oncolytic virus to the tumor in the presence of neutralizing antibodies, recombinant VSV were generated using envelope proteins from other vesiculoviruses or from a beta-retrovirus. As expected, switching the glycoprotein with those from distinct serogroups of VSV or modifying the immunodominant neutralizing epitope was sufficient to achieve immune evasion in vitro, but in vivo immune evasion was superseded by the non-vesiculovirus related beta-retrovirus env protein. Altogether these findings indicate that envelope protein shuffling can be an effective strategy for immune evasion for oncolytic viruses, however serotype distinctions based on *in vitro* neutralization assays are poor predictors of *in vivo* immune evasion and clinical efficacy. These data supports the notion that to evade VSV IND neutralizing serum *in vivo* requires the expression of a more evolutionary distinct envelope protein, a difference that may not be dictated based on protein sequences alone.

Chapter 3: Non-neurotropic recombinant rhabdoviruses.

Introduction

VSV has the potential to be an effective oncolytic agent to treat human cancer. However, the neurotropism of this virus has serious consequences, and unless this is eliminated, VSV will only remain a pre-clinical tool.

The neuropathogenesis of VSV has been extensively studied in both immune competent and nude mice and has been used as a model for CNS infection, pathogenesis and immunology[178,179,326,392]. In short, the etiology of the neuropathogenesis involves two separate issues: 1) the ability of VSV to target brain tissue, termed neurotropism; and 2) the fact that VSV, which is exquisitely sensitive to interferon[368], is able to replicate seemingly unabated in normal brain tissues. Similar to other rhabdoviruses, the VSV G protein has been implicated in the neuropathogenesis[77,142,184,246,326,388]. Futhermore it is implicated in the neurotropism as G proteins produce an attenuated virus[65,351], and furthermore because lentivirus pseudotyped with VSV G targets brain[408]. Whereas the ability of vesiculoviruses to replicate in the CNS is still unclear, it may likely be to an inability of the neurons to produce type I and II interferon in a timely manner to inhibit virus replication despite the fact that neurons are responsive to type I IFN and that exogenous type I IFN strongly inhibits VSV replication in neurons both in vitro and in vivo[321,386,387]. Alternative noncytolytic means of clearing viral infections in neurons such as antibody-mediated clearance have been

demonstrated in other neurotropic viral infections[91,223]. However, since neither antibodies to VSV nor B cells infiltrating the CNS are observed before day 10, this mechanism is unlikely to be essential in clearance of VSV infection in the CNS in immunocompetent mice[33]. Overall, these studies provide direction for novel VSV attenuation strategies.

The following VSV attenuation strategies have been employed: reorganizing the viral genome through gene rearrangements[15,118,119,412], G protein truncations[65,308,309,335], nucleotide substitution or deletion within the M gene[188,369] or a combination thereof[65]. Despite developing these attenuated recombinants, they are all neurotoxic when administered intracranially in animal models, except for the combination of an M mutant with a truncated G protein expressing the HIV gag – the only VSV recombinant that reportedly has not demonstrated any neurotoxicity when administered at 1e7 PFU IC[65].

Given that the VSV G protein appears to be the main determinant of neurotropism, I sought to eliminate it altogether and replace it with one of two novel candidate genes: 1) The JSRV envelope protein with a truncated cytoplasmic tail, from the JSRV beta-retrovirus, is non-oncogenic and binds to Hyal-2 receptors found in various organs except for adult human brain[220,272,370]; 2) The p14 reovirus fusogenic peptide, FAST (Fusion Associated Small Transmembrane), which is considered the least complex protein-based membrane fusion machine, being both necessary and sufficient for membrane fusion[385].

In this study I generated 2 novel recombinant VSV viruses that did not cause any neurotoxicity when administered intracranially in mice. The strategy was based on the hypothesis that the VSV G protein is responsible for the neurotropism. Therefore by replacing the envelope protein of the VSV virus bearing the wild type M with a non-neurotropic JSRVenv gene from the JSRV beta-retrovirus, or deleting the G gene and replacing it with a non-structural cell-cell FAST fusion protein from reovirus, I eliminated any neurotoxic side effects in our murine models.

Material and Methods

Viruses and Cells:

Vero cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA 20108 USA) and propagated in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% fetal calf serum (Cansera, Etobicoke, Ontario, Canada). Human primary astrocytes were obtained from ScienCell Research Laboratories (San Diego, CA, USA) and were propagated in prescribed Astrocyte medium. Crystal violet staining was performed on adherent cells after removing culture media, incubating with a 1% crystal violet stain in 100% methanol for 10 minutes, followed by 3 washes in ddH_2O.

Recombinant viruses bearing the wild type VSV M gene were utilized for all experiments in this study. Recombinant viruses were produced as previously described in chapters 1 and 2. The JSRVenv-VSV G FL chimera bears a truncated cytoplasmic tail JSRVenv fused to the transmembrane/cytoplasmic tail of VSV G which replaces the VSV G gene, in addition it contains a firefly luciferase (FL) gene between the inserted chimeric gene and L gene. The VSVΔG/FAST recombinant, although lacking the G gene which is replaced by an mRFP1 gene, is pseudotyped with the G protein yet can spread because of the FAST fusion gene inserted upstream of the L gene. The VSV/GFP and VSV/FL are used as positive controls. For production of virus used in animal experiments Vero cells were infected at an MOI of 0.01. Culture supernatants were collected 24 or 48

hours later and cleared by centrifugation and filtration through a 0.2 μm filter. Stocks were titered on Vero cells.

