Role of MicroRNAs in Pathogenesis of Venezuelan Equine Encephalitis Virus and Development of Antiviral Strategies.

THESIS

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CERTIFICATE

This is to certify that the thesis entitled **"Role of MicroRNAs in Pathogenesis of Venezuelan Equine Encephalitis Virus and Development of Antiviral Strategies."** and submitted by Manish Bhomia ID No 2005PH29108P for award of Ph.D degree of the Institute embodies original work done by him under my supervision.

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Abstract

Venezuelan equine encephalitis virus is a member of the *alphavirus* family and genus *togaviridae.* These viruses are mosquito-borne viruses and can cause fatal human diseases. VEEV can be easily spread through aerosol and has been weaponized making it a potential biothreat agent. MicroRNAs (miRNA) are a class of small (19-28nt) endogenous RNA molecules which have been proven to be key regulators of gene expression. MiRNAs modulate gene expression either by transcriptional blocking or by translational repression and have been shown to play an important role in early development and differentiation.

Role of microRNAs in the pathology of alphaviruses and more specifically VEEV has not been studied. In this research work we have studied the role of microRNAs in both *in vitro* and *in vivo* system. We identified miRNAs which can play crucial role in regulating the inflammation, viral entry and virus replication from our *in vitro* and *in vivo* results. MiRNAs modulation specific for cells of blood brain barrier indicated a role of these cells in viral entry into brain. Detailed studies revealed that VEEV infects endothelial cells of blood vessels and may present an additional viral entry route in the brain. Based on the leads from *in vitro* miRNA expression data, we further studied the role of phosphatidylinositol 3-Kinase-AKT pathway in VEEV infection. It was concluded that this pathway is very critical in the initial phase of the virus replication and blocking the pathway results in impaired virus replication.

Moreover, currently there are no FDA approved antivirals or vaccines are available for therapeutic and prophylaxis respectively. We have used various antiviral approaches including known antiviral compounds siRNA and artificial miRNAs and evaluated their therapeutic potential in response to VEEV infection. Data demonstrated that siRNA and miRNA effectively inhibited VEEV replication *in vitro*. The protection in case of artificial miRNAs appears to be

better in comparison to siRNA mediated transcriptional inhibition. Our studies with FDA approved antiviral and anti-inflammatory compounds suggests that anti-viral and anti-inflammatory drugs alone does not offer any protection against VEEV but have a marginal protection effect when administered together in mouse model. However, a marginal increase in virus replication was observed upon treatment with anti-inflammatory drug alone suggesting a dual role of inflammation in VEEV infection.

Finally, in a pilot study we have engineered a novel live attenuated VEEV vaccine candidate using miRNAs. Complementary sequences of host miRNAs were cloned in the viral genome which then inhibits the replication of virus in the host cells expressing those miRNAs. Initial data suggests a miRNA based attenuation of VEEV which can be further tested for a potential vaccine.

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List of Abbreviations

1. Ago: Argonaute protein.
2. BBB: Blood brain barrier.
3. BMEC: Brain microvascular endothelial cells.
4. BSA: Bovine serum albumin.
5. CNS: Central Nervous System.
6. CP: Capsid
7. CPE: Cytopathic effect
8. CSE: Conserved sequence element.
9. CTP: Cytidine triphosphate.
10. DCs: Dendritic cells.
11. DMEM: Dulbecco's modified Earle's medium
12. EEE: Eastern Equine encephalitis.
13. ICAM-1: Intercellular adhesion molecule I.
14. IFN: Interferon.
15. MEM: Minimal essential medium.
16. MiRNA: MicroRNA.
17. MMP: Matrix metalloproteases.

18. MREs: miRNAs recognition elements.

19. MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

20. NK cells: Natural killer cells.

21. NSAIDs: Non-steroidal anti-inflammatory drugs

22. Nsp: Non Structural Proteins.

23. PBS: Phosphate buffered saline.

24. PEG: Poly ethylene glycol.

25. PFU: plaque forming units.

26. RdRP: RNA dependent RNA polymerase.

27. RISC: RNA inducing silencing complex

28. RISC: RNA inducing Silencing complex

29. SiRNAs: short interfering RNAs.

30. TLRs: Toll like receptor.

31. UTR: Untranslated Regions.

32. VEEV: Venezuelan equine encephalitis virus.

33. VLPs: Virus like particles.

34. WEE: Western Equine encephalitis.

Introduction

History

Venezuelan equine encephalitis virus (VEEV) is an arthropod borne virus which belongs to the family *Togaviridae* and genus *alphavirus*. *Alphavirus* genus comprises of two geographically different group of virus known as New World alphavirus and Old World alphavirus. The Old World alphaviruses include chikungunya, O'nyong-nyong, and Ross River viruses, which causes flu like symptoms and polyarthritis. The new-world alphaviruses, including Venezuelan equine encephalitis, eastern equine encephalitis (EEE), and western equine encephalitis (WEE) viruses, cause disease in both equines and humans which causes severe flu like symptoms which may also result in neurological complications (Calisher et al 1994, Weaver et al 2004, Steele et al 2010).

Of the New World alphaviruses, VEEV is one of the most important human and equine pathogen. VEEV often causes massive outbreaks in equines and spillover epidemics in humans due to their close proximity with equines. Although EEEV and WEEV are more lethal than VEEV, they typically are limited to individual cases in both humans and equines (Calisher et al 1994). VEEV has caused periodic outbreaks of febrile and neurological disease in both humans and equines, primarily in Latin America, during the past century. Since equines are very important in Latin America for agricultural and transportation purposes, outbreaks of diseases infecting equines have a large social and economic impact. The first outbreak of VEEV was reported in 1935 in central river valleys of Colombia. The virus was first isolated in 1938 from the brain of fatal equine cases in Yaracuy state in Venezuela (Beck and Wykoff, 1938). Since then, periodic outbreaks of VEEV have occurred from 1938 to 1973 in various parts of South America. One of the major outbreaks of VEEV on record occurred during the 1960s in central

Colombia, where over 200,000 human cases and more than 100,000 equine deaths of VEEV infection were reported. The last major outbreak of VEEV occurred in 1995 in which almost 75,000 to 100,000 humans were infected with the VEEV (Weaver et al 2004). The last reported case of VEEV occurred recently in Peru where two human deaths were reported due to VEEV infection along with several other unreported cases (Wilcarromero et al 2010).

Taxonomy

VEEV is one of the members of group A arboviruses. VEEV antigenic complex is categorized among six different subtypes based on hemagglutination inhibition and neutralization test which differ in terms of epidemiology and pathogenecity for both humans and equines (Weaver 2005). VEEV subtypes are further classified as epizootic and enzootic strains. Enzootic strains of VEEV, designated as the subtypes ID, IE, IF, II, III, IV, V, and VI, cycle between mosquitoes, especially Culex sp. and small mammal host mainly rodents. These enzootic strains do not replicate to high titers in equine and are generally avirulent for equines. However, horses are highly susceptible to the epizootic strain subtypes IAB and IC, which are believed to have evolved predominantly from genetic mutations of enzootic strains. Horses are not only susceptible to epizootic strains but these strains amplify to high titers causing epizootics. Humans are susceptible to both epizootic and enzootic strains of VEEV. During epizootics, accidental spillover of virus occurs which gets transmitted to humans by mosquitoes which have previously fed on infected equines resulting in epidemics (Weaver et al 1996). Rare human cases caused by enzootic strains of VEEV are also documented (Johnson et al 1968). These different subtypes along with the vector responsible for transmission are listed in the Table 1.

Table 1: Antigenic complexes of Venezuelan equine encephalitis virus.

Species	Serotype	Transmission pattern	Equine virulence	Location	Vector
Subtype I					
VEE virus	AB	Epizootic	Yes	Central, South and North America	*Ochlerotatus, Psorophora spp.*
VEE virus	C	Epizootic	Yes	South America	*Ochlerotatus, Psorophora spp.*
VEE virus	D	Enzootic	No	Central and South America	*Culex (Mel.), Alkenii s.I. (Ocossa, Panocossa), Vomerifer, Pedroi, Adamesi*
VEE virus	E	Enzootic	Variable	Central America and Mexico	*Culex (mel.)taeniopus*
Mosso das Pedras virus	F	Enzootic	Unknown	Brazil	Unknown
Subtype II					
Everglades virus		Enzootic	No	Southern Florida	*Culex (Mel.) cedecei*
Subtype III					
Mucambo virus	A	Enzootic	No	South America	*Culex (Mel.) portesi*
Tonate virus	B	Enzootic	No	South and North America	*Unknown, Oeciacus vicarius*
Mucambo virus	C	Enzootic	Unknown	Western Peru	Unknown
Mucambo virus	D	Enzootic	Unknown	Western Peru	Unknown
Subtype IV					
Pixuna virus		Enzootic	Unknown	Brazil	Unknown
Subtype V					
Cabassou virus		Enzootic	Unknown	French Guyana	Unknown
Subtype VI					
Rio Negro virus		Enzootic	Unnown	Northern Argentina	*Culex (Mel.) delpontei*

Information extracted from Weaver et al 2004

Virus structure

VEEV is an enveloped alphavirus with a non-segmented positive strand RNA genome of 11.4kb (Passeler and Weaver 2009). The genome is enclosed in capsid which is spherical in shape with 70nm diameter and have T=4 icosahedral symmetry (Paredes et al 2001). Towards the 5'end of the genome are 4 non-structural proteins (nsp1-4) which are central in the genomic replication and protein processing. The 3'end encodes for four structural proteins: the capsid, E1 E2 and E3 glycoprotein (Figure 1). Crystal structure of VEEV has been resolved recently to a resolution of 4.4 A° (Zhang et al 2011). Structural studies have revealed that E2 glycoprotein forms spikes on the viral surface (Figure 1) whereas E1 protein lies adjacent to the host cell–derived lipid envelope (Pletnev et al 2001)

Clinical symptoms of the disease

An epizootic strain of VEEV causes wide range of clinical symptoms ranging from unapparent disease to acute encephalitis in both humans and equines (Johnson and Martin 1974). Mortality rates in equines during epizootics have been estimated at 19%–83%, (Walton et al 1973). In equines, the clinical symptoms of the disease usually appear about 2-5 days after infection with the epizootic VEEV. The disease symptoms includes, fever, tachycardia, depression, and anorexia. Some animal develop encephalitis after 5-10 days of infection with signs of circling, ataxia and hyper excitability which is followed by death of the animal (Johnson and Martin 1974).

In case of human, fatalities occur less frequently, with neurological disease appearing in only about 4%–14% of cases. In humans, children are more susceptible to develop fatal encephalitis than adults. In case of pregnant women, VEEV infection may cause severe birth defects or stillbirths. The incubation period is approximately 2-5days.The onset of disease is

characterized by variety of clinical symptoms which includes malaise, fever, chills, and severe retro-orbital or occipital headache. Myalgia typically centers in the thighs and lumbar region of the back. Other symptoms usually include leucopenia, tachycardia, and fever and are frequently accompanied by nausea, vomiting, and diarrhea. Central nervous system (CNS) involvement is less common in humans which otherwise is characterized by convulsions, somnolence, confusion, and photophobia. The illness is occasionally biphasic with the recurrence of the disease after 4-8 days of the onset of the initial infection. Human encephalitis is less common (< 1% of the cases) since the virus infection is contained by the immune system at the lymphoid phase of infection (de la Monte et al 1985).

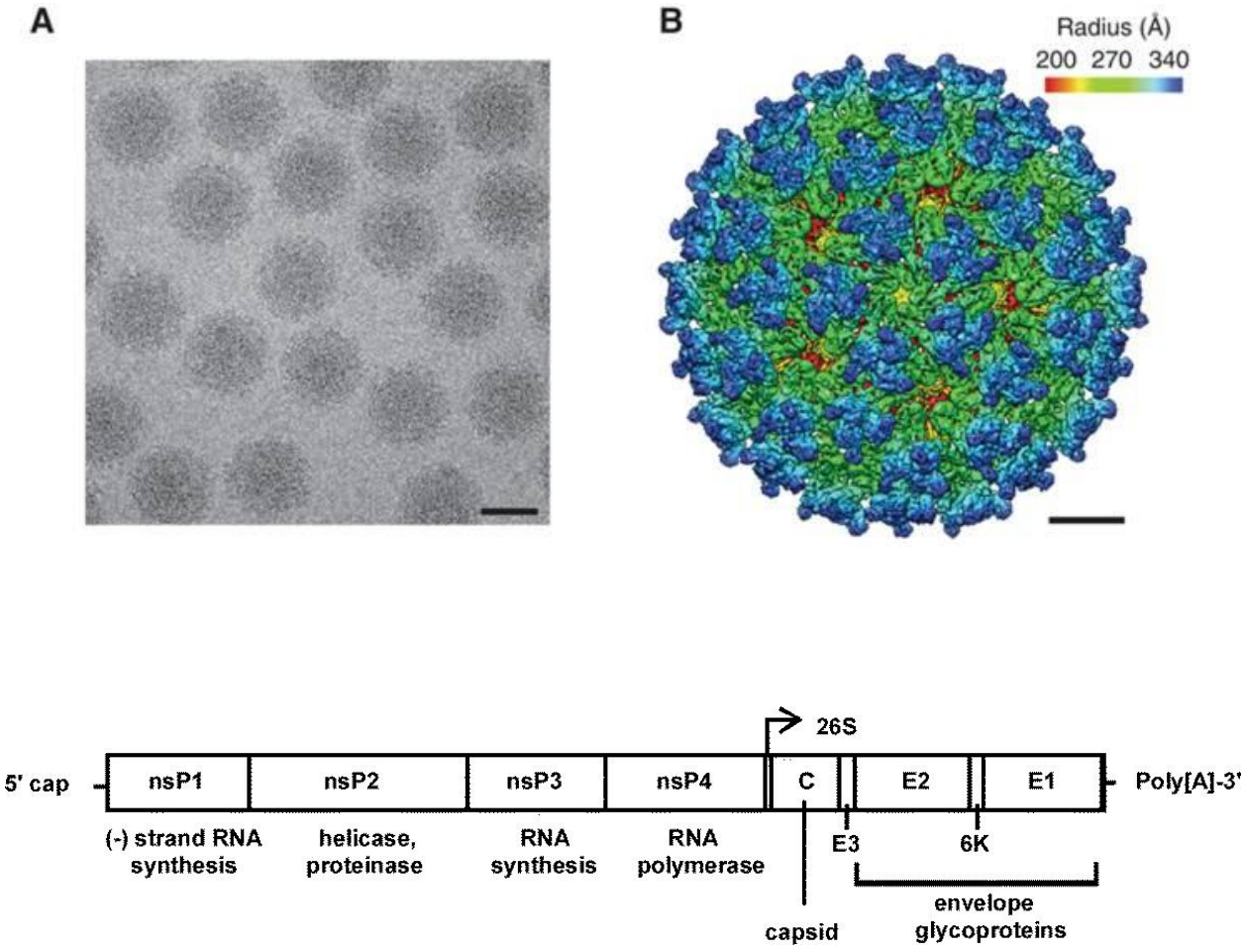


Figure 1: Structural and genomic organization of VEEV.

(A) CCD image of VEEV TC-83 strain embedded in vitreous ice. Scale bar: 50 nm. (B) Radially coloured 3D reconstruction of VEEV, showing the E1 basal triangle (green) (adapted from (Zhang et al 2011)and E2 central protrusion (blue) for each spike. Scale bar: 10 nm. (C) Organization of structural and non-structural genes in VEEV genome (Adapted from Weaver et al 2004).