Mouse Experiments:

Female, 8 to 10 week-old Balb/c mice were obtained from Charles River Laboratories (Wilmington, MA), and B6 nude mice were obtained from Taconic (Hudson, NY). All experiments were conducted with the approval of the University of Ottawa Animal Care and Veterinary Services. Mice were anesthetized with 3% induction and 2% maintenance of isoflurane for intranasal (IN) instillation of 10u or 50ul of virus and allowed to recover under oxygen. For intracranial injections, mice were anesthetized with 20ul IP cocktail containing acepromazine(10 mg/ml – 0.5 ml) xylazine(20 mg/ml – 1.5 ml) and ketamine (100 mg/ml – 1.5 ml). A total volume of 5ul of PBS or virus was injected into the brain. Mice were monitored daily and euthanized at indicated time points or upon signs of morbidity by carbon dioxide narcosis. Viral content of homogenized tissues was determined by plaque assay on Vero cells. All titered tissues were done immediately after tissue harvest, and tissue weight was measured after homogenization.

In vitro IVIS imaging:

In vitro tissue culture 6 well plates were incubated with 10ul of d-luciferin (10mg/ml in PBS) (Molecular Imaging Products Company, Ann Arbor, MI) for Firefly luciferease imaging where applicable. Plates were imaged with the in vivo

imaging system 200 Series Imaging System (Xenogen Corporation, Hopkinton, MA). Data acquisition and analysis were performed using Living Image v2.5 software. For each experiment, images were captured under the most sensitive exposure settings to allow detection of a bioluminescent signal.

Results

The JSRVenv-VSV G chimera or VSVΔG/FAST does not infect mouse brain

To evaluate the susceptibility of mouse brain to infection with the chimeric recombinant, freshly harvested Balb/c mouse brain was collected and infected at various doses with 1e6 PFU of the JRVenv-VSV G chimera. Unlike the VSV/FL and chimera infected Vero controls, only VSV/FL infected mouse brains (Fig. 41). To confirm that the chimera did not infect in the in vivo setting, Balb/c mice were administered intranasal instillations of virus and observed for any signs of neurotoxicity. No sequeleae were observed for the duration of the study (Table 8). Furthermore, B6 nude mice were repeatedly administered VSVΔG/mRFP1/FAST intranasally and did not develop any overt neurological symptoms over a 36 day period (Table 8). To confirm the presence of VSVΔG/mRFP1/FAST, mice bearing lung tumors were sacrificed and visualized for RFP expression by microscopy to confirm replicating virus (Figure 27E-F).

Intracranial injection of JSRVenv-VSV G or VSVΔG/FAST is not toxic

To confirm the recombinants were being exposed to the brain tissues, direct intracranial injections were performed in Balb/c mice. All mice receiving VSV/GFP or VSV/FL suffered toxic neurological sequelae within 8 days (Fig. 42). Symptoms included: dehydration, hunched posture, isolation, blindness, and eventually all developed limb paralysis, whereas the mice receiving either

Figure 41. JSRVenv-VSV G chimera does not infect ex-vivo mouse brain.
To confirm that the chimeric recombinant did not infect mouse brain tissue, mouse brains were harvested and infected with 1e6 PFU of the recombinant or VSV/FL, both bearing the firefly luciferease gene. Tissues were kept in tissue culture media and observed under IVIS at 24h after infection. Control Vero cells were infected simultaneously with the exact same dose.

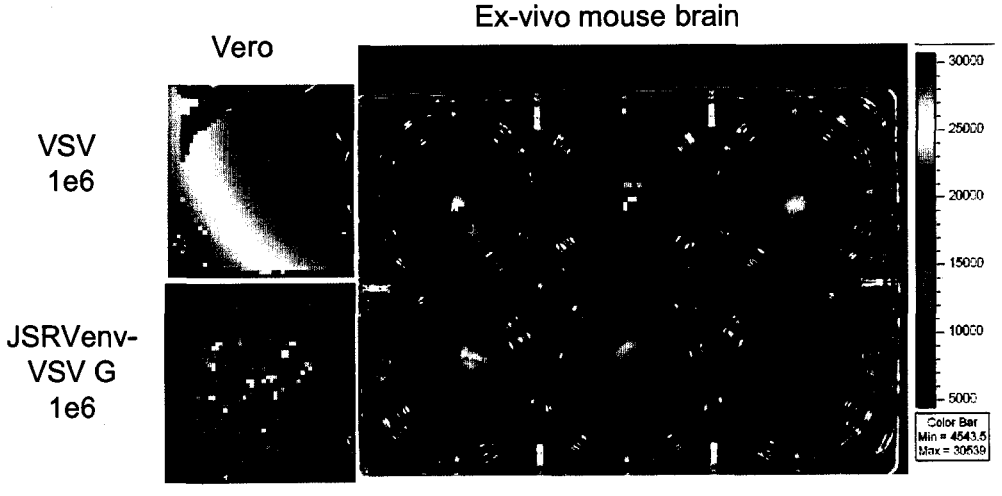

Ex-vivo mouse brain
Vero
VSV
1e6
JSRVenv-
VSV G
1e6
30000
25000
20000
15000
10000
5000
Color Bar
Min = 4543.5
Max = 30539

Table 8. Intranasal instillation of putative non-neurotoxic VSV recombinants.
To determine if the JSRVenv-VSV G chimera or the VSVΔG/mRFP1/FAST were toxic in vivo, mice were administered intranasal instillation of virus as indicated and observed for any sequelae. Balb/c mice receiving the JSRVenv-VSV G chimera were given 1 dose of virus, whereas VSVΔG/mRFP1/FAST was administered to B6 mice 6 times over a period of twelve days. None of the mice developed any neurotoxic symptoms for the entire course of the studies as indicated. To confirm the presence of VSVΔG/mRFP1/FAST, mice bearing lung tumors were sacrificed and visualized for mRFP1 expression by microscopy to confirm replicating virus (Figure 27E-F).

Recombinant	PFU	IN Volume	Duration of Study
JSRVenv-VSV G	1e5	10ul	17d
JSRVenv-VSV G	1e6	10ul	17d
JSRVenv-VSV G	1e7	10ul	17d
VSVΔG/FAST	3e4	50ul	36 d

JSRVenv-VSV G chimera, VSVΔG/mRFP1/FAST or PBS were all asymptomatic for the duration of the study (Fig. 42). Brains of mice that developed limb paralysis were titered and confirmed the presence of virus in the brain tissues: VSV/GFP 1.6e7 PFU/g and VSV/FL 5.7e7 PFU/g.

Primary human astrocytes survive a JSRVenv-VSV G chimera infection

To evaluate the susceptibility of human brain tissue to infection with the putative non-neurotropic chimera, primary human astrocytes were cultured at 1e5 cells/well and subsequently infected with VSV/FL or the chimeric recombinant. After 48h incubation with the viruses, one week old primary human astrocytes were stained with crystal violet and observed under the microscope for viability (Fig. 43A). In comparison to VSV/FL infected wells which were sparsely populated with healthy looking astrocytes, the JSRVenv-VSV G FL chimera were still confluent. Similar results were obtained with two week old primary human astrocytes (Fig. 43B). Vero cells infected with the same PFU of virus confirmed that an adequate amount of virus was administered to susceptible cells (Fig. 43C).

Figure 42. Intracranial injection of non-neurotoxic VSV recombinants.
Balb/c mice were injected intracranially with 5ul at indicated PFU and observed for any sequelae. Mice receiving VSV/GFP or VSV/FL developed symptoms indicative of neutrotoxicity (dehydration, hunched, isolation, blindness and eventual limb paralysis) within 8 days and were subsequently euthanized. Control mice and mice receiving either of the putative non-neurotoxic VSV recombinants were free of symptoms for the entire duration of the study. The number of mice per group and dose injected is documented on the figure. Log rank test describing the difference between the best and worst prognostic group is 34.24 and $p<0.0001$. Note on graph legend VSV=VSV FL.

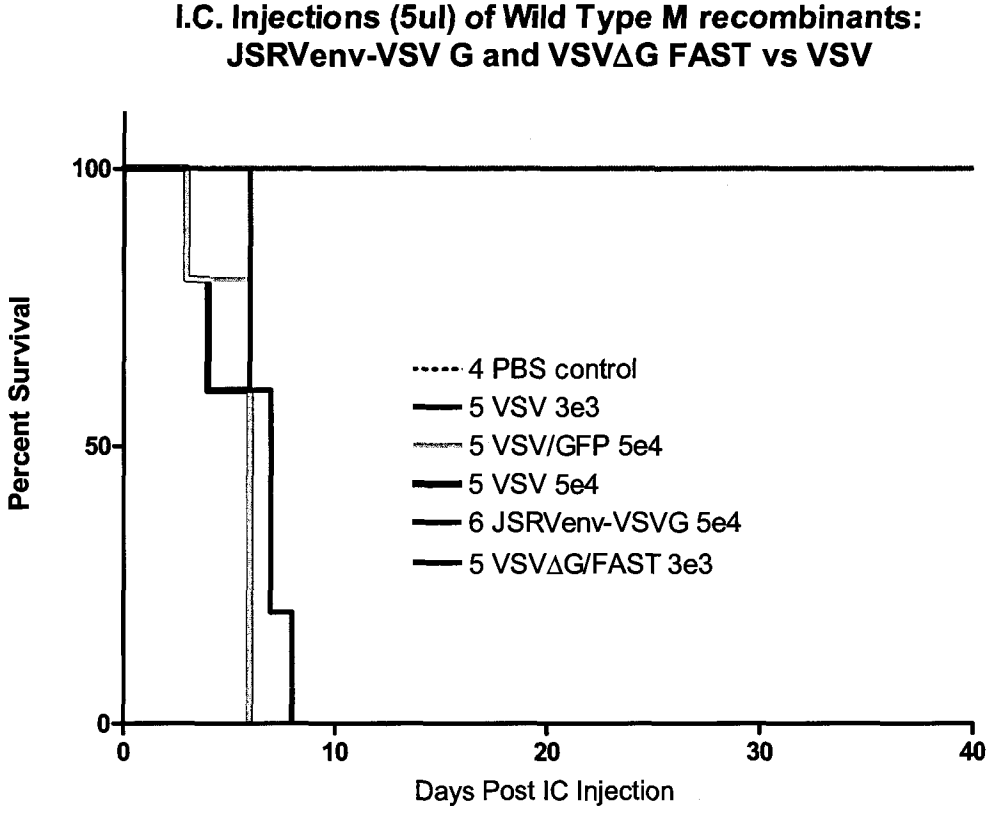
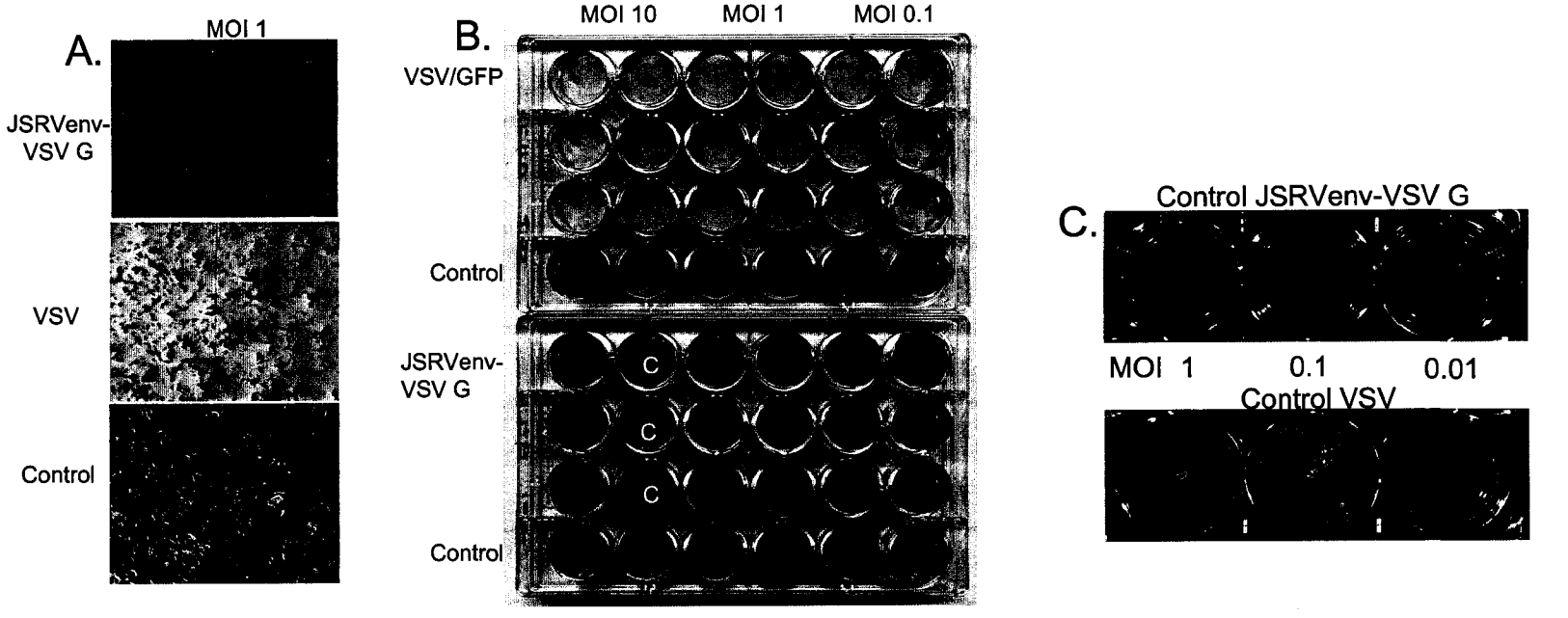