Pathogenesis

VEEV pathology in case of humans in not very well studied due to the lack of clinical samples from fatal human cases during the VEEV outbreak. However, it was observed that VEEV infection in humans share many common characteristics with VEEV infection of many different animal species. Gross findings in human cases of VEEV indicated a widespread edema in the brain along with mild meningitis (Johnson et al 1968). Vasculitis and perivascular hemorrhage were also evident in the lethal cases of VEEV (De la Monte et al 1985). VEEV infection was also evident in lymphoid organs including spleen and lymph nodes where main pathological changes included lymphocyte degeneration, lymphoid depletion, and follicular necrosis, accompanied by infiltrates of neutrophils (Steele et al 2010). Lung and liver lesions were also observed in majority of fatal cases. All these observations are based on tissue examination and not by histopathological examination or electron microscopy (Steele et al 2010).

Due to the lack of data on pathogenesis of VEEV in humans, mouse model of VEEV infection has been widely used to study due to the similarity of the disease course to humans and equines. Both the natural route of infection and the one relevant to biological warfare i.e. aerosol route (Steele and Twenhafel, 2010) are studied. A cDNA clone of VEEV i.e. V3000 has been widely used to study the pathogenesis of wild type and other mutant strains of VEEV (Davis et al 1991). Mice infected with epizootic strains of VEEV have a median lethal dose of 1-30 plaque forming units (PFU) depending on the strain, age of mice and route of inoculation (Ludwig et al 2001).

After inoculation with virus either through subcutaneous route or footpad injection which mimics the natural infection (mosquito bite), the dermal dendritic cells (DCs) are first infected

with VEEV. The virus along with the DCs then reaches to the draining lymph nodes (Davis et al 1994). Once in the lymph node, virus replication is detected as early as 4h and by 12h virus enters the blood reaching titers upto $1x10^6$ plaque forming units (PFU). Virus then spreads to various lymphatic organs and is detected in B cell region of the spleen on day 1 and 2 after infection (Jackson et al 1991). During the lymphatic stage of the disease, lymphoid damage occurs with observable necrosis of the outer cortex of follicles in lymph nodes. Presence of neutrophils is also observed in the follicle of the lymph nodes. At this stage of the infection, VEEV also spreads to other organs and tissues including pancreas, liver, chemosensory epithelium of the vomeronasal organs, Bowman's glands, epithelial cells in the medulla of the thymus, cells of the peyer's patches of the distant small intestine and the odontogenic and ameloblastic epithelia and dental pulp of the teeth (Steele et al 1998, Vogel et al 1996, Jackson et al 1991). By 3-4 days post infection, VEEV is effectively cleared off from the peripheral system but the virus has already entered the brain by 36-48h post infection.

The virus first appears in the brain though the olfactory neuroepithelium and then enters the olfactory bulb. The virus in the blood escapes through fenestrated capillaries beneath the olfactory mucosa of the nasal tract directly into the unmyelinated axons of the olfactory nerves (Charles et al 1995, Steele et al 2010). By 36-48h post infection the virus spreads to other parts of the brain which receives efferent projections from the olfactory bulb which include the lateral olfactory tracts, pyriform cortex, hypothalamus, amygdale, hippocampus, dentate gyrus and supraoptic nucleus (Charles et al 1995). Direct invasion of the virus into brain has been suggested before (Ryzhikov et al 1991) but the main entry route appears to be through the olfactory neuroepithelium. Alternatively, infection of trigerminal nerve is also believed as a major route of CNS invasion (Charles et al 1995).

In case of an aerosol infection, no viremia is detected and the virus enters the brain as early as 16h post aerosol exposure (Ryzhikov et al 1991). VEEV infection of olfactory neuroepithelium is specific and neither the respiratory nor squamous epithelia of the nasal tracts are infected by VEEV. The olfactory neurons are in direct contact with air where they contract the virus infection in the form of aerosol. These neurons synapse directly with the neurons of the olfactory bulb and thereby providing a direct route for virus transmission to the brain (Steele et al 2010).

Once the virus finds it way inside the brain through the olfactory route, it rapidly spreads to the various parts of the brain, though in case of aerosol exposure the virus spread in the brain appears to be faster than the peripheral infection route. The virus first infects the lateral olfactory tract and pyriform cortex followed by infection of thalamus, midbrain and cerebellum. During VEEV infection neurons are the primary target which includes several subtypes of neurons including motor neurons which can cause paralysis, neurons of hippocampus, cerebellum and spinal cord (Jackson et al 1991, Vogel et al 1996 and Steele et al 1998, 2010). Other cell types of the brain also appeared positive for VEEV infection including brain macrophages, and microglial cells (Sharma et al 2008). Astrocytes though show infection with VEEV *in vitro*, but *in vivo* infection of astrocytes does not appear significant (Schoneboom et al 1999, Steele et al 2010). Brain microvascular endothelial cells are widely damaged but their infection with VEEV is not clear. Earlier reports have indicated presence of virus in the endothelial cells using electron microscopy but others have suggested the damage of endothelial cells as a result of widespread inflammation due to VEEV infections (Gorelkin 1973, Steele 2010). In addition, cells of the choroid plexus and ependymal cells are not considered as a main target of VEEV.

Role of host factors in VEEV pathogenesis has been studied in last few years using mouse model of infection. Some of these studies have identified that immunopathology plays an important role in pathogenesis of VEEV infection in the mouse brain which if not controlled can change the outcome of the disease. In one such study, it was shown that the survival times of immunocompromised mice are longer than the immunocompetent animals (Charles et al 2001). Similar study showed that mice treated with antithymocyte serum survived longer than saline treated animals (Woodman et al 1975). In both these cases the increased survival time of the mice was attributed to the less number of inflammatory cells inside the brain. In particular CD8+ T-cells since infection in immunocompetent animals lead to a high virus titer in brain. Neuronal cell death has been reported in areas of brain with no virus infection but a high degree of reactive astrogliosis has been associated for the neuronal death (Schoneboom et al 2000). Our laboratory has also previously reported increased expression of genes related to immune response, apoptosis and inflammation (Sharma et al 2008). VEEV infection also increased the expression of toll like receptor (TLRs) molecules in the mouse brain. TLRs recognize microbial peptide or nucleotide sequence and induce a cascade of proinflammatory immune response. In response to VEEV infections, TLR 1, 2, 3, 7 and 9 were upregulated along with several chemokines, inflammatory cytokines and interferon (Sharma et al 2009).

Cell adhesion molecules on the endothelium regulate the passage of activated CD8+ T-cells from the peripheral system to the brain. Though infection of endothelial cells lining the microvessels in the brain with VEEV is not clear, increased expression of intercellular adhesion molecule I (ICAM-I) and vascular cell adhesion molecules was observed along with cadherins, intergrins and matrix metalloproteases (MMPs). Further, ICAM-1 knockout mice showed increased protection against the disease severity against VEEV infection (Sharma et al 2011).

TC-83, is a live attenuated vaccine strain of VEEV, which does not cause a lethal disease in C57BL/6J mice, but causes a lethal infection in C3H/HeN mice. Examination of brain of both the strains of mice indicated a higher load of natural killer (NK) cells in C3H/HeN mice. When these mice were artificially depleted of NK cells, their susceptibility towards TC-83 infection was diminished (Taylor et al 2012). These studies suggests that though infection of neurons is critical in VEEV infection, but host factors also play an important part in the development of overall pathology of the disease. Moreover, it also opens another avenue for the development of antiviral by targeting important host protein critical for virus survival.

Inflammation and neuron infection by VEEV collectively causes neuronal cell death which can occur both by apoptosis and necrosis that ultimately lead to the death of the animal. Typically larger neurons appear to be necrotic whereas smaller granule-type neurons, which are found in hippocampus and the cerebellum shows apoptotic cell death (Griffin 1994 and 1994, Steele et al 2010).

Replication of VEEV

The genomic of organization of both the old world and new world alphaviruses is quite similar. Replication of alphaviruses is mainly studied using laboratory strains of Sindbis and Semliki Forest viruses which are widely used to study pathogenesis of alphaviruses. In the following section, a common replication mechanism which applies to all the viruses in alphavirus family is discussed.

Virus Entry: The first step in the viral entry into the host cells begins with the attachment of the viral glycoproteins with host cell receptors. E2 glycoprotein is predominately the most important receptor involved in the binding of the virus to the host proteins and role of E1 protein is not fully characterized in virus binding (Jose et al 2009). Alphaviruses are capable of

infecting wide variety of vertebrate and invertebrate hosts. The mechanism of virus entry in all these cells types is not fully understood. Studies have suggested that alphaviruses might use a protein receptor which is highly conserved and is found on almost all the cell types. In case of VEEV and sindbis virus, laminin binding receptor which is present in both vertebrate and invertebrates has been suggested as the possible receptor to which the virus binds during infection (Wang et al 1992, Ludwig et al 1996). After virus attachment, the E1 and E2 glycoprotein undergoes a conformational change and virus is internalized into the cell via clathrin mediated endosomal pathway (Helenius et al 1980). Inside the endosomal vesicle, the pH drops and becomes mildly acidic which triggers the dissociation of the E1 and E2 glycoprotein and exposes a fusion peptide at the distal end of the E1 glycoprotein (Ahn et al 1999, Hammar et al 2003). This fusion peptide trimerizes with the endosomal membrane to form a pore in the vesicle through which the viral nucleocapsid is released in the cytoplasm of the host cell where its disassembly occurs followed by delivery of the viral genome in the cytoplasm (Wahlberg et al 1992, Helenius et al 1984).

Translation of viral non-structural proteins: For their genomic replication, RNA viruses must first translate their non-structural proteins (nsps) using the host translational machinery. Alphavirus RNA contains a 4-60 nucleotide repeat sequence element upstream of ploy(A) tail towards the 3' end of the genome. This sequence recruits the host protein to the viral RNA for translation of non-structural proteins (Ou et al 1982). Translation of the nsps leads to the formation of two polyprotein species, P123 and P1234 where the number indicates the nsps. In some alphaviruses including VEEV translational read through of an opal codon after non-structural protein 3 leads to the formation of P1234 but P123 remains the predominant polyprotein (Li et al 1993). Alphaviruses encodes for four different nsps i.e. nsp1-4.

Nsp-1 has the properties of guanine-7-methyltransferase and guanyltransferase which is important for capping and methylation of newly synthesized viral RNA copies (Cross et al 1983, Mi et al 1991). Additionally, nsp-1 is a membrane associated protein due to the presence of a patch of basic and hydrophobic residues. This helps in anchoring the viral RNA replication complex to the cellular membranes (Laakkonen et al 1996, Ahola et al 1999).

Nsp-2 is a multifunctional protein with several known enzymatic properties. The N-terminal of nsp-2 has the helicase activity which is necessary for the unwinding of the RNA important for its replication of the genome (Gomez et al 1999). The C-terminal end harbors the papain-like cysteine protease along with enzymatically inactive methyltransferase activity (Strauss et al 1992, Vasiljeva et al 2001).

Nsp-3 role in viral replication and pathogenesis is not very well understood. Mutational analysis experiments have indicated a role of nsp-3 protein in the minus strand synthesis of the genome during replication (Tuitilla and Hinkannen, 2003). Further, a virus with mutated nsp-3 can also regulate viral pathogenesis in mice (Strauss and Strauss, 1994).

Nsp-4 gene encodes the viral RNA dependent RNA polymerase (RdRP) which is the primary mediator of viral genomic replication. Nsp-4 protein has a conserved GDD domain which is required for it activity which is conserved across nsp-4 of several RNA viruses (Kamer and Argos, 1984). The C- terminal region of nsp-4 is highly conserved and shares homology with other RdRPs of other viruses whereas the N-terminal region is not conserved (Hahn et al 1989). In addition to the polymerase activity, nsp-4 has also been shown to have terminal adenyltransferase activity which may be useful for maintaining viral poly(A) tail (Tomar et al 2006). The cellular concentration of nsp-4 is relatively low in comparison to other nsps because

of the presence of opal codon which allow only partial read through. Additionally the presence of a primary destabilizing tyrosine residue also rapidly degrades the free nsp-4 inside the cell (De Groot et al 1991).

Replication of viral genomic RNAs: Once the nsps are translated using the host machinery, replication of viral RNA takes place in the cytoplasmic vacuoles from endosomes and lysosomes (Froshauer et al 1988). Both the viral nsps and host proteins forms the replicase complex whereas, the viral structural proteins are also implicated in its activity (Kujala et al 2001, Barton et al 1991). Early in the infections, the processing of viral non-structural polyprotein by nsp-2 yields P123 and nsp-4 in the replicase complex is required for the minus strand synthesis using genomic RNA as template (Shirako and Strauss, 1994). A 51 nucleotide long conserved sequence element (CSE) found at the 5' end and spanning into nsp-1 acts as a promoter sequence for the replication of minus strand RNA (Frolov et al 2001). As the infection progress, the processing of P123 occurs to form individual nsps (1-4) which slows down the formation of minus strand. The replicative is complex now composed of cleaved nsps and host proteins which favor the formation of plus strand synthesis and sub genomic RNAs (Strauss and Strauss, 1994). Another CSE at the 5' end (first 44 nucleotides of the genome) is believed to serve as a promoter sequence in minus strand to facilitate transcription of plus strand (Frolov et al 2001). Plus strands are either transcribed from genomic or subgenomic promoters, however, transcription from subgenomic promoter makes three fold excess sub genomic RNA than plus strand RNA (Raju and Huang, 1991).

Translation of structural proteins and nucleocapsid assembly: Structural proteins in alphaviruses are translated from subgenomic RNA as a polyprotein in the order of Capsid (CP)-pE2-6K-E1. CP is released from the polyprotein very early by autoproteolysis leaving a new N-

terminal to the polyprotein with a signal sequence for its translocation to endoplasmic reticulum membrane (ER) (Garoff et al 1990, Strauss and Strauss, 1994). Translation of CP protein initiates the process of nucleocapsid assembly Nucleocapsid of alphaviruses including that of VEEV contains a single positive strand RNA and 240 copies of CP protein (Jose et al 2009). The assembly of nucleocapsid is a multistep event which is initiated by a nucleation event when residues 81 to 112 are recognized by the signal sequence on the viral RNA which leads to the interaction between CP and viral RNA and formation of nucleocapsid (Weiss et al 1989, Weiss et al 1994, Linger et al 2004).

The presence of signal sequence on the N-terminal translocates the pE2-6K-E1 to ER. Inside the ER, the pE2 and E1 protein undergoes a series of folding, disulphide bond formation and post translational modifications which include addition of mannose chain to all N-linked glycosylation sites (Sefton 1977). The pE2-E1 complex after reaching the trans-Golgi network is cleaved by furin at the cleavage site to form E3 and E2 glycoprotein. This cleavage is critical for the new virus since uncleaved pE2 incorporation in the virus envelop will reduce the virulence (Heidner et al 1994, Salminen et al 1992). The glycosylated forms of E1 and E2 proteins are then transported to plasma membrane (Jose et al 2009).

Virus Budding: After the formation of nucleocapsid and transportation of glycopreotein to the plasma membrane, the final step in the life cycle of virus is budding of virions from the host cell plasma membrane. Nucleocapsids which were assembled in the cytoplasm transit to the plasma membrane where they interact with the E2 glycoprotein. This interaction along with the lateral interaction of the E1 and E2 glycoproteins results in an increase of free energy which then propels the virus out of the plasma membrane. During this process, the virions also acquire a complement of glycoprotein spikes from the plasma membrane (Garoff and Simons, 1984). The

virus thus released has a complete set of envelope glycoprotein, CP and a single copy of RNA and is highly infectious. The replication and budding mechanism of VEEV is illustrated in Figure 2.