I.C. Injections (5ul) of Wild Type M recombinants:
JSRVenv-VSV G and VSVΔG FAST vs VSV
Percent Survival
100
50
0
0
10
20
30
40
Days Post IC Injection
4 PBS control
5 VSV 3e3
5 VSV/GFP 5e4
5 VSV 5e4
6 JSRVenv-VSVG 5e4
5 VSVΔG/FAST 3e3

Figure 43. JSRVenv-VSV G does not kill primary human astrocytes in vitro. Wells bearing (A) 1 week old or (B) 2 week old human primary astrocytes were infected with either the JSRVenv-VSV G FL chimera or VSV/FL at an MOI of 0.1, 1 or 10 and observed for cytopathic effects using crystal violet staining of wells. (C) Confirmation of virus infection was performed in parallel on Vero cells that were observed at 24 and 48h after infection using IVIS. Wells in (A) are magnified 10X. Wells in (B) marked with "C" are additional control wells. Note on graph legend VSV=VSV FL.


A.
MOI 1
JSRVenv-VSV G
VSV
Control
B.
MOI 10
MOI 1
MOI 0.1
VSV/GFP
Control
JSRVenv-VSV G
C
C
C
Control
C.
Control JSRVenv-VSV G
MOI 1
0.1
0.01
Control VSV

Discussion

In this study I generated 2 novel recombinant VSV viruses that did not cause any neurotoxicity in mice. Unlike previous approaches to attenuating the neurovirulence of VSV, my strategy was based on the hypothesis that the VSV G protein is responsible for the neurotropism. I replaced the G protein gene of the VSV virus with protein genes from 2 different virus species each with a distinct virulence mechanism. JSRVenv binds to the human Hyal2 receptor, not expressed in human adult brain, whereas p14 FAST is a non-structural fusion protein that induces syncytia formation.

JSRVenv-VSV G recombinant is not toxic to murine and human brain tissues

It is reassuring that the JSRVenv-VSV G chimeric recombinant did not infect mouse brain (Fig. 41), given that the murine homologue Hyal2 receptor does not bind to JSRVenv[417]. Furthermore, the chimeric recombinant did not induce CPE in 1 and 2 week old primary human astrocyte cultures (Fig. 43). These findings substantiate the evidence that VSV G is responsible for the neurotropism. When coupled with data that mice are asymptomatic after in vivo intranasal and intracranial administration (Table 8 & Figure 42), this study provides proof of principle that a recombinant VSV infection can be targeted away from brain tissue. It should be noted that human embryonic brain tissues do express Hyal2 but this fades as development progresses[219,220].

VSVΔG/FAST recombinant pseudotyped with VSV G is not neurotoxic

Although the VSVΔG/mRFP1/FAST recombinant does not express VSV G, it is pseudotyped with the VSV G. Furthermore, previous studies have shown that this recombinant is able to spread in tumor cells in vitro and in vivo due to the expression of the fusogenic FAST gene (Chapter 1). It is therefore intriguing that this recombinant did not cause any neurotoxicity when administered repeatedly intranasally or after a single intracranial dose, as the administered recombinant was suited with the G protein and was also able to spread (Table 8 & Fig. 42). Although experiments are required to evaluate if ex-vivo brain is infected by this recombinant, this study suggests that it is the expression of VSV G that is responsible for the neurotoxicity.

Limitations and extrapolations

A shortcoming of the in vivo studies in this work was confirming the presence of the intranasal or intracranial administered virus. In previous studies where either the JSRVenv-VSV G or the VSVΔG/FAST were administered systemically or intranasally in mice bearing tumors, the mice never developed neurological symptoms over a 3 or 4 week period respectively, despite evidence of viral replication in the tumors (Chapter 1 & 2). However, their brains were never evaluated for the presence of virus infection.