Antiviral for VEEV

At present, there is no FDA approved antiviral treatment or vaccine for the treatment and prophylaxis of VEEV respectively. Several approaches for the development of novel antiviral have been reported in literature. Many attempts to design novel antiviral drugs have focused on development of antivirals directed against the virus. Among these efforts some antiviral approaches which have shown great potential are discussed here. Peptide-conjugated phosphorodiamidate morpholino oligomers were also found to be protective in cell culture and in animals upon pretreatment before virus challenge. However, their protective efficacy was significantly reduced after a treatment post VEEV infection (Passeler et al 2008). In a similar way, random short interfering RNAs (siRNAs) have been tested against VEEV which were found to be protective against various strains of VEEV but their efficacy in animal models is not tested (O'brien 2007). Another study tested the efficacy of carbocyclic cytosine which inhibits cellular cytidine triphosphate (CTP) synthetase. CTP synthetases convert UTP to CTP thus indirectly inhibit the replication of virus by reducing the CTP pools. Treatment with carbodine was shown to be effective in reducing the disease severity and mortality in mouse model (Julander et al 2005).

Antiviral effect of interferon (IFN) has been very widely studied in various viral infections. The efficacy of peglyated alpha interferon on VEEV infection was tested in animal models. Treatment of VEEV-infected BALB/c mice with polyethylene glycol-conjugated alpha interferon (PEG IFN-alpha) results in a greatly enhanced survival from either a subcutaneous or

an aerosol infection. Virus is undetectable within PEG IFN-alpha-treated animals by day 30 post infection (Lukaszewski et al 2000). Other efforts to develop antiviral against VEEV include generation of humanized monoclonal antibodies against VEEV. Humanized monoclonal antibody against VEEV was used to treat mice post VEEV challenge. Treatment with these antibodies after 4 days post VEEV infection offered a complete protection in mice (O'brien et al 2010, Goodchild et al 2011). High throughput screening of small chemical molecules library identified compound thieno[3,2-b]pyrrole, which showed a very robust anti-VEEV activity *in vitro*, however, its efficacy in *in vivo* system is yet to be established (Peng et al 2009). Though a lot of studies have evaluated the antiviral potential of many different molecules and compounds in preclinical studies but none of these have made their way to the clinic for practical purposes. Therefore, there is an urgent need to identify novel drug candidates which can be used to treat VEEV infections and should also be easily licensable.

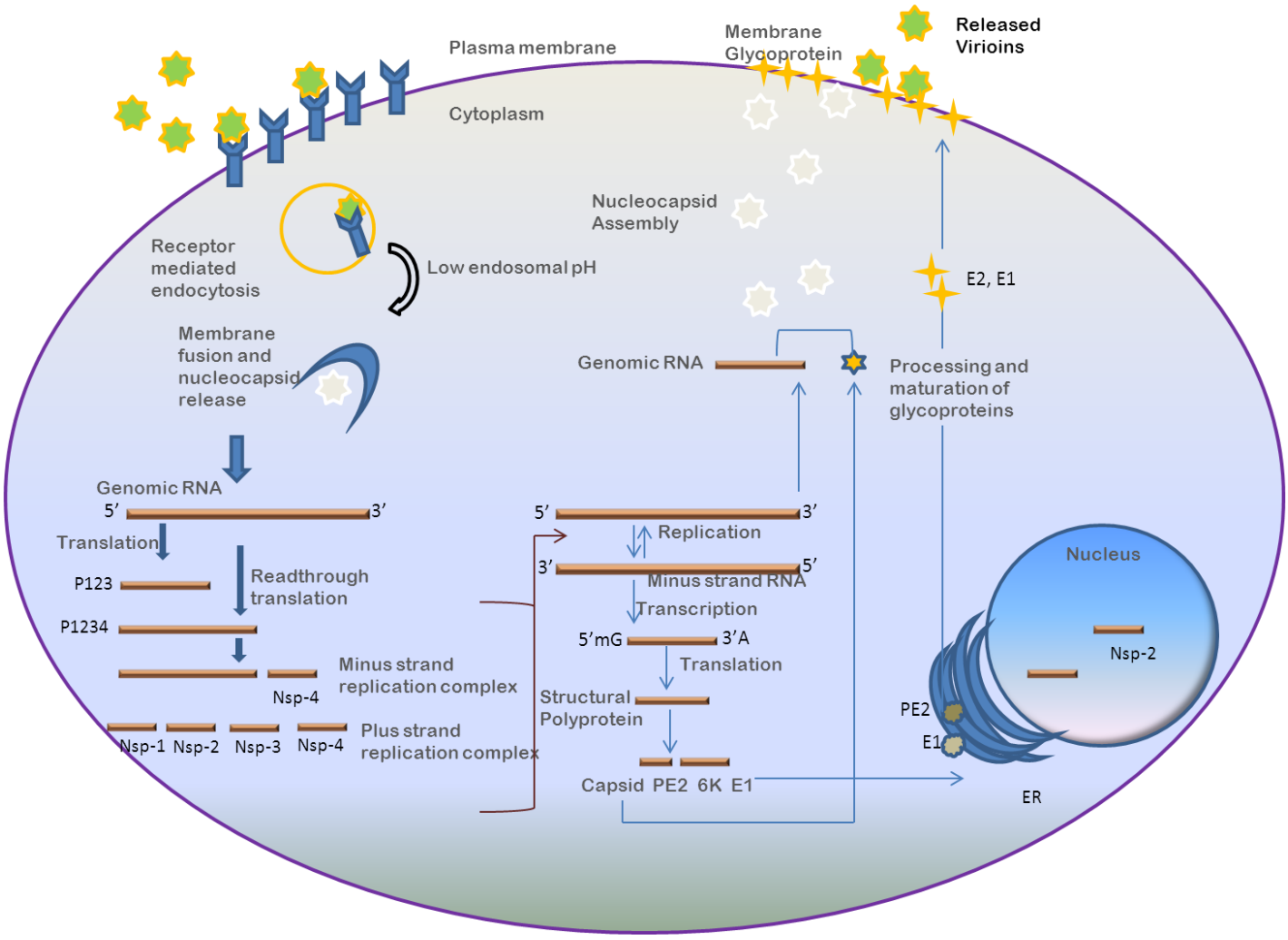


Figure 2 : Replication and budding mechanism of VEEV inside the host cell. (*Information extracted from Jose et al 2009).*

Vaccine

Similar to the antiviral agents, currently there is no licensed FDA approved vaccine for VEEV. Numerous approaches to develop both live attenuated and inactivated vaccines have been tried but all of them suffer from issues of safety and immunogenicity. The first live attenuated vaccine strain for VEEV was developed by serial passaging of VEEV in guinea pig heart cell which is called TC-83 (McKinney et al 1963). TC-83 has been shown to be very effective in mouse model against the virulent VEEV infection. Currently, it is used as a vaccine for personnel involved in VEEV research but is not licensed for common use due to non- responders and it failure to elicit a long lasting immunity (Pittman et al 1996).

The reactogenicity and limited immunogenicity of TC-83 is attributed to the presence of two point mutations which were incurred in parent Trinidad donkey strain during the *in vitro* passages. RNA viruses have a high inherent mutation rate which presents a risk of reversion of TC-83 to wild type strain during amplification in the vaccinee which can cause epizootics (Passelar and Weaver, 2009). To avoid this reversion, a cDNA clone of virulent strain was generated which was then subjected to point mutations in PE2 cleavage-signal along with an E1 gene resuscitating mutation. This new V3526 was found to be safe and immunogenic in both rodents and non-human primates (Pratt et al 2003, Hart et al 2000). Despite these encouraging results in animal models, V3526 failed in the Phase I human clinical trial due to its residual toxicity and no longer been used as a live attenuated vaccine strain.

Other live attenuated vaccine approaches involved introduction of internal ribosomal entry site in the structural genes of TC-83 along with 12 synonymous mutations to inactivate the sub-genomic promoter. This modification resulted in a complete block of replication of the virus

in the mosquito cells and hence reducing chances of reversal to the virulent strain making TC-83 more safe to use.

Due to the safety concerns with live attenuated vaccines for RNA viruses, several approaches have been tested to inactivate the VEEV virus to generate inactivated vaccine. An initial attempt to inactivate the virus was done by using formalin which completely inactivated the virus and is currently used to immunize equines but failed to elicit sufficient immunity in humans (Randall et al 1949). The loss of immunogenicity is mainly due to the damage to the surface epitopes which are critical for antibody response. Our laboratory has recently used chemical compound 1,5-iodonapthyl azide which sequesters in the hydrophobic domain of the viral envelope thereby inactivating the virus without damaging the surface protein. This inactivation strategy elicited a strong immune response in mouse model (Sharma et al 2011). Recently, our laboratory have also inactivated V3526 using gamma irradiation in presence of a radio-protective manganese complex which prevents oxidative damage of the surface proteins due to radiation but allows the radiation to completely denature the genome. Virus was successfully inactivated with this method and protein integrity was preserved (Gaidamakova et al 2012). The protective efficacy of this vaccine remains to be tested. Other strategies to develop novel vaccine candidates which include DNA vaccine, alphavirus replicons and SIN/VEEV chimeric vaccines have also been tested which have shown great promise to protect animals from lethal VEEV challenge (Passelar and Weaver, 2009). Despite all these efforts, a safe and efficacious vaccine against VEEV for humans still remains a challenge.

MicroRNA Biogenesis

MicroRNAs (miRNA) are a class of small (19-28nt) endogenous RNA molecules which are evolutionary conserved across all the eukaryotic organism species. MiRNAs regulate gene

expression at post transcriptional level either by translational repression by binding to the partially complementary sites in the 3' untranslated region (UTR) in the target mRNA. MiRNAs are encoded by their genes which are present in the intronic region of the protein coding genes and found in both introns and exons in the non-protein coding genes (Rodriquez et al 2004). These miRNA coding regions are often transcribed by RNA polymerase II to form primary miRNA (pri-miRNA) transcript which is often polycistronic in nature (Borchert et al 2006). The Pri-miRNA transcripts have a stem loop configuration with a 5' cap and 3' poly A tail (Lee et al 2004).In the nucleus, the pri-miRNA transcripts is cleaved by type III RNase called drosha and double stranded RNA binding protein co-factor called DGCR-8. This processing yields a 70 nucleotide long precursor miRNA (pre-miRNA) with a 5' monophosphate and a 3' hydroxyl group. The pre-miRNA is then transported out of nucleus for further processing in cytoplasm by exportin-5/RanGTPase complex (Lee et al 2003, Bohnsack et al 2004).

In the cytoplasm, the pre-miRNA is further processed by another RNase III called dicer to generate a short RNA duplex with 2 nucleotide over hank at the 3'end (Bernstein et al 2001). This resulting miRNA duplex contains two strands, a functional strand termed as the guide strand and the complementary strand termed as the passenger strand. The strand whose 5' end is loosely paired with the complementary pair is selected as the guide strand by the Argonaute protein (Ago) (Mourelatos et al 2002). The other strand is rapidly degraded by the cellular RNAses but in some cases both the strands form functional miRNAs which are designated as miRNA-5p and 3p (Ro et al 2007). MiRNA along with ago proteins constitutes the RNA inducing silencing complex (RISC) which target mRNAs for hydrolysis or translation repression (Figure 3).

MicroRNA mechanism of gene regulation: Regulation of translation of mRNA by miRNAs occur after the binding of RISC complex with the target mRNA. MiRNAs binds to their target mRNA in miRNAs recognition elements (MREs) predominantly but not exclusively found in the 3'UTR of mRNA (Dugas and Bartel 2004). MiRNAs bind to their target mRNA by base pairing matching of proximal 2-8 nucleotides called as the seed region (Lewis et al 2003). There are four different types of Ago proteins which are found in eukaryotes and each of which contains multiple domains which includes N-terminal, PAZ, MID and PIWI domains (Jinek and Doudna 2009). Only Ago 2 out of the four ago proteins is catalytically active in mammals (Liu et al 2004). If Ago2- along with the guide miRNA strand binds with the target mRNA with substantial complementarity then the mRNA undergoes endonucleotic cleavage (Hutvagner and Zamore, 2002, Elbashir et al 2001). However, if the base pairing with the target mRNA occurs only with the seed region of the miRNA, translation repression or mRNA decay occurs. Translational repression by miRNAs which does not involve mRNA cleavage may occur by inhibition of translational initiation or elongation steps, premature termination of translation, co-translational degradation of nascent peptide, and miRNA-mediated mRNA decay (Eulalio et al 2008).

The exact mechanism of translational repression by miRNAs is not completely clear. However, several studies have found that mRNA-RISC complex colocalizes with discreet cytoplasmic loci known as processing bodies (p-bodies) which harbors enzymes responsible for mRNA deadenylation, decapping and degradation. Currently, these p-bodies are considered as sites for mRNA decay or degradation by miRNAs. It is proposed that when Ago-bound miRNA recognizes its mRNA target; GW182 protein binds to mRNA–RISC complex and marks mRNA for decay and thereafter the mRNA–RISC–GW182 complex is delivered to P-bodies where it is

stored for later translation or deadenylated, decapped, and degraded (Eulalio et al 2007, Bratkovič et al 2012).

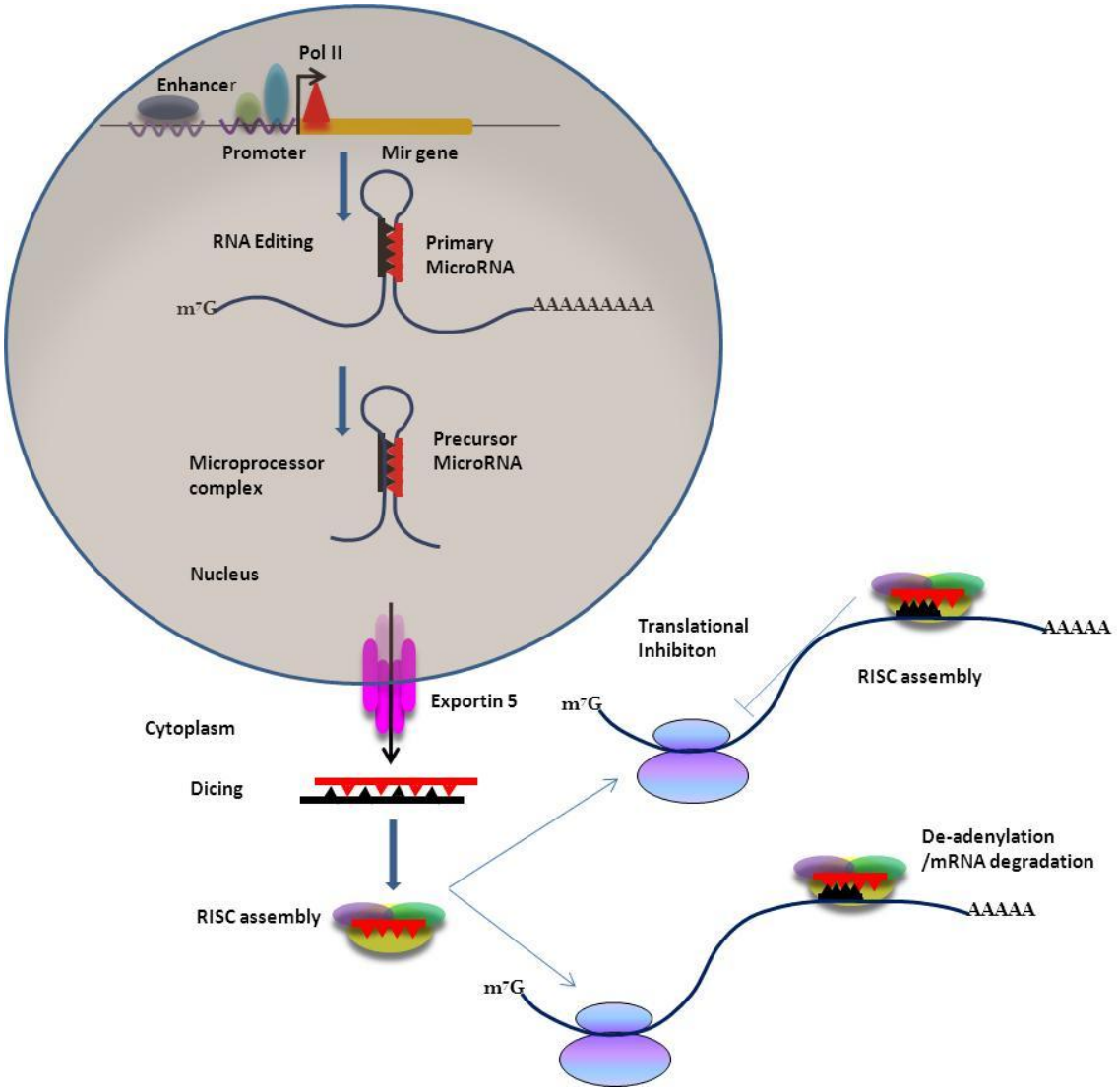


Figure 3: Biogenesis of MicroRNAs.