Due to the increased sensitivity of young mice to the neurotoxic effects of VSV[325,326], it could be argued that the age and type of mice used for the intranasal

model was not appropriate since many studies utilize 3-4 week old Swiss–Webster mice. However, gold standard intracranial injections were performed with appropriate controls, and coupled with the fact that I administered at least 300 fold more virus intracranially compared to other studies[65] this vindicates my findings. Neurotoxicities have also been reported in mice receiving less than 100 PFU of VSV intranasally[15] further supporting the the idea that 300 fold more intranasal doses given administered depite the lack of a positive control (Table 8).

As previously mentioned, the discrepancy between the degree of neurotoxicity between peripherally (IV) and centrally (IC) administered VSV[326] is thought to be due to a more robust peripheral antiviral interferon response[386]. When administered peripherally some attenuated viruses are cleared from the central nervous system, as the bulk of the anti-viral respsonse has been initiated[29,118], but are neurotoxic when given intracranially. It is possible that an immune compromised cancer patient may not mount a peripheral antiviral response, thereby validating the use of truly non-neurotropic VSV.

Summary

VSV is neurotoxic and the VSV G is responsible for neurotropism. In this study I developed two non-neurotoxic recombinant VSV viruses whereby the VSV G gene is replaced with a novel envelope protein gene. JSRVenv is a beta-retrovirus envelope protein whose receptor, Hyal2, is not expressed in mice or human brain. The p14 FAST fusion protein induces cell-cell fusion, and allows the spread of VSV lacking the G gene. Ex-vivo and in vivo murine models as well as human primary astrocytes do not demonstrate susceptibility to infection. Evaluating the susceptibility of primary human neurons, human ex-vivo samples, followed by intracranial injections into non-human primates will determine the fate of this endeavour. Shortcomings of these studies lie in the fact that there is no direct evidence that the intracranial virus is viable.

General Concluding Remarks

The increase in cancer rates has revived the need for an oncolytic virus platform. Despite the integrity of current clinical trials, the outcomes are reminiscent of those performed half a century ago, the significant difference being the level of understanding of the biology. These clinical trials are pivotal in determining the focus of future research and have identified poor tumor oncolysis, antiviral antibodies and safety as barriers to success for oncolytic virotherapy.

Using the VSV oncolytic virus platform, this thesis demonstrates that modulating viral proteins is a strategy whereby each of the aforementioned barriers can be addressed. The novel VSV/FAST recombinant, encoding the reovirus p14 FAST fusogenic protein, has demonstrated increased tumor spread in animal models, and confirms that FAST is not only a virulence factor but an oncolytic agent. Envelope protein shuffling between VSV and vesiculoviruses or a beta-retrovirus can also be an effective strategy for host immune evasion, and finally VSV neurotoxicity is vanquished when the VSV G gene is replaced with non-rhabdovirus envelope genes.

As demonstrated, barriers to oncolytic virotherapy are being identified and overcome, with advancements beginning at the level of the basic sciences.

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Contributions of Collaborators

The following are recognized for their contribution as collaborators to this work. Brian Lichty and Kyle Stephenson are collaboraters on the work with the p14 FAST, specifically they provided the original recombinant p14 FAST genome constructs and performed the Wester blot to detect the presence of FAST as well as the in vivo serial brain sections and IHC found in figure 7b and 12. With regard to the immune evasion work, Anthony T. Power generated the N-Gly VSV G mutant and performed the Western blot demonstrated in figure 36. Dan McManus provided the pCI-Neo driven Maraba N, P and L plasmids which facilitated virus rescue.

Appendix I Oncolytic virus review article

Christopher W. Brown and John C. Bell. Oncolytic Viruses: A New Weapon to Fight Cancer. The Journal of Medical Imaging and Radiation Sciences. 39 (3):115-127, September 2008.

http://www.jmirs.org/article/S1939-8654(08)00105-7/abstract

Appendix II FAST as a neurovirulence factor

Christopher W. Brown, Kyle Stephenson, Stephen Hanson, Michael Kucharcyk, Roy Duncan, John C. Bell, Brian D. Litchty. The p14FAST protein of resptilian reovirus increases vesicular stomatitis virus neuropathogenesis. Journal of Virology. Submitted Sept. 2008.

Appendix III Novel Ewing sarcoma cell line

Georges Maire, Christopher W. Brown, Jane Bayani, Carlos Pereira, John C Bell, Jeremy A Squire, Maria Zielenska. Molecular characterization of a new Ewing sarcoma cell line: EWS-ERG fusion gene hidden within a complex three chromosomes rearrangement, associated with RB1 loss and polyploidyzation. Cancer Genetics and Cytogenetics, 181, 81-92, 2008.

http://www.cancergeneticsjournal.org/article/S0165-4608(07)00744-3/abstract

Appendix IV Ex-vivo samples with JX-963

Steve H. Thorne, Tae-Ho H. Hwang, William E. O'Gorman, David L. Bartlett, Shizuko Sei, Femina Kanji, Christopher Brown, Joel Werier, Jin-Han Cho, Dong-Ewon Lee, Yaohe Wang, John Bell, and David H. Kirn, Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. Journal of Clinical Investigation, 117 (11):3350-3358, 2007.

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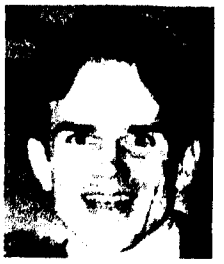
Appendix V Rescue of Let-7 recombinant VSV

Rob E. Edge, Therasa J. Falls, Christopher W. Brown, Brian D. Lichty, Harold Atkins, John C. Bell. A let-7 MicroRNA-sensitive Vesicular Stomatitis Virus Demonstrates Tumor-specific Replication. Molecular Therapy (2008) 16 8, 1437–1443.

http://www.nature.com/mt/journal/v16/n8/abs/mt2008130a.html

Curriculum Vitae

Christopher (Cricho) Waclaw Brown



Education:

Orthopaedic Surgical Residency, 2002-Present
Ottawa Hospital & University of Ottawa, Ottawa, Ontario

Completion of Clinical Investigator Program
Royal College of Physician and Surgeons of Canada
2005-2008

Ph.D., Oncolytic Viruses 2005-2008
Department of Microbiology & Immunology
University of Ottawa, Ottawa, Ontario

M.D., Faculty of Medicine, 2002
University of Ottawa, Ottawa, Ontario

M.Sc., Anatomy and Neurobiology, 1998
University of Ottawa, Ottawa, Ontario

B.Sc., Biochemistry & Minor in Mathematics, 1996
Magna Cum Laude
St. Bonaventure University, St. Bonaventure, NY

Awards:

W.E. Collins Surgical Day Research Award for Surgical Resident Research
University of Ottawa, Department of Surgery, May 2006 & 2007.

H.K. Uhthoff 2nd Place Research Award for Orthopaedic Surgery Resident Research, University of Ottawa, Department of Surgery, Division of Orthopaedics, April 2006 & 2007.

Ottawa Health Research Institute Research Day
Postdoctoral Research Poster Award, September 2005.

The Canadian Society for Clinical Investigation (CSCI) Award
Young Investigators Forum, September 2005.

Physicians Services Incorporated (PSI) Resident Research Prize
University of Ottawa, Faculty of Medicine, July 2005.

W.E. Collins Surgical Day 1st Place Research Award for Surgical Resident Research, University of Ottawa, Department of Surgery, May 2005.

H.K. Uhthoff 1st Place Research Award for Orthopaedic Surgery Resident Research, University of Ottawa, Department of Surgery, Division of Orthopaedics, April 2005.

Medical Student Award for Excellence in Research,
University of Ottawa, December 1999.

Recipient of the Biology Award,
St. Bonaventure University May 1996.

John B. Skehan Award for Academic & Athletic Excellence
St. Bonaventure University May 1996.

Research Grants & Scholarships:

NCIC Clinical Research Fellowship
Terry Fox Foundation
Three Years of Salary Support Totaling 140,000.00 $
July 2006 - 2009

Admission/Tuition Scholarship for Doctoral Program
Department of Microbiology & Immunology
University of Ottawa, February 2005.

Full athletic scholarship
St. Bonaventure University,
St. Bonaventure New York, 1992-1996

Patents:

Oncolytic Rhabdoviruses - Provisional Patent Filed with the US Patent Office August 2007.

Peer Reviewer:

Arthritis & Rheumatism, John Wiley & Sons 2005

Research Experience & Research Projects:

Doctoral Thesis – Supervisor: Dr. John Bell, Senior Scientist, Ottawa Regional Cancer Center, Ottawa, Ontario.
Thesis: Enhancement of Vesiculovirus Oncolysis by Expression of Exotic Envelope Proteins. Feb 2005 - June 2008.

Research Assistant – Supervisor: Dr. John Bell, Senior Scientist, Ottawa Regional Cancer Center, Ottawa, Ontario. Project: Vesicular Stomatitis Virus as a potential oncolytic virus in human sarcomas. Sept 2004 - Feb 2005.

Biomechanical Research – Supervisor: Dr. Peter Thurston, Orthopaedic Surgeon Department of Orthopedics, Ottawa General Hospital, Ottawa, Ontario. Project: The impact of the sequential mixing technique on working time and porosity in comparison to the manufacturers recommended technique for Simplex Type polymethylmethacrylate - A pilot study. May 2003 –April 2005.

Clinical Research – Supervisor: Dr. James Jarvis, Orthopaedic Surgeon Department of Orthopedics, Children's Hospital of Eastern Ontario, Ottawa, Ontario. Project: The treatment regimen and outcome of children with vertebral Langerhans cell histiocytosis at the Children's Hospital of Eastern Ontario. May 2004 – June 2004.

Clinical Research - Supervisor: Dr. Geoff Dervin, Orthopaedic Surgeon Department of Orthopedics, Ottawa General Hospital, Ottawa, Ontario. Project: Comparing the outcome of pinning displaced subcapital femoral head fractures versus hemiarthroplasty at the Ottawa Hospital. May 2001- 2005.

Research Assistant - Supervisor: Dr. John J. Wysolmerski, Associate Professor of Medicine, Section of Endocrinology / metabolic bone disorders, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT, USA. Project: Studying the role of PTHrP in the metastasis of breast cancer to bone as well as its role in mammary gland development. June to Sept. 1999 & 2000.

Masters Thesis - Supervisor: Dr. Marie-Andrée Akimenko, Associate Professor, Department of Medicine, Loeb Medical Research Institute & University of Ottawa, Ontario. Thesis: Migration and in vivo transfection of cells in the regenerating caudal fin of Zebrafish. Sept. 1996 to Aug. 1998

Project Student - Supervisor: Dr. John Kupinski, Department of Biology, St. Bonaventure University, NY, USA. Thesis: Investigating the mechanism of T-DNA transfer and insertion from the prokaryote *Agrobacterium tumefaciens* into the plant cells of *Arabidopsis thaliana*. Sept. to June 1996.

Research Assistant – Supervisor: Dr. Clint Chapple, Department of Biochemistry, Purdue University, IN, USA. Project: Isolation and characterization of *Arabidopsis thaliana* mutants to better understand the role of plant cell wall-biosynthetic enzymes. May - Sept., 1995.

Publications and Presentations:

(1) Full Papers:

Christopher W. Brown, Hesham Abdelbary, Rebecca Auer, Joel Werier, David Kirn, Harry Atkins and John C. Bell. From the Bedside to the Bench and Back – Cancer Therapy À La Carte. Submitted September 2008.

Christopher W. Brown, Kyle Stephenson, Stephen Hanson, Michael Kucharcyk, Roy Duncan, John C. Bell, Brian D. Litchty. The p14FAST protein of resptilian reovirus increases vesicular stomatitis virus neuropathogenesis. Journal of Virology. Submitted Sept. 2008.