(Information adapted from Kanellopoulou and Monticelli, 2008)

MiRNAs and its role in viral replication

Role of miRNAs in response to virus infection has been studied in last few years. Recent studies have shown that cellular miRNAs play a very important role in the pathogenesis of viral infections (Cullen 2011). The most highlighted example is the remarkable positive regulation of HCV replication by the liver-specific miR-122. MiR-122 enhanced the translation of viral genome and colony formation efficiency of the HCV replicon (Jopling et al 2005, Chang et al 2008). Mir-122 is constitutively expressed in uninfected hepatocytes but its expression is substantially increased after infection with HCV. A clear case of miRNA induction due to virus infection which facilitates virus replication was found in Epstein bar virus (EBV) where virus induces a very high upregulation of mir-155 which helps in cell immortalization by the virus (Gatto et al 2008). Other herpes viruses like KSHV and hCMV induces upregulation of mir-132 which is known to suppress interferon stimulated genes and hence support viral infection (Lagos et al 2010). In HIV infected CD4+ T cells, it was observed that mir28, mir125b, mir150, mir223 and mir382 were upregulated. Inhibition of these miRNAs resulted in active replication on HIV in CD4+ T cells. It was suggested that HIV uses these miRNA to down regulate viral gene expression and establish viral latency (Huang et al 2007). HIV also down regulates mir17/92 and, this is needed for its replication. Latency is achieved by repression of histone acetyl transferase Tat cofactor PCAF that is needed for transactivation of the latent virus (Triboulet et al, 2007). Expression of mir32 in HeLa cells inhibited the replication of primate foamy virus type-1 by interacting with the 3'UTR of the viral genome (Lecellier et al, 2005).

Most of the studies have suggested that virus induces the expression of certain miRNAs to facilitate its own replication but the reports on suppression of virus replication due to the presence of specific miRNA in the cells are few. In HIV, mir-29b has been shown to inhibit its

replication in T cells due to the presence of a mir-29b binding site in the viral genome (Nathans et al 2009). One reason for this less number of reports of inhibition of virus replication by miRNAs may be because of the high susceptibility of the RNA viruses to mutate and to adapt to the environment favorable to their replication. However, it is known that viruses do not infect all types of cells and different miRNAs are also enriched in specific cell types, it is proposed that this enrichment of few miRNAs which can target the genome of the virus leads to tissue tropism of the virus (Cullen et al 2012).

MicroRNAs as potential antiviral therapeutics

Each miRNA regulates many genes and hence they possess a great potential to be used both as therapeutic target and intervention tool. MiRNAs have been shown to be modulated in various cancers, tissue injury, viral infection and other disorders. Many studies have shown that targeting these aberrantly expressing miRNAs significantly reduced the disease severity. In this regard, modified locked nucleic acid also termed as “antagomirs” or “anti-mirs” which contains sequences complementary to the miRNA have been shown to inhibit the over expressing miRNAs (Ørom et al 2006, Soifer et al 2007). Similarly, synthetic miRNAs containing binding sites of miRNA have been used as miRNA “decoy or” “miRNA sponge” (Liu et al 2008). In a recent study in vivo silencing of miR-21 by a specific antagomirs in a mouse pressure-overload-induced disease model reduces cardiac ERK-MAP kinase activity, inhibited interstitial fibrosis and attenuates cardiac dysfunction. This study showed that mir-21 can contribute to cardiac dysfunction and established that antagomirs can be successfully used as an *in vivo* miRNA inhibitor (Thum et al 2008). Intravenous injections of antagomirs specific of mir-122 significantly reduced the viral loads of HCV in non-human primate model and currently anti-mir-122 is tested in phase II clinical trial in humans against HCV (Lanford et al 2010, Cullen et

al 2012). On the other hand in many cases a reduced expression of miRNAs has been observed and which has been correlated with the progression of the disease. In case of hepatocellular carcinoma (HCC), an expression level of mir-26a is greatly reduced. Systemic administration of this miRNA in a mouse model of HCC using adeno-associated virus results in inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression without toxicity (Kota et al 2009). These studies indicate the underlying potential of miRNA as a potential therapeutic target and intervention tool. The stability and non-toxicity of antagomirs have made them a valuable tool to antagonize the aberrantly expressing miRNA. The availability of stable and non-toxic plasmid based vectors has enabled delivery of under expressed miRNAs.

MicroRNA based live attenuated vaccines

As mentioned before, certain miRNAs are enriched in specific tissue and are not expressed elsewhere. This property of miRNAs has been utilized to develop live attenuated viral vaccine strains. In this strategy, complementary sequences of the miRNAs are cloned in the viral genome which results in the binding of the miRNA to its target sites in viral genome inside the cell leading to cleavage of the viral transcript by RNAi machinery (Lauring et al 2010). This strategy harnesses the property of cell tropism of miRNAs in which a miRNA is specifically enriched in only one cell type. This could be utilized to induce miRNA mediate tissue tropism in viruses which restrict the virus replication in the tissues like brain where it can cause severe disease but at the same time allow its replication in the peripheral system for the induction of a robust immune response. This strategy has been successfully used to generate live attenuated vaccine for polio, dengue, adenoviruses and oncolytic virus (Barnes et al 2008, Kelly et al 2008, Cawood et al 2009 and Cullen et al 2012).

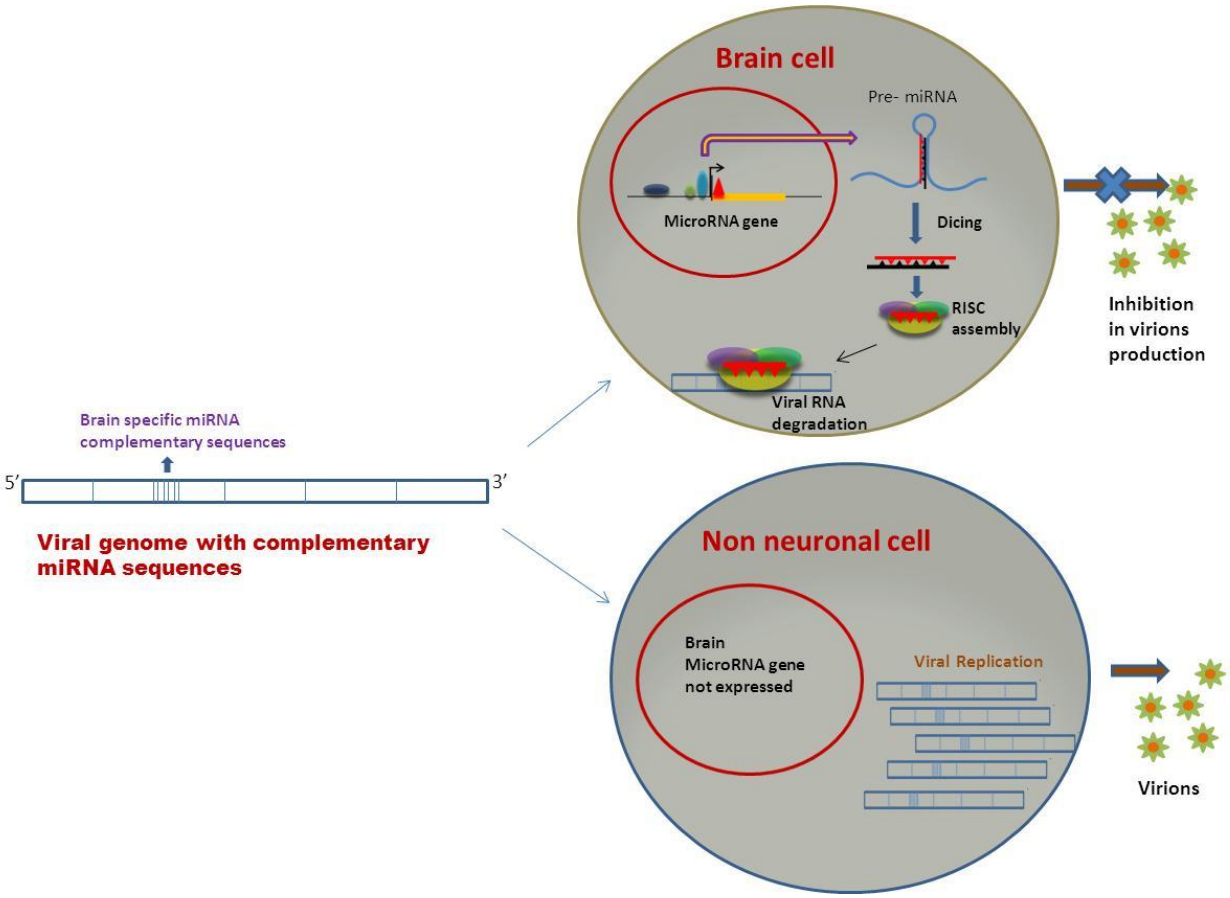


Figure 4: MicroRNA based development of live attenuated vaccine for viral pathogens. (Information extracted from from Brown and Naldini, 2009).

Chapter 1

Analysis of MicroRNAs induced by Venezuelan Equine Encephalitis virus infection.

Abstract

MicroRNAs (miRNA) are small non- coding RNA (~22nts) molecules that are expressed endogenously in cells and play an important role in regulating gene expression. Recent studies have shown that cellular miRNA plays a very important role in the pathogenesis of viral infection. Role of miRNAs in VEEV pathology is however not studied so far. In this study, we report for the first time that VEEV infection in mice brain causes modulation of miRNA expression. Pathway analyses showed that majority of these miRNAs are involved in the neuronal development and function. Many miRNAs which are modulated have already been shown to play a critical role in increasing expression of inflammatory and proinflammatory cytokines. Further, miRNAs specific to endothelial cells are also modulated early in the infection which suggest an active involvement of these cells during VEEV pathogenesis. Target gene prediction of the modulated miRNAs correlates with our recently reported mRNA expression in VEEV infected mice brain. VEEV infection also caused modulation of miRNAs in fibroblasts cells in which VEEV is known to replicate efficiently early during the lymphoid phase of the infection. Computational analysis of modulated miRNAs indicated the role of these miRNAs in various host pathways which may be critical for virus replication. Together these studies have enhanced our present understanding of role of miRNAs in VEEV pathogenesis.

Introduction

MiRNA are the post-transcriptional regulators that bind to the complementary sequences in the mRNA and blocks the translation and/or accelerates mRNA decay (Brown and Naldini 2009). MiRNAs play an important role in cellular processes such as development, differentiation, cell proliferation, tumorigenesis, neuronal development and hematopoiesis (Ambros 2004).

There is a growing body of evidence that demonstrates key regulatory roles for cellular miRNA during viral infection and altered cellular miRNA expression during the virus infection may be an important determinant of virulence (Ghosh et al 2009, Gottwein et al 2008 and Dykxhoom, 2007). Cellular miRNAs may aid for the progression or suppression of viral replication. For example, mir-122 has been shown to positively regulate hepatitis C virus (HCV) replication by enhancing colony formation efficiency of HCV (Chang et al 2008). Moreover, knocking down mir-122 in non-human primate model leads to long lasting suppression of HCV viremia (Lanford et al 2010). Many other studies have also reported important regulatory role of miRNAs in viral infection as discussed previously. Though studies have reported host miRNAs modulation during DNA viruses such as Epstein Barr virus (Godshalk et al 2008) and retroviruses such as HIV and PFV (Godshalk et al 2008, Lecellier et al 2005) , only a few studies have shown host miRNA modulation in RNA virus infections (Chan et al 2010).

In central nervous system (CNS), VEEV targets neurons and glial cells and induces inflammation and neurodegeneration (Grieder et al 1997). VEEV induced inflammation in CNS plays an important role in the disease outcome and is often believed to be associated with the secondary neuronal damage in brain (Charles et al 1995, Schoneboon 2000. Earlier, we have reported that VEEV causes modulation of genes that are involved in host innate immune

responses such as apoptosis, inflammation and antigen presentation in VEEV infected brain (Sharma et al 2008, 2009). However, the regulatory pathways which control the expression of these genes are poorly understood. Since, miRNAs are believed to play a critical role in neurodegenerative disorders (Bak et al 2005, Kosik 2006, Krichevsky 2007, Bilen et al 2006 and Kim et al 2006). To evaluate the the miRNAs that are modulated in the brain after VEEV infection, a global miRNA profiling was carried out in the brain infected with VEEV. Further, a computational correlation of the modulated miRNAs with earlier reported genes expression studies was carried out. To understand the cellular miRNA changes which are induced by VEEV in the peripheral infection during its initial replication, we have studied the miRNA modulation in an *in vitro* L-11 cell line post VEEV infection. L-11 cells are fibroblasts and represent a suitable model to study VEEV infection mechanism since fibroblasts are productively infected during the initial phases of VEEV replication.

Data reported in this study have demonstrated the modulation of several miRNAs in VEEV infected mice brain and L11 cell line. Computational analysis of the predicted targets of these miRNAs revealed a possible regulation of the downstream genes by the miRNAs. Specifically, our data in the *in vivo* study suggest that miRNA such as mir-155 is persistently upregulated in VEEV infection and may be a contributing factor to the inflammation. We also observed miRNA modulation of miR-126 which is specific to endothelial cells indicating a role of blood brain barrier (BBB) early in the course of infection. Our *in vitro* data indicates that the modulated miRNAs may be directly influencing the cellular ability to support growth and differentiation by altering the various cellular pathways. To our knowledge, this is the first study to evaluate the miRNA modulation *in vitro* and *in vivo* following alphavirus infection.

Material and Methods

Animals: Six to eight weeks old male CD-1 mice were obtained from Charles River Laboratories, Wilmington, MA. All the experiments with live virus challenge were carried out at the Uniformed Services University of the Health Sciences (USUHS) bio-safety level 3 (BSL-3) facilities in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care And Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86–23, revised 1996).

Cell culture: Mouse L-11 cells, a subclone of mouse LB cells were maintained in Eagles's MEM media (Cell Grow Inc) supplemented with 10% new born calf serum and 1% solution of penicillin and streptomycin. Virus infection of mouse L 11 cells was done with TC-83 (alive attenuated form of V3000) . Cells were infected with a multiplicity of infection (MOI) of 5 in phosphate buffered saline (PBS) and incubated for 1h at 37 ºC and 5% CO_2. Virus was washed after 1h and cells were supplemented with fresh medium.

Virus challenge: V3000, derived from an infectious full length cDNA clone of the wild type Trinidad donkey strain subtype IA/B was used for these experiments. Virus challenge was done as described before (Sharma et al 2008). Six animals were used for each group. Briefly, mice were anesthetized with 5% isoflurane and 1000 plaque forming units (pfu) of V3000 were injected in the left rear footpad. Control animals were injected with saline.

Brain isolation: Following the infection with VEEV, animals were sacrificed at 48 and 72 h post infection (p.i) and brains were isolated and immediately snap-freezed over dry ice and stored at -80ºC until further use.

RNA isolation: Frozen brain section were minced over ice and total RNA was isolated using TriZol reagent (Invitrogen Inc, Carlsbad, CA) according to the manufacturer's protocol. Briefly, brain tissue were minced in 1ml of trizol reagent and incubated at RT for 10min. Half volume of chloroform was added to this and mixed by vortexing. Aqueous phase was separated by centrifugation followed by RNA precipitation by isopropanol. The RAN pellet was washed with 70% ethanol and resuspened in nuclease free water. For cellular RNA, cells were harvested using Trizol reagent and RNA was isolated using manufacturer's protocol. RNA was quantitated using Nanodrop (Thermo Scientific, Wilmington, DE) and the presence and integrity of miRNA in the total RNA samples was analyzed on 6% Tris borate EDTA urea gel.

Real Time PCR: cDNA pool was synthesized from the total RNA by reverse transcription reaction using a stem-loop multiplex miRNA primer pool as per the manufacturer's protocol (Applied Biosystems Inc, Foster city, CA). The stem-loop multiplex miRNA primer pool specifically binds only to miRNA to generate a corresponding miRNA-cDNA pool. The expression profile of miRNAs in the infected tissues or cells and the uninfected control tissues or cells were measured by quantitative real-time polymerase chain reactions (qPCR) using the Low Density miRNA Taqman array as per manufacturer's protocol (Applied Biosystems Inc, Foster city, CA). From here onwards these arrays are referred as miRNA array cards. The miRNA array card contains 821 unique rodent miRNAs including 6 endogenous controls and 1 negative control. Briefly, 6µl of total cDNA was mixed with 450µl Taqman PCR master mix without Uracil N-glycosylase (UNG) (Applied Biosystems Inc, Foster city, CA) and 444µl nuclease free water. 800µl of this mix was loaded on the miRNA array cards (Applied Biosystems Inc, Foster city, CA) and the cards were sealed following centrifugation at 2000 rpm for 2 min and qPCR was carried out on Applied Biosystem 7900HT real time PCR machine.

Statistical Analysis: qPCR data obtained from the miRNA array cards was analyzed for the modulation of statistically significant miRNAs using Statminer software (Integromics Inc, Philadelphia, PA). Cycle threshold (Ct) values of >35 was considered to be non-specific [28] and miRNAs with raw Ct value of >35 were excluded from the analysis. Detectability threshold of miRNAs in all the samples was set as ≥4. A computationally predicted endogenous control calculated using Genorm scoring method was used for normalization. All the 6 endogenous controls present on the miRNA cards were used for Genorm scoring. A parametric limma test was used to compute the statistically significant miRNAs in infected and control samples. MicroRNAs with a p value score of ≤0.05 and fold change of ≥1.5 were considered statistically significant.

Target prediction and pathway analysis: MiRNA target prediction was done by using TargetScan Mouse 5.1(http://www.targetscan.org/). These predicted gene target of miRNAs were then used for network and functional analysis through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity Systems Inc., Redwood City, CA).

RESULTS

MicroRNA modulation in mouse brain upon VEEV infection

VEEV have been shown to cause severe neurodegeneration in the CNS, which is also considered to be the cause of death following VEEV infection. Modulation of genes involved in the host cellular and immune regulatory pathways such as apoptosis, antigen presentation and inflammation in the brain following VEEV infection play a major role in neurodegeneration (Weaver et al 2004, Sharma et al 2008, 2009 and 2011). VEEV appears in the brain at around 36-48 h following footpad infection and rapidly spreads throughout the brain (Steele et al 2010). Therefore, to understand the miRNA changes occurring at or around the time of VEEV appearance and during its active replication in brain, we evaluated miRNAs expression in brain tissue at 48 and 72 h p.i.

After isolation of RNA, we analyzed the quality of the RNA using gel electrophoresis (Figure 5). Following we analyzed the modulation miRNAs using real time PCR. Overall, 32 miRNAs were found to be significantly modulated upon VEEV infection at 48 h p.i., out of which 20 were upregulated and 12 were down regulated (Table 2). At 72 h p.i, a total of 36 miRNAs were significantly modulated, however, in contrast to 48 h p.i., 31 of these miRNAs were down regulated and only 5 miRNAs were upregulated (Table 3). MicroRNAs such as mir-155, mir-27a, rno-mir-381, mir-154* and mir-801 were found to be modulated at both the time points. Since miRNA negatively regulate gene expression, this pattern corroborate with our previously reported gene expression study where gene expression for many genes increases along with the progression of the disease (Sharma et al 2009) where, the number of genes upregulated increased from 48 h to 72 h p.i.

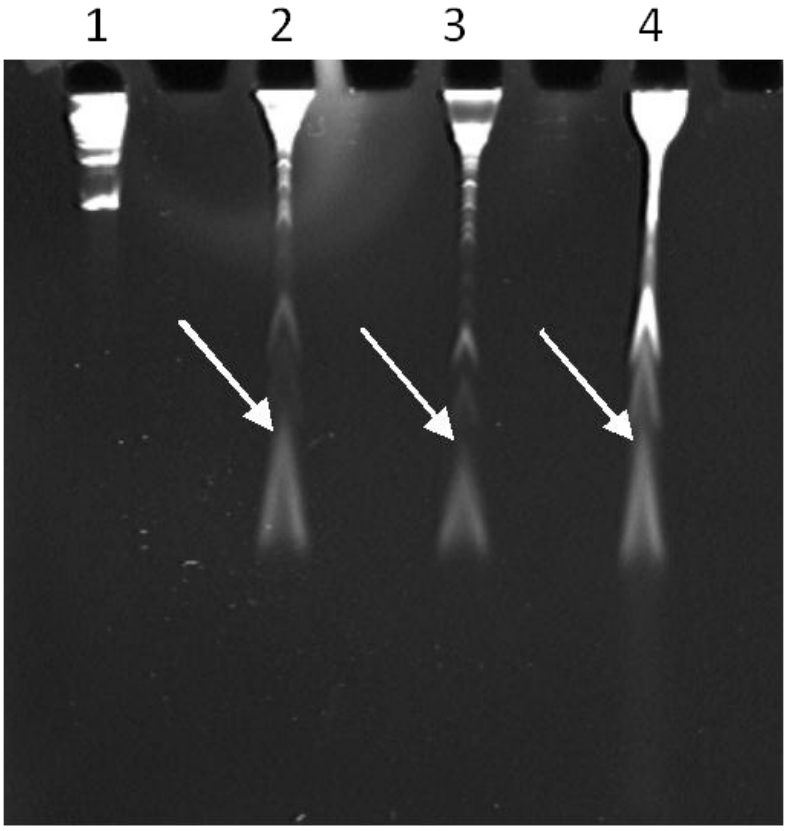


Figure 5: Gel Picture showing the presence of small RNA species brain samples.

Lane 1: RNA ladder, 2: Brain RNA from VEEV infected 72h p.i, 3: Brain RNA from uninfected sample, 4: Control RNA from VERO cells.

Table 2: Modulation of miRNAs in VEEV infected mouse brain at 48h p.i.

miRBase Accession Number	Upregulated MicroRNAs	Fold Increase	miRBase Accession Number	Downregulated MicroRNA	Fold decrease
MIMAT0000165	mmu-miR-155	5.05	MIMAT0003742	mmu-miR-455	13.97
MIMAT0003171	mmu-miR-542-5p	4.83	MIMAT0003388	mmu-miR-376b*	3.14
MIMAT0004578	mmu-miR-300*	3.75	MIMAT0004544	mmu-miR-193*	2.66
MIMAT0000236	mmu-miR-203	3.33	MIMAT0003469	mmu-miR-690	2.57
MIMAT0004842	mmu-miR-879	3.29	MIMAT0000209	mmu-miR-129-5p	2.35
MIMAT0000666	mmu-miR-320	2.73	MIMAT0004520	mmu-let-7i*	2.33
MIMAT0004633	mmu-miR-27a*	2.69	MIMAT0000660	mmu-miR-181a-1*	2.12
MIMAT0000148	mmu-miR-136	2.69	MIMAT0004537	mmu-miR-154*	2.09
MIMAT0004531	mmu-miR-135a*	2.68	MIMAT0003199	rno-miR-381	2.02
MIMAT0001542	mmu-miR-449a	2.38	MIMAT0004624	mmu-miR-15a*	1.83
MIMAT0003735	mmu-miR-672	2.32	MIMAT0000537	mmu-miR-27a	1.45
MIMAT0003480	mmu-miR-297b-5p	2.23		[#]mmu-miR-801	3.61
MIMAT0004746	mmu-miR-409-3p	2.19			
MIMAT0000138	mmu-miR-126-3p	2.18			
MIMAT0004522	mmu-miR-27b*	2.18			
MIMAT0004893	mmu-miR-574-3p	2.12			
MIMAT0000133	mu-miR-101a	1.96			
MIMAT0000612	mmu-miR-135b	1.89			
MIMAT0004643	mmu-miR-331-3p	1.87			
MIMAT0000584	mmu-miR-339-5p	1.72			

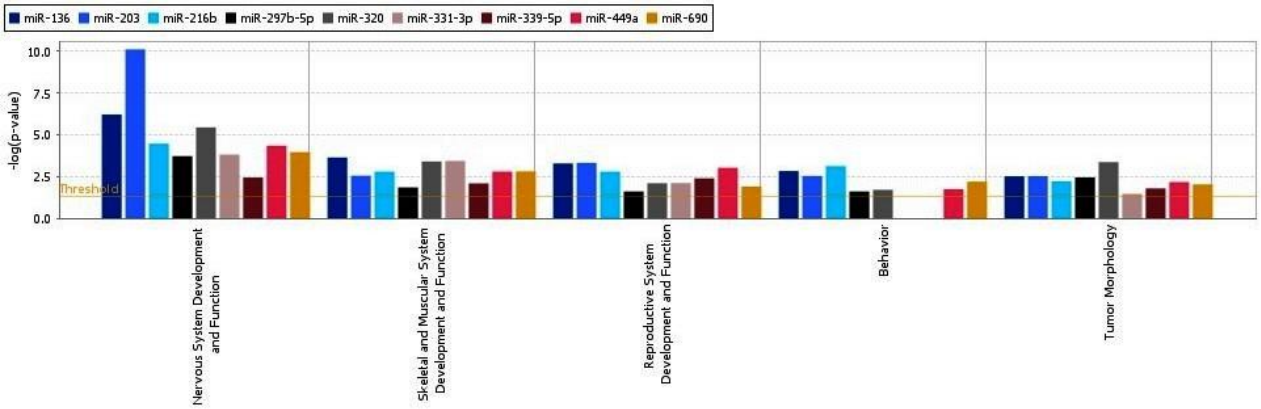
Statistical analysis was done using Statminer (Integromics Inc). MicroRNAs with $p<0.05$ and fold change of >1.5 were considered significant. (* are miRNA strands that are the complementary strands of the functional single-stranded miRNAs, [#] mmu-miR-801 is no longer present in miRBase database since miR-801 appears to be a fragment of U11 spliceosomal RNA and hence removed from the database.)

Table 3: Modulation of miRNAs in VEEV infected mouse brain at 72h p.i.

miRBase Accession Number	Upreglated MicroRNA	Fold Increase	miRBase Accession number	Downregulated MicroRNA	Fold Decrease
MIMAT0000165	mmu-miR-155	10.66	MIMAT0004706	rno-let-7e*	8.34
MIMAT0003494	mmu-miR-704	9.59	MIMAT0003732	mmu-miR-668	5.99
MIMAT0005334	rno-miR-743a	6.81	MIMAT0004582	mmu-miR-106b*	5.27
MIMAT0004746	mmu-miR-409-5p	2.24	MIMAT0000239	mmu-miR-206	4.38
MIMAT0003509	mmu-miR-501-3p	1.81	MIMAT0000569	mmu-miR-330*	4.09
			MIMAT0000746	mmu-miR-381	3.93
			MIMAT0000249	mmu-miR-30e*	3.76
			MIMAT0000129	mmu-miR-30a*	3.73
			MIMAT0000548	mmu-miR-322	3.61
			MIMAT0004636	mmu-miR-93*	3.15
			MIMAT0003484	mmu-miR-720	3.13
			MIMAT0004942	mmu-miR-598	2.93
			MIMAT0004537	mmu-miR-154*	2.93
			MIMAT0003202	rno-miR-382*	2.92
			MIMAT0000648	mmu-miR-10a	2.87
			MIMAT0004666	mmu-miR-33*	2.82
			MIMAT0004722	rno-miR-30d*	2.81
			MIMAT0000136	mmu-miR-125b*	2.61
			MIMAT0004630	mmu-miR-26b*	2.36
			MIMAT0000537	mmu-miR-27a	2.22
			MIMAT0004583	mmu-miR-130b*	2.18
			MIMAT0000534	mmu-miR-26b	2.10
			MIMAT0003450	mmu-miR-488	2.04
			MIMAT0000248	mmu-miR-30e	1.96
			MIMAT0000137	mmu-miR-126-5p	1.90
			MIMAT0003199	rno-miR-381	1.80
			MIMAT0000679	mmu-miR-217	1.80
			MIMAT0000224	mmu-miR-194	1.78
			MIMAT0000225	mmu-miR-195	1.72
			MIMAT0000219	mmu-miR-24	1.68
				[#]mmu-miR-801	4.62

Statistical analysis was done using Statminer (Integromics Inc). MicroRNAs with $p<0.05$ and fold change of >1.5 were considered significant. (* are miRNA strands that are the complementary strands of the functional single-stranded miRNAs, [#] mmu-miR-801 is no longer present in miRBase database since miR-801 appears to be a fragment of U11 spliceosomal RNA and hence removed from the database.)

a



b

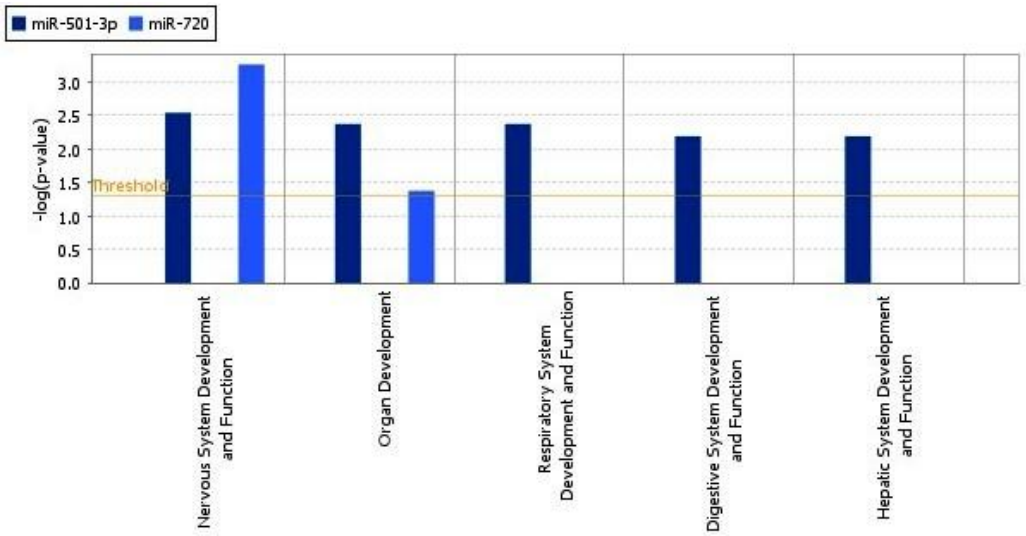


Figure 6: Biofunctional analysis using modulated miRNAs using IPA: Analysis showed Nervous System Development and Function as topmost biofunction both at 48h (a) and 72h (b) p.i. in Physiological Systems and Development and Functions category post VEEV infection. The analysis was done based on target prediction by TargetScan and Ingenuity pathway analysis.