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Rob E. Edge, Therasa J. Falls, Christopher W. Brown, Brian D. Lichty, Harold Atkins, John C. Bell. A let-7 MicroRNA-sensitive Vesicular Stomatitis Virus Demonstrates Tumor-specific Replication. Molecular Therapy (2008) 16 8, 1437–1443.

Georges Maire, Christopher W. Brown, Jane Bayani, Carlos Pereira, John C Bell, Jeremy A Squire, Maria Zielenska. Molecular characterization of a new Ewing sarcoma cell line: EWS-ERG fusion gene hidden within a complex three chromosomes rearrangement, associated with RB1 loss and polyploidyzation. Cancer Genetics and Cytogenetics, 181, 81-92, 2008.

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Christopher W. Brown, M.D., M.Sc., James G. Jarvis, M.D., FRCSC and R. Mervyn Letts, M.D., FRCSC, Blair Carpenter, M.D. The treatment regimen and outcome of children with vertebral

Langerhans cell histiocytosis at the Children's Hospital of Eastern Ontario. *Canadian Journal of Surgery*, 48(3):230-6, 2005.

Dunbar M. E., Dann P, Brown C. W., Dreyer B, Philbrick W. P., Wysolmerski J. J. Temporally-regulated overexpression of PTHrP in the mammary gland reveals distinct fetal and pubertal phenotypes. *J Endocrinol* 171, 403-16, 2001.

Poleo, G., Brown, C. W., Laforest, L., and Akimenko, M.-A. Cell proliferation and movement during early fin regeneration in zebrafish. *Developmental Dynamics* 221, 380-390, 2001.

Laforest, L., Brown, C. W., Poleo, G., Géraudie, J., Tada, M., Ekker, M., Akimenko, M.-A. Involvement of the sonic hedgehog patched1 and BMP2 genes in patterning of the zebrafish dermal rays. *Development* 125, 4175-4184, 1998.

(2) Symposium Presentations:

Christopher W. Brown, Joel Werier, John Bell. A novel targeted oncolytic virus approach for diffuse metastatic disease. Combined COA/AOA, Quebec city, June 2008.

Hesham Abdelbary, Christopher W. Brown, Joel Werier, Jay Wunder, John Bell. Treating resistant Ewing Sarcoma using virotherapy – From the bedside to the bench and back. Combined COA/AOA, Quebec city, June 2008.

Hesham Abdelbary, Christopher W. Brown, Joel Werier, Jay Wunder, John Bell. Oncolytic viral therapy: A novel biotherapeutic approach in treating sarcomas. Connective Tissue Oncology Society meeting Seattle November 2007.

Christopher W. Brown, M.D., M.Sc. and John Bell, PhD. Switching Rhabdovirus G Proteins – A strategy for Immune Evasion and Tissue Tropism. 4rth International Conference on Oncolytic Viruses as Cancer Therapeutics, Scottsdale Arizona, March 14-17, 2007, HKU research day 2007, Collins Day 2007, Connective Tissue Oncology Society meeting Seattle November 2007, Cold Spring Harbor Laboratory – In vivo barriers to gene therapy meeting November 2007.

Georges Maire, Jane Bayani, Carlos Pereira, Christopher W. Brown, John C Bell, Jeremy A Squire, Maria Zielenska. Molecular characterization of a new Ewing sarcoma cell line: EWS-ERG fusion gene hidden within a complex three chromosomes rearrangement, associated with RB1 loss and polyploidyzation. The 11th Annual DPLM Symposium and 5th Laurence Becker Symposium "Advances in Pediatric Pathology", Toronto, June 14th, 2007.

The rhabdovirus G protein is critical in establishing tumor tropism. Location: HKU research day 2006, Collins Day 2006.

Christopher W. Brown, M.D., M.Sc., Jay Wunder M.D., FRCSC, Peter Ferguson M.D., FRCSC, Robert Bell M.D., FRCSC, John Bell, PhD. and Joel Werier, M.D., FRCSC. Vesicular Stomatitis Virus (VSV) demonstrates 'in vitro' Oncolytic Activity in High Grade Bone and Soft Tissue Sarcoma. Musculoskeletal Tumor Society Meeting, Tennessee, USA, May 2005; Canadian Society for Clinical Investigators / Royal College of Physicians and Surgeons of Canada Annual Meeting, Vancouver, BC, September 2005; 5th Annual Ottawa Health Research Institute Research Day, October, 2005; Connective Tissue Oncology Society Meeting, Florida, USA, November 2005.

The role of Vesicular Stomatitis Virus as an Oncolytic agent in the Treatment of Human Sarcoma. Sept.2004 until present. Location: HKU research day 2005 1rst Prize, Collins Day May 2005.

Christopher W. Brown, M.D., M.Sc., Ben Deheshi, M.D., Anna Conway, M.A. and Geoff Dervin, M.D., M.Sc. Outcome of Internal Fixation of Un-Displaced Femoral Neck Fractures in the Elderly at A Tertiary Teaching Hospital in Ontario. Canadian Orthopaedic Association annual meeting, Montreal, June 2005.

Standard versus the Sequential Mixing Methods: Effect on Working Time and Porosity. May 2003 – April 2005.
Location: HKU research day April 2005.

The treatment regimen and outcome of children with vertebral langerhans cell histiocytosis at CHEO.
Location: Department of Surgery Annual Research Symposium – Children's Hospital of Eastern Ontario, June 2004, HKU research day April 2004, Collins Day May 2004.

Anatomy, Physiology and the Role of Physiotherapy in the Management of Diseases of the Spine
Location: The Dr. HIJ Kellam Annual Nursing Seminar, The Ottawa Hospital, Civic Campus. November 1st 2004.

Outcome of Internal Fixation of Un-Displaced Femoral Neck Fractures in the Elderly at A Tertiary Teaching Hospital in Ontario. Location: COA June 2005, HKU research day April 2003, Collins day May 2003.