Table 4: List of genes which were commonly observed between the targets of modulated miRNA at 48 and 72h p.i.

S. No	Functional Grouping	Genes at 48 h P.I	Unigene ID	Genes at 72 h P.I	Unigene ID
1	Antigen presentation	CALR	Mm.1971	CSF1R	Mm.22574
		CSF1	Mm.795	DDR1	Mm.5021
		STAT3	Mm.249934		
2	Inflammatory Response	CALR	Mm.1971	BCR	Mm.333722
		CSF1	Mm.795	CSF1R	Mm.22574
		STAT3	Mm.249934	CXCL2	Mm.4979
				DDR1	
3	Nervous System Development and Function	ANK3	Mm.235960	ANK3	Mm.235960
		APLP2	Mm.19133	ATN1	Mm.333380
		CAMK4	Mm.222329	ATP1B1	Mm.4550
		CASP2	Mm.3921	ATP2B2	Mm.321755
		CHL1	Mm.251288	BCL11B	Mm.254329
		CHRM1	Mm.240607	BCR	Mm.333722
		CITED2	Mm.272321	CAMK2G	Mm.235182
		CLCN3	Mm.259751	CD200	Mm.245851
		CNTN2	Mm.260861	CDH2	Mm.257437
		CSF1	Mm.795	CLCN3	Mm.259751
		EBF3	Mm.258708	CTNNB1	Mm.291928
		ENAH	Mm.87759	CTNND2	Mm.321648
		ERBB4	Mm.336982	CXCL10	Mm.877
		ETV1	Mm.4866	DKK1	Mm.214717
		GNAI3	Mm.271703	EPHA4	Mm.3249
		IGF1R	Mm.275742	FOXG1	Mm.4704
		APK8IP3	Mm.43081	GJC2	Mm.40016
		MAPT	Mm.1287	GRIA2	Mm.220224
		NAP1L2	Mm.347470	IGF1R	Mm.275742
		NEFM	Mm.390700	LINGO1	Mm.246605
		EUROD1	Mm.4636	MAPK8IP3	Mm.43081
		EUROD2	Mm.4814	MAPT	Mm.1287
		NFIA	Mm.374797	NCAM1	Mm.4974
		S1PR1	Mm.982	NFIX	Mm.9394
		SCN1A	Mm.142733	ODZ2	Mm.339846
		SEMA5A	Mm.260374	PRKCD	Mm.2314
		SLITRK1	Mm.257268	RTN4R	Mm.40149
		STMN2	Mm.29580	SMARCA4	Mm.286593
		SV2A	Mm.200365	SOCS3	Mm.3468
		TCF4	Mm.4269	STXBP1	Mm.278865

		TGFB2	Mm.18213	SV2A	Mm.200365
		UGCG	Mm.198803	SYP	Mm.223674
				TIAM1	Mm.124100
				UBE4B	Mm.288924

To analyze the direct and indirect miRNA-gene interactions, we performed IPA with mRNA expression data imported from the earlier study (Sharma et al 2008) and significantly modulated miRNAs. The miRNAs involved in these networks were mir-155, mir-126 and mir-300 at 48 h p.i and mir-155, mir-126, mir-195 and mir-206 at 72 h p.i. The miRNA-gene networks showed that miR-155 targets IL1β indirectly through intermediate molecules. IL1β then regulates many other downstream genes which were observed to be modulated upon VEEV infection (Figure 7&8). These miRNA-gene networks clearly showed that miRNA may indirectly regulate gene expression during VEEV infection through intermediate molecules. Moreover, mir-155 was upregulated at 48 h p.i and its expression increased by 72h p.i, whereas miR-126 was upregulated at 48 h p.i and its expression was down regulated at 72h p.i. These differentially regulated miRNAs thus may play an important role in VEEV infection of brain.

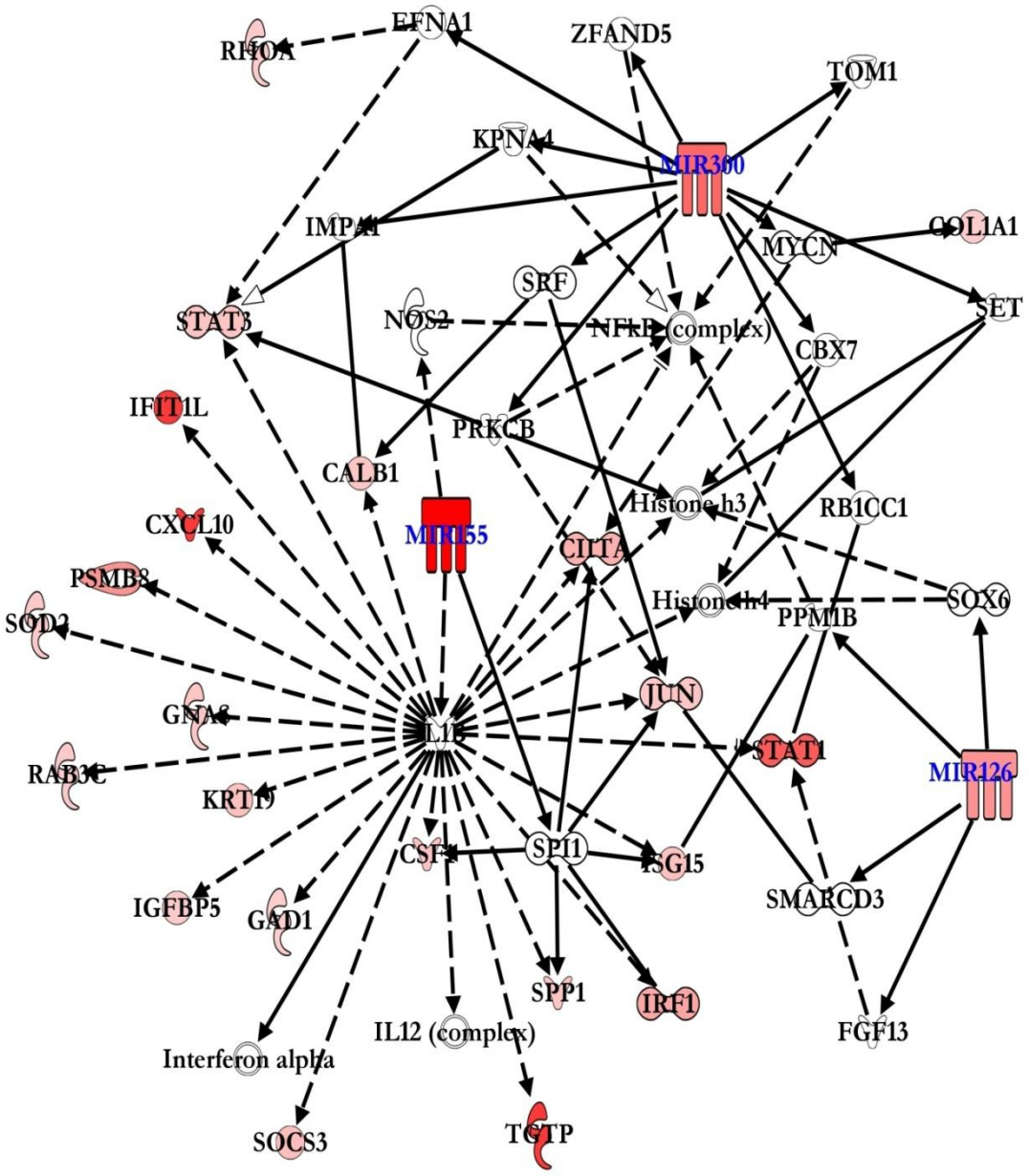


Figure 7: Prediction of mRNA regulation by miRNAs using IPA at 48h p.i.: Network analysis with mRNA expression data of topmost network molecules with modulated miRNAs at 48h p.i with VEEV. Solid lines indicate a direct interaction whereas a broken line indicates an indirect interaction. Green color of the molecules indicates low expression or down regulation whereas red color indicates upregulation. The expression levels indicated here are based on the experimental data of miRNA and mRNA expression analyses

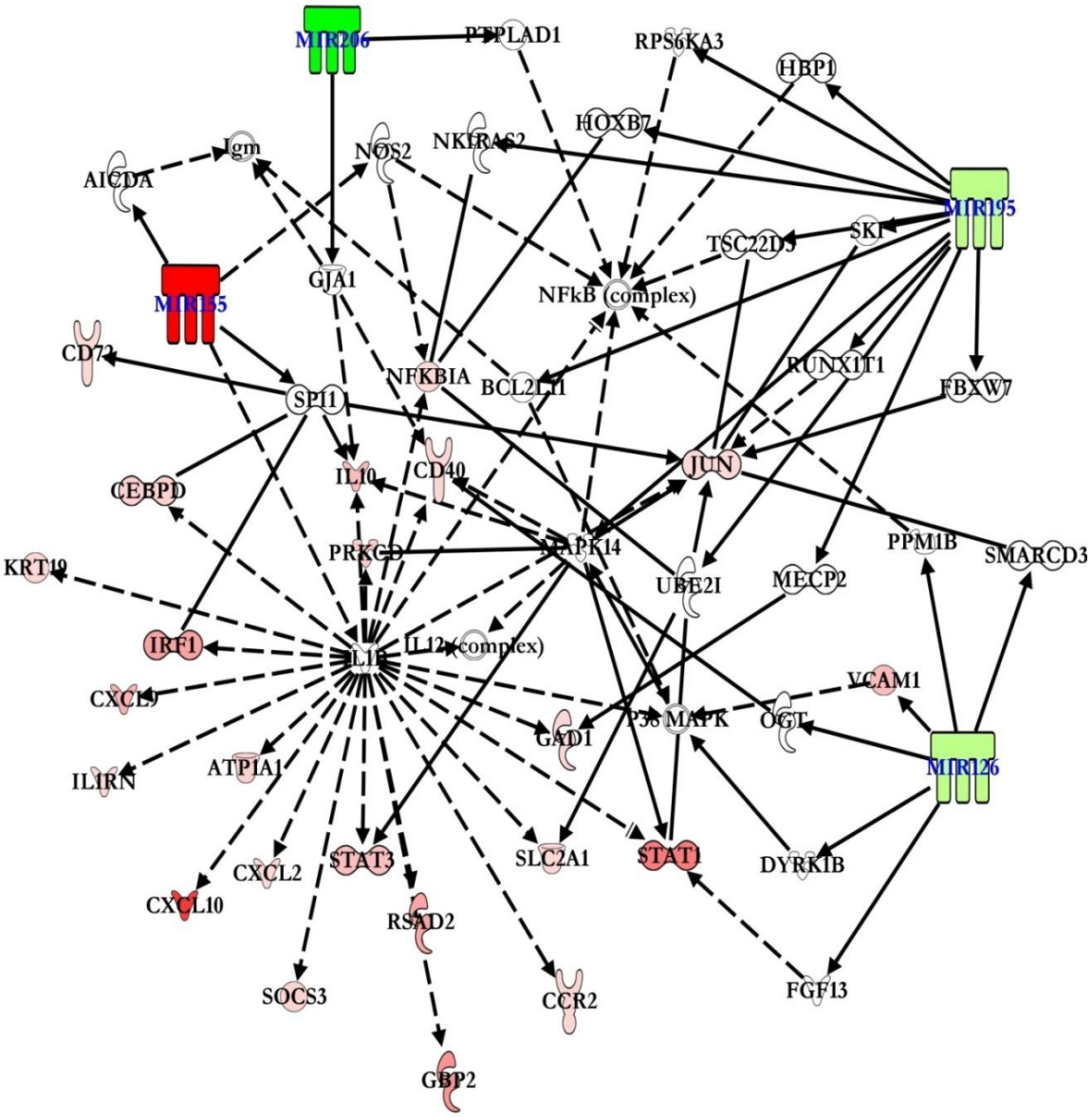


Figure 8: Network analysis with mRNA expression data showing topmost network molecules with modulated miRNAs at 72h p.i with VEEV.Solid lines indicate a direct interaction whereas a broken line indicates an indirect interaction. Green color of the molecules indicates low expression or down regulation whereas red color indicates upregulation. The expression levels indicated here are based on the experimental data of miRNA and mRNA expression analyses.

MiRNA modulation in mouse fibroblast cells after VEEV infection.

Mouse fibroblasts were infected with TC-83 (an attenuated strain of VEEV) which productively infects these cells and miRNA modulation was analyzed 12h p.i. This time point was chosen since VEEV replicates very efficiently in the cells and virus starts to appear in the supernatant around 8-12h p.i. This time point would help in analyzing the cellular miRNAmodulation in response to VEEV replication inside the cells. After statistical analysis, 23 miRNAs were found to be significantly modulated post VEEV infection. Among these, 12 miRNAs were upregulated out of which 6 miRNAs which include mir-184, mir-196a, mir-452, mir-467a, mir-96 and mir125b were only detected in the infected cells. The remaining 11 miRNAs were down regulated among which expression of 5 miRNAs were completely suppressed in infected cells and were not detected (Table 5).

Functional analysis of miRNAs modulated in fibroblast cells after VEEV infection.

Expression data of the miRNAs was used for biofunctional analysis using IPA as previously described. Network analysis revealed that the modulated miRNAs targets molecules involved in regulation of cell death and survival inflammatory molecules. Six miRNAs that were only expressed after the VEEV infection were shown to play an important role in pathways which promote cellular function and maintenance, hematopoesis and post transcriptional modifications. Analysis of the 5 miRNAs which were suppressed by VEEV infection indicated their involvement in regulation of molecules involved in cell survival and apoptosis and inflammation. This analysis indicated that the miRNA modulation post VEEV infection can be induced either by virus to propagate itself by suppressing host antiviral factors or by host, as a defense mechanism.

Further, computation analysis was done to elucidate the possible regulation of protein molecules which are critical mediators of host pathway for viral entry and replication. Data meta-analysis showed that modulated miRNAs may regulate several molecules involved in phosphatidylinositol 3-kinases (Pi3Kinase) (Table 6). Interestingly, some of these predicted relationships of miRNA and proteins have already been validated in previous published studies which showed a direct relationship of miR199 and 214 trageting host pi3-AKT pathway to facilitate virus replication (Santhakumar 2010). In addition to Pi3kinase pathway, protein molecules of downstream mTOR pathway were also predicted to interact with modulated miRNAs. Other important pathways predicted by IPA, which may interact with miRNAs include ERK/MAPK signaling. These predicted pathways have been implicated in replication of various viruses including alphaviruses including sindbis virus and semliki forest virus. (Dunn et al 2012, Ji et al 2008, Patel et al 2012).

Table 5: Modulated miRNAs in L11 fibroblast after 12h of VEEV infection.

Serial No	MicroRNA	Fold Change (Log 10)
1	mmu-miR-1188	-0.435385289
2	mmu-miR-128a	0.871944633
3	mmu-miR-1896	-0.43333949
4	mmu-miR-29c	0.364771103
5	mmu-miR-339-5p	-0.393000868
6	mmu-miR-463	-0.758308143
7	mmu-miR-466g	0.322475636
8	mmu-miR-503	0.375225875
9	mmu-miR-669n	-0.500839747
10	mmu-miR-700	-0.312299994
11	rno-miR-345-3p	0.318026864
12	rno-miR-7	0.384350395
13	mmu-miR-184	Only expressed in infected cells
14	mmu-miR-196a	Only expressed in infected cells
15	mmu-miR-452	Only expressed in infected cells
16	mmu-miR-467a	Only expressed in infected cells
17	mmu-miR-96	Only expressed in infected cells
18	rno-miR-125b	Only expressed in infected cells
19	mmu-miR-107	Only expressed in infected cells
20	mmu-miR-191	Only expressed in infected cells
21	mmu-miR-376b	No expression in control cells
22	mmu-miR-671-3p	No expression in control cells
23	rno-miR-20a	No expression in control cells

Table 6: Computational targets of modulated miRNAs showing involvement of phosphoinositidyl-3-phosphate and AKT pathways.

MiRNA	Log Ratio (p Value)	Source	Confidence	Predicted gene target
mmu-miR-107	-1.982	TargetScan Human	Moderate (predicted)	AKT2
mmu-miR-107	-1.982	miRecords	Experimentally Observed	CRKL
mmu-miR-107	-1.982	TargetScan Human	High (predicted)	GNAI3
mmu-miR-107	-1.982	TargetScan Human	Moderate (predicted)	IFNAR1
mmu-miR-107	-1.982	TargetScan Human	High (predicted)	KPNA3
mmu-miR-107	-1.982	TargetScan Human	High (predicted)	PIK3R1
mmu-miR-128	0.872	TargetScan Human	High (predicted)	CRKL
mmu-miR-128	0.872	TargetScan Human	Moderate (predicted)	IFNA1/IFNA 13
mmu-miR-128	0.872	TargetScan Human	Moderate (predicted)	PIK3C2A
mmu-miR-128	0.872	TargetScan Human	High (predicted)	PIK3R1
mmu-miR-184	1.584	miRecords	Experimentally Observed	AKT2
mmu-miR-29c	0.365	TargetScan Human	High (predicted)	AKT2
mmu-miR-29c	0.365	TargetScan Human	High (predicted)	AKT3
mmu-miR-29c	0.365	TargetScan Human	Moderate (predicted)	IFNG
mmu-miR-29c	0.365	Ingenuity Expert Findings,TargetScan Human,miRecords	Experimentally Observed,High (predicted)	PIK3R1
mmu-miR-29c	0.365	TargetScan Human	High (predicted)	PIK3R3
mmu-miR-29c	0.365	TargetScan Human	High (predicted)	ZNF346
mmu-miR-339-5p	-0.393	TargetScan Human	High (predicted)	GNAI2
mmu-miR-339-5p	-0.393	TargetScan Human	Moderate (predicted)	KPNA3
mmu-miR-96	1.518	TargetScan Human	High (predicted)	CASP9
mmu-miR-96	1.518	TargetScan Human	High (predicted)	CRKL
mmu-miR-96	1.518	TargetScan Human	High (predicted)	GNAI3
mmu-miR-96	1.518	TargetScan Human	Moderate (predicted)	IFNA8
mmu-miR-96	1.518	TargetScan Human	High (predicted)	KPNA3
mmu-miR-96	1.518	TargetScan Human	High (predicted)	MAP2K1
mmu-miR-96	1.518	TargetScan Human	High (predicted)	PIK3C2A
mmu-miR-96	1.518	TargetScan Human	High (predicted)	PIK3R1

Discussion:

Mortality of VEEV infection is mainly due to VEEV infection of the CNS. VEEV induced neuronal damage has been well documented by our laboratory as well as by others (Sharma et al 2008 and 2009, Grieder et al 1997). Following neuroinvasion VEEV targets neurons and glial cells and induces massive neurodegeneration and inflammation. VEEV induced inflammation in brain also contributes to the neurodegeneration (Grieder et al 1997). Molecular mechanisms of VEEV induced neurodegeneration are poorly understood. Viruses are known to hijack host cellular machinery for their survival and replication which includes transcriptional and translational machinery. Recently, many reports have identified modulation of host miRNAs by virus infection which can be utilized by virus like respiratory syncytial virus (RSV), Influenza A virus, Hepatitis C virus, Marek's disease virus and many others (Baek et a 2012, Loveday et al 2012, Tian et al 2012, Peveling-Oberhag et al 2012). Role of miRNAs in VEEV infection is unknown and hence in this study the miRNA modulation in both *in vitro* and *in vivo* system was evaluated following VEEV infection.

In the *in vivo* study of miRNAs modulation in mouse brain post VEEV infection, we selected two time points to analyze the immediate and the later effects of VEEV infection on miRNA expression. From our data, it is clear that with increasing infection of the brain, miRNA expression decreases. This type of miRNA modulation of viruses have been previously reported in case of vaccinia virus where the virus suppressed the miRNA machinery within 24h p.i by ablating the expression of the enzyme dicer-1, which catalyzes important step in miRNA maturation (Grinberg et al 2012).

Biofunctional analysis of the modulated miRNAs indicated a collective role of several miRNAs in nervous system development and functions. Among the several miRNAs modulated,

up regulation of miRNA-155 was observed at both 48 and 72h p.i. Mir-155 has been suggested as a pro inflammatory miRNA. Up regulation of mir-155 has been associated with increased inflammation in arthritis by suppressing SHIP-1 and increasing expression of proinflammatory cytokines (Kurowska-Stolarska et al 2011). Similarly, depletion of mir-155 resulted in a reduced inflammatory condition in mouse model of experimental autoimmune encephalomyelitis (Murugaiyan et al 2011). However, in an attempt to decrease the inflammatory damage using anti-inflammatory drug naproxen in mice, a marginal increase in the virus load was observed (Sharma et al 2011).

Another miRNA which is of special attention is mir-126 which is highly enriched in the endothelial cells and regulates endothelial cell inflammation and senescence (Quin et al 2012). Mir-126 has been shown to directly target vascular cell adhesion molecule-1 (VCAM-1) to down regulate its activity. In our previous studies we have shown that VCAM-1 expression on the brain endothelial cells is up regulated following VEEV infection which contributes to the breach of BBB and increased inflammation in the brain (Sharma et al 2011). Our data indicates a reduced expression of mir-126 in the brain at 72h p.i which may contribute to the increased expression VCAM-1 and hence may play a role in increased inflammation. Similarly, another endothelium specific miRNA mir-10a was down regulated post VEEV infection. Mir-10a is suppressor of proinflammtory events that takes place in case of pathologic conditions in the endothelial cells (Urbich et al 2008, Fang et al 2010). This indicates that miRNA plays an important role in curbing the inflammation which is suppressed by the virus for its survival and disease progression. However, targeting these using anti sense oligonucleotides may result in successful suppression of inflammation and a subsequent decrease in the viral of brain cells.

MiRNA modulation in the brain post VEEV infection indicates a role of miRNAs in VEEV pathogenesis. VEEV is a neurovirulent virus but natural cases of VEEV often does not result in neurological complication, however due to efficient replication of the virus in the peripheral lymphoid system a severe flu like clinical symptoms are presented (Weaver 2005). *In vivo* VEEV is known to efficiently replicate in fibroblasts and epithelial cells during the lymphatic phase of the disease (Gardener et al 2008). Role of miRNAs in VEEV replication in the peripheral system is not known. Hence we further studied the modulation of miRNAs in mouse fibroblasts. MiRNA modulation in mouse fibroblast cells showed a complete different profile in comparison to miRNA modulation in VEEV infected brain. Target prediction and computational analysis revealed that many of these miRNAs may play a role in down regulation of Pi3 kinase and mTOR pathway. These pathways have been shown to play an active role in replication of several viruses including that of alphaviruses (Mohankumar et al 2011, Patel et al 2012). Further *in vitro* studies are needed to conclusively determine the role of these miRNAs in virus replication.

These experiments have for the first time have shown modulation of miRNAs which may play a critical role in regulation of inflammation during VEEV infection in both *in vitro* and *in vivo* systems which improve our present understanding of VEEV pathogenesis. The major finding of these studies is a possible role of brain micro vascular endothelial cells early during the infection since endothelial specific mir-126 was found to be modulated at 48h p.i. Additionally, miRNAs also indicated a involvement of important cellular pathways in VEEV pathology. The role of these factors has been discussed in detail in subsequent chapters.

Chapter 2

Brain Microvascular Endothelial Cells are infected by Venezuelan Equine Encephalitis Virus Infection.

Abstract: VEEV is a neurotropic virus which causes severe encephalitis in both humans and equines. The fatal outcome of the disease depends whether or not virus enters the neurotropic phase of infection. The route of entry to the brain hence plays a critical role in the outcome of the disease. The present understanding of VEEV pathogenesis is that in VEEV enters the brain via the olfactory neuroepithelium of the olfactory bulb and from there spread throughout the brain causing excessive inflammation and neuronal cell death. Few studies believe that this excessive inflammation results in the breach of BBB which then allows a second inflow of virus from the peripheral system without causing any detectable infection in the endothelial cells of BBB. In chapter one, we observed modulation of endothelial cells specific miRNAs in brain after VEEV infection indicating its role in BBB breach. In our previous studies we found the breach of blood brain barrier in the areas of the brain without any significant inflammation. In this study we have identified the role of microvascular endothelial cells lining the blood vessels for their susceptibility to infection with VEEV and possible role in BBB breakdown. We have found that endothelial cells of the blood vessels in the brain are infected by VEEV exhibiting delayed cytopathic effect (CPE). Further, this infection also leads to the change in gene expression of endothelial cells. Specifically, genes responsible for maintenance of BBB are modulated including MMP-9, Agt-1 and Adam-17. We therefore conclude that VEEV infect endothelial cells and may in part lead to BBB breach thus facilitating additional virus entry to the brain along with the olfactory tract.

Introduction

Brain microvascular endothelial cells (BMECs) play a dynamic role in maintaining the BBB integrity. BBB comprises of BMECs and perivascular astrocytes along with pericytes which are separated by a basement membrane (Figure 9). BBB constantly maintains a dynamic selective transport between the blood and the brain thereby maintaining chemical composition of the neuronal "milieu," which is required for proper functioning of neuronal circuits, synaptic transmission, synaptic remodeling, angiogenesis, and neurogenesis in the adult brain (Zlokovic et al 2008). Endothelial cells of the BBB are unique because of the presence of junctional complex proteins which helps in sealing the gap between the cells and selectively allowing transport of essential nutrients to the brain and restricting the entry of harmful compounds, invading pathogens and infiltrating leukocytes (Roe et al 2012). Junctional proteins are mainly tight junctions located at the apical side of the cells which include occludins, claudins and junctional adhesion molecules. The adherent junction proteins like ve-cadherin, caveolin-1 and pecam-1 are located on the basolateral side of the cells. Together, both tight junction proteins and adherent junction proteins confers the low para-cellular permeability and high electrical resistance of the BBB. Gap junction proteins are also identified on endothelial cells but their role is not clear in maintenance of BBB (Zlokovic et al 2008, Bazzoni and Dejana, 2004).

Endothelium of the BBB is highly sensitive and reactive since it is both a source and target for inflammatory proteins and reactive oxygen species (Grammas et al 2011). BBB plays a key role in many neurodegenerative disorders. Dysfunction of BBB leads to the infiltration of inflammatory molecules which might be originated from endothelium itself, immune cells and other immune mediators which then lead to neurodegeneration (Palmer et al 2011). Endothelium dysfunction and BBB damage has been implicated in various neurological disorder including,

alzhiemer's disease, parkinson's disease and multiple sclerosis (Frakas et al 2001, Zlokovic 2008, Engelhardt and Ransohoff, 2005). In most of neuroinflammatory cases, BBB breakdown and tight junction opening is attributed to increased production of cytokines, chemokines, reactive oxygen species (ROS), glutamate, and mmps that may alter the expression and function of tight junctions (Persidsky and Gendelman 2003).

Alphaviruses are known to infect a wide range of host cells including vertebrate and invertebrate cells (Wang et al 1992, Patel et al 2012). Many alphaviruses are known to cause encephalitis in the host where they replicate in neurons and other CNS resident cells. VEEV initially replicates in the peripheral system causing viremia followed by entry into the brain either via olfactory tract or blood brain barrier (Charles et al 1995). In some cases, like SFV and sindbis virus, the cells lining the brain microvessels are infected causing a breach of BBB by the virus (Soilu-Hänninen et al 1994, Rust et al 2012). In case of VEEV, the virus entry occurs predominantly through the olfactory route where the virus first appears as early as 36h p.i (Steele et al 2010). BBB disruption is also observed during VEEV infection in mouse model which is considered as a result of a storm of inflammatory molecules (Sharma et al 2011). Infection of endothelial cells of the blood vessels has been long debated with first reports suggesting the presence of virus in the endothelial cells using electron microscopy (Gorelkin 1960). Later many other studies used immunostaining against the viral surface proteins and were not able to confirm the initial findings. A recent study using virus like particles (VLPs) for VEEV showed the breach of blood brain barrier in response to VLP infection but the endothelial cells were not found to be infected. Interestingly, the same study reported an upregulation of mmp-9 on the endothelium lining as early as 18h p.i when the virus was still in the blood (Schäfer et al 2011). During previous studies from our lab, we have observed endothelial cuffing in the mouse brain tissues

with very less inflammatory damage or cellsin the vicinity. This indicates that inflammation in the CNS may not only be responsible for BBB disruption.

In this study, we have evaluated the role of endothelial cells during VEEV infection. We have examined the infection potential of virulent as wells attenuated strains of VEEV in *in vitro* and *in vivo* conditions. Further, we have also evaluated the role of important genes of endothelial cells during VEEV infections. These studies are important since these may explain the dynamic role of endothelial cells during VEEV infection.

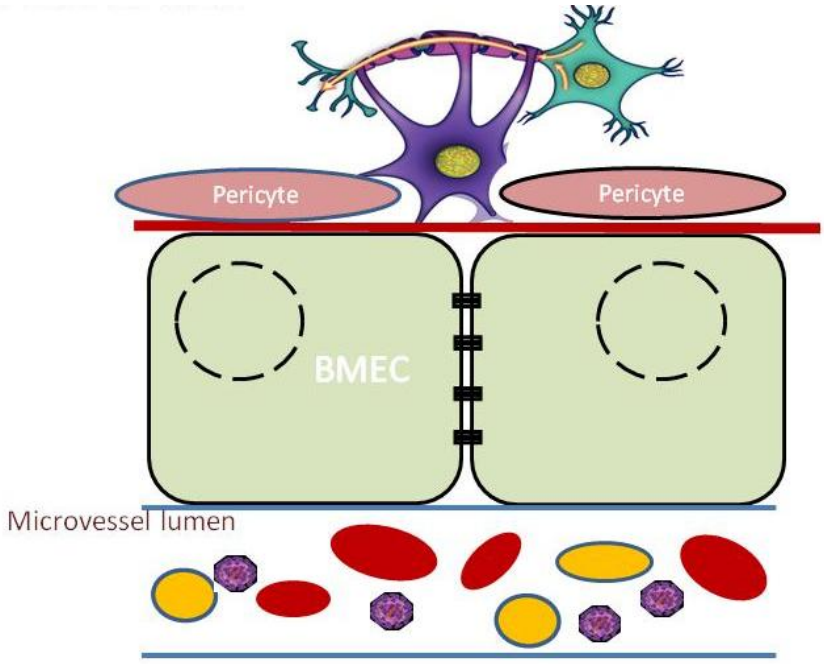


Figure 9: Schematic of the Neurovascular Unit. Endothelial cells and pericytes are separated by the basement membrane. Pericyte processes sheathe most of the outer side of the basement membrane. (Information extracted from Zlokovic 2008)

Materials and Methods

Virus: For cell culture studies TC-83 strain was used which is a live attenuated vaccine strain of VEEV (Kinney et al 1989). TC-83 with cherry red protein expresses a red fluorescent protein upon infection and was a kind gift from Dr Frolov (University of Texas). Studies with TC-83 strain were done in BSL-2 laboratory. Animal infection studies were carried out with a full length cDNA clone of Trinidad donkey strain, V3000.