Laforest, L., Brown, C. W., Poleo, G., and Akimenko, M.A. Dual Effects of Retinoic Acid on *Sonic Hedgehog* signaling pathway in zebrafish. 6th International Limb Development and Regeneration Conference, Sun Valley, Idaho. May 17-21,1998.

Laforest, L., Brown, C. W., Poleo, G., and Akimenko, M.A. La cascade de signalisation de *Sonic Hedgehog* au cours du développement et de la régénération des nageoires du danio. Presented at the 66th Congrès de l'ACFAS. Université de Laval, Québec. May 17-21, 1998.

Laforest, L., Brown, C. W., Poleo, G., and Akimenko, M.A. Dual Effects of Retinoic Acid on *Sonic Hedgehog* signaling pathway in zebrafish. Presented at the Third Cold Spring Harbor Conference on Zebrafish Development and Genetics, Cold Spring Harbor Laboratory, New York, April 29- May 3, 1998.

Laforest, L., Brown, C. W., Poleo, G., Ekker, M., Akimenko, M.-A. (1997). "Development and regeneration of the fin dermal skeleton in zebrafish: role of the *shh* signaling pathway.", International Symposium - Mechanisms of Metamorphosis and Regeneration: Keys to Tissue Restoration, IUPUI, Indianapolis, Indiana, August 1997.

Akimenko, M.-A., Laforest, L., Brown, C. W., Poleo, G., Beyers, M., Ekker, M. (1997). "*Hedghog* signaling pathway in regenerating fins of zebrafish.", Canadian Society of Zoologists 36th Annual Meeting, University of Western Ontario, London, Ontario, May 1997.

Laforest, L., Brown, C. W., Poleo, G., Ekker, M., Akimenko, M.-A. (1997). "*Hedgehog / Patched* signaling pathway in regenerating fins of zebrafish.", 17th Great Lakes Mammalian Development Meeting, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, April 1997.

Akimenko, M.-A., Laforest, L., Beyers, M., Brown, C. W., Tada, M., Ekker, M. (1997). "Genetic control of fin development and regeneration in zebrafish.", Taniguchi Symposium on

Developmental Biology: Pattern formation in developing/regenerating body axis and appendages, Mandarin Oriental Hong Kong, Hong Kong, January 1997.

Career & Personal Development :

American Orthopaedic Association - OREF-Zimmer Resident Leadership Forum, Quebec City, June 3-5, 2008.

American Academy of Orthopaedic Surgeons - Clinician Scientist Development Program, Quebec City, June 1-3, 2008.

Teaching & Volunteer Experience:

Teaching:

Mentor and supervisor to summer students and new graduate students doing oncolytic virus research: Andrew Stern, Ramya Krishnan, Rhea Ferguson, Al Walid Hamam, Kirsty Malone 2005-2008.

Guest Lecturer – Care of the Spine: physiotherapy, surgery & rehabilitation. The Dr. HIJ Kellam Annual Nursing Seminar, November 1, 2004.

PBL Tutor for Musculoskeletal block – University of Ottawa Medical School, 2003, 2004 & 2005.

Teaching Assistant – Supervisor: Dr. J.-M. Renaud, Department of Cellular and Molecular Medicine, University of Ottawa. Anatomie et Physiologie. Sept. 1997 - April 1998.

Guest Lecturer – Supervisor: Dr. M. Ekker, Department of Cellular and Molecular Medicine, University of Ottawa. Current Topics in Cell and Developmental Biology. April 1998.

Volunteer Work:

Member of Postgrad committee for Orthopaedic Residents, University of Ottawa, 2004-2005
Junior Chief Orthopaedic Resident 2004-2005
Local representative at the Canadian Orthopaedic Residence Association 2004
Smoking Cessation for elementary school children, 1999 – 2000
Volunteer Queensway Carleton Hospital, Ottawa Summer of 1995
Healthy Sexuality Program for High School Students, 1994 – 2000
Queensway Carleton Hospital Volunteer, Summer of 1997
Fundraising participant for the Children's Hospital of Eastern Ontario, 1985-1992

Other Work: Process Engineer - Spero Manufacturing Incorporated. Implemented a quality assurance procedure, updated the process control documents so that chemical conversion process would surpass standards, and ensured that the chemical waste levels were in accordance with municipal code. June – Sept. 1996.

Extracurricular:

Swimming: Competitive swimmer from 1983 – 1996
National record holder in 1987 & Provincial record holder in 1990
Olympic Trial participant in 1992
Full athletic scholarship to St. Bonaventure University, 1992-1996
Varsity record holder in 3 events, 1992-1996

Outdoors: Canoeing, boating, camping, gardening

Other: Weight training, home renovations, vintner, cooking

Personal Information:

I am originally from Ottawa, the oldest of three brothers. My wife, three sons and I currently live outside the Ottawa area along the Rideau River, in the farming community of Kemptville.

I have spent 13 years as a competitive swimmer, which allowed me to travel across Canada and the United States for competitions, as well as to obtain a university scholarship. My wife, an elementary school teacher is originally from Connecticut and also swam competitively; we met at university in 1992 and married in the summer of 2000.

Description of my current, 5 and 10-year career goals:

Currently I have completed a PhD in field of oncolytic viruses and have returned to complete my surgical residency. To keep my research interests in line with my surgical skills, I have decided to pursue a fellowship, and I have accepted a 1 year Musculoskeletal Surgical Oncology Fellowship at the University of Toronto, to begin in the summer of 2010. I will then pursue a year of additional spine Fellowship training.

Following my Fellowships, I would like an academic appointment that will allow me to dedicate more than 50 percent of my time towards clinical trials and basic science research.

My professional ambitions are to seek an academic appointment as a Surgeon Scientist with sub specialization in spinal musculoskeletal oncology and to lead translational research in the field of oncolytic viruses as a cancer therapeutic.