Cell culture: BMECs from C57BL/6J mice were acquired from Cell Biologics Inc, Chicago IL. Frozen cells were seeded in endothelial cell culture media (Cell Biologics, Inc) along with 5% fetal bovine serum and growth factors (bullet kit, Cell Biologics). The culture flask was coated with 0.1% gelatin for 30m at room temperature before seeding the cells. The cells upon confluency, cells were cultured at ratio of 1:2 to avoid growth of contaminating cells. Cells were discarded after 5^{th} passage.

Animals: Six to eight weeks old male CD-1 mice were obtained from Charles River Laboratories, Wilmington, MA. All the experiments with live virus challenge were carried out at the Uniformed Services University of the Health Sciences (USUHS) bio-safety level 3 (BSL-3) facilities in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care And Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86–23, revised 1996). Virus infection was done by injecting 1000 pfu of VEEV in right footpad.

Virus titer: Supernatant from the cells infected with TC-83 or TC-83 cherry red were harvested and stored at -80˚C. 50% tissue culture infectious dose ($TCID_{50}$) was performed to estimate the virus titer. Briefly, Vero cells (ATCC) were seeded in 96 well plates and grown to a

confluency of 70-80%. 100 µl of cell supernatant was serially diluted and added to the Vero cells. The cells were then incubated with virus for 72h and $TCID_{50}$ was calculated.

Immunofluorescence: 10,000 BMECs were seeded in 8 well chamber slides (BD falcon) and grown till 80% confluence at 37˚C, 5% CO_2 . Cells were then infected with TC-83 cherry red at a MOI of 20. After 1h incubation, cells were washed with saline and replenished with fresh medium. Supernatant were harvested at 12, 24, 48and 96h. The cells were fixed with 4% paraformaldehyde at 4 ˚C for 15m. Paraformaldehyde was preferred over other fixatives to preserve the red fluorescence from the virus infection. Cells were washed with 1X phosphate buffer saline (PBS) for 5min followed by blocking. Blocking was done with 5% bovine serum albumin (BSA) in 1X PBS for 1h at 37˚C. The cells were then incubated with primary antibody for VE-cadherin (goat polyclonal, Santa Cruz Biotech, CA). Primary antibody (stock 20ug/ul) was diluted in 5% BSA at a dilution of 1:100 followed by incubation for 1h at 37˚C. Cells were then washed with 1X PBS for 3 times 5m each and then secondary antibody was added. Fluorescein isothiocyanate (FITC) conjugated donkey anti-goat secondary antibody was diluted to 1:100 (stock 1ug/ul) in 5%BSA and incubated at 37˚C for 30m. Cells were again washed with 1X PBS 3 times for 5m each and then mounted with mounting media with 4',6-diamidino-2-phenylindole (DAPI) (Vector laboratories Inc). The slides were then observed under fluorescent microscope.

Immunohistochemistry: Animals were perfused first with 5 ml ice-cold 1× PBS (Gibco) followed by 10 ml cold 10% buffered neutral formalin (BNF) (VWR). The whole brain was removed and fixed in 10% BNF for 3–4 weeks. Tissues were embedded in paraffin blocks and 5 µm-thick sections were prepared. For VEEV-specific staining, rabbit polyclonal antiserum (1:10000), raised against VEEV, eastern equine encephalitis virus, western equine encephalitis

virus and sindbis virus (kindly provided by Dr Cindy Rossi and Dr George Ludwig, United States Army Medical Research Institute for Infectious Diseases, Frederick, MD), was used as described previously (Sharma *et al.*, 2008). An indirect avidin-biotin-immunoperoxidase technique was used for IHC using Vectastain ABC Elite kit (Vector Laboratories Inc) as specified by the manufacturer. As a negative control, separate sections from each test were incubated with species-matched normal serum. Tissues were counter-stained with Gill modified haematoxylin (EMD Chemicals) or haematoxylin QS (Vector Laboratories).

RNA isolation: Total RNA was isolated from BMECs using standard Trizol method (Invitrogen Inc). Post virus infection, cells were harvested using 500ul of Trizol LS (Life technologies, CA) followed by addition of 0.5 volume of chloroform (Sigma Inc). The cell suspension was incubated for 15m at room temperature (RT). The suspension was then centrifuged at 12000g for 15m at 4˚C to separate the aqueous and the inorganic phase. The aqueous phase was collected in a fresh tube and equal volume of isopropanol was added followed by centrifugation at 12000g for 15m. Pellet obtained was washed with 70% ethanol at 10000g for 10m twice. Decant excess ethanol and air dry pellet for 10m. Resuspend the pellet in 50ul of DNase & RNase free water.

cDNA synthesis: cDNA synthesis was performed using superscript III first strand cDNA synthesis kit (Life technologies Inc). Briefly, 1ug of total RNA was used to perform cDNA synthesis. 10ul of RT reaction mix was added along with 2ul of RT enzyme and RNA. The total volume of the reaction was made up to 20ul with DEPC treated water. The reaction was mixed by tapping and a brief centrifuge followed by incubation at 25˚C for 10min. The reaction was then started by incubation at 50˚C for 30m. The reaction was terminated at 85˚C for 5m. 1ul of

RNase H was added and incubated at 37˚C for 20m to remove DNA-RNA hybrids. The cDNA product obtained was either used immediately or stored at -20˚C for later use.

Gene Expression: Modulation of endothelial specific gene expression after infection with TC-83 in BMECs was studied using endothelial specific PCR arrays (Qiagen Inc). These PCR arrays profiles expression of 84 genes which are central to the endothelial cells function. These genes are shown to play an important role in angiogenesis, vasoconstriction, vasodilation, inflammatory response, apoptosis, cell adhesion, coagulation and platelet activation. To analyze gene expression, cDNA was used to perform real time PCR using sybr green chemistry. Briefly, master mix was prepared by adding 1350ul of 2X RT reaction mix with sybr green along with 102 ul of diluted cDNA product. Water was added to make up the final reaction volume to 2700ul. 25ul of this master mix was then added to each well of PCR array and the wells were sealed using the cap strips. The PCR arrays was centrifuged briefly and loaded on the applied bio systems 7900 HT real time PCR systems. Data analysis was done using a web based tool for analysis of PCR array data from Qiagen.

Western blotting: Cell lysates were harvested using 1X RIPA buffer (G-Biosciences, Inc) supplemented with EDTA free protease inhibitor and phosphatase inhibitors. The lysates were then briefly sonicated to remove clumps due to the presence of genomic DNA. Total protein in the lysates was quantitated using protein assay kit (Biorad Inc). 4-12% Bis- Tris gel (Life Technologies, Inc) was used to run the protein samples. To prepare the protein samples, 20ug protein was used along with gel loading buffer and reducing agent. The samples were then heated at 95˚C for 10m. Samples were loaded on the gel and gen was run at 180V for 45m with 1X MOPS-SDS gel running buffer (Life Technologies, Inc.). Proteins were electrostatically transferred to a nitrocellulose membrane using a transfer assembly for 90m at 75V (Biorad, Inc).

Following transfer, membrane was blocked in 5% fat free milk for blocking for 1h at RT with light agitation. Anti-mouse primary antibody for MMP-9 (Santa Cruz Biotech, inc) diluted in 5%BSA (1:1000) and the membrane was incubated overnight at 4˚C. Membrane was washed with Ix tris buffer saline with 0.1% tween-20 (TBST) for 5m, 3 times. Horse radish peroxidase (HRP)conjugated goat anti-rabbit secondary antibody was diluted (1:5000) in 2% fat free milk and incubated for 1h at RT. Membranes were again washed with 1X TBST and then incubated for 1m in 10ml of lightning fast substrate for HRP(Perkin Elmer, Inc) and developed on a X-ray film (Bioexpress, Inc).

RESULTS

BMECs are infected by VEEV in an *in vivo* mouse model: We wanted to study whether VEEV infect BMECs *in vivo*. CD-1 mice were infected with 1000 pfu of virulent V3000 strain of VEEV. Brains were collected from infected animals at 24h, 48h, 72h, 96h and 120h post infection. Immunohistochemical analysis was performed on the formalin fixed and paraffin embedded sections of these mouse brains for VEEV specific antibody. It was observed that during later phase of infection at 96h and 120h many cells lining the blood vessels which appeared as BMECs are positive for VEEV staining though not all the vessels were found to be positive for VEEV staining. The vessels which did show positive staining for VEEV infections also appeared leaky due to the presence of edema and infiltrating lymphocytes (Figure 10). VEEV specific staining was also observed in majority of the brain cells but in some cases neuronal cells adjoining the infected BMECs were found negative for VEEV staining. This observation is peculiar since it indicates that the virus infection of BMECs might have occurred due to the presence of virus in the blood rather than virus form an infected brain cell. However, infection of BMECs of the brain at 72h was much less whereas at 48h we did not found any micro vessel to be positive for VEEV infections.

BMECs are infected by TC-83 in *in vitro* conditions: From the *in vivo* data it was clear that VEEV infects BMECs of BBB. We further wanted to validate whether VEEV can infect pure culture of BMECs. Brain endothelial cells from C57BL/6J mice were purchased from Cells Biologics, Inc. To determine whether VEEV can infect BMECs under *in vitro* conditions, cells were infected with an MOI of 5 with TC-83. Cell supernatants were harvested at 24h, 48h, 72h, 96h and 120h p.i and virus titer was estimated using $TCID_{50}$. Virus titer from the supernatant indicated a productive but persistent infection of endothelial cells with peak virus titer reaching

at 24h pi and remained same throughout the remaining course of infection. A hall mark of VEEV infection is appearance of CPE in the cells within 24-48h of infection, however, in case of BMECs a clear CPE was not observed until 120 h (Figure 11).

There is a possibility that the low level infection and the absence of CPE might be because of the infection of the contaminating cells, which may include macrophages, astrocytes and oligodendrocytes in the primary cells culture. To rule out the possibility of infection of contaminating cells, we performed immunostaining with ve-cadherin, an endothelial cells specific marker. Immunostaining of the cultured BMECs indicated the presence of pure population of endothelial cells. These cells were also infected with TC-83 expressing cherry red protein which produces red fluorescence in the infected cells. Fluorescent imaging of these cells confirmed the infection of ve-cadherin positive cells by TC-83 due to the presence of red fluorescence in these cells as early as 24h pi. However, we found that not all the cells were positive for VEEV infection and approximately only 50% of the total cells population was found positive for infection (Figure 12). To confirm the replication of VEEV inside these cells we performed western blot on the infected BMEC lysates with an antibody for VEEV E2 surface glycoprotein. Western blot analysis confirmed the presence of VEEV specific E2 glycoprotein at 48h p.i. These results suggested that VEEV infects BMECs in cells culture however, the infection of these cells is low but persistent which unlike other host cells which show productive virus replication resulting in CPE (Figure 13).

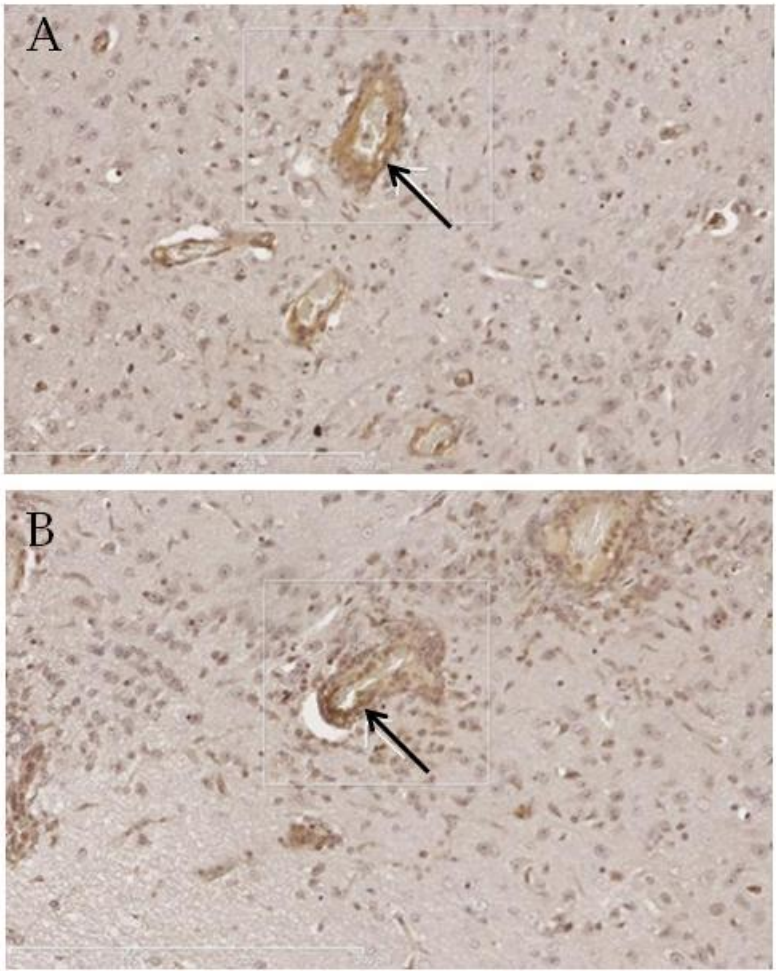
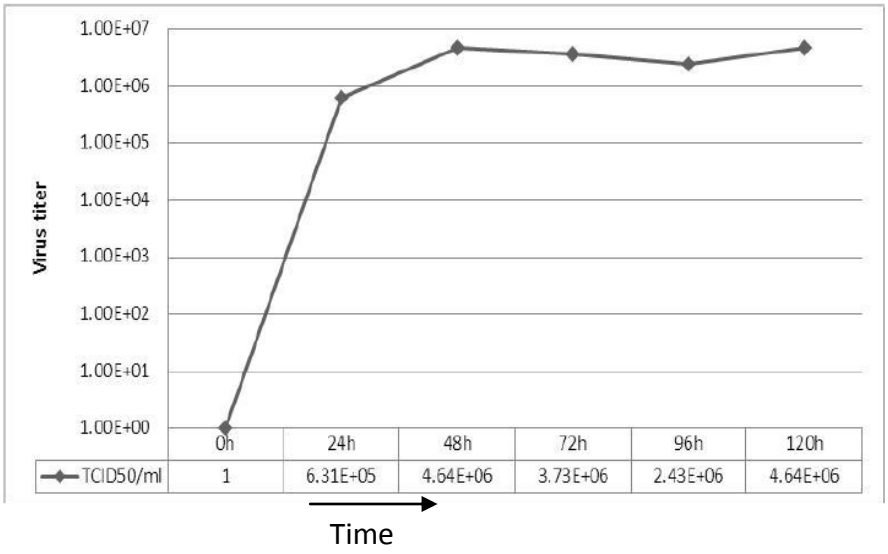


Figure 10: Immunohistochemistry of VEEV infected brain sections stained for VEEV antigen showing presence of virus in endothelial cells. a). Brain sections infected with V3000 strain were stained with VEEV specific antibody. Staining shown is for 96h b. 120 h post infection. Positive staining was observed at 96 and 120h p.i in BMECs.

A.



B.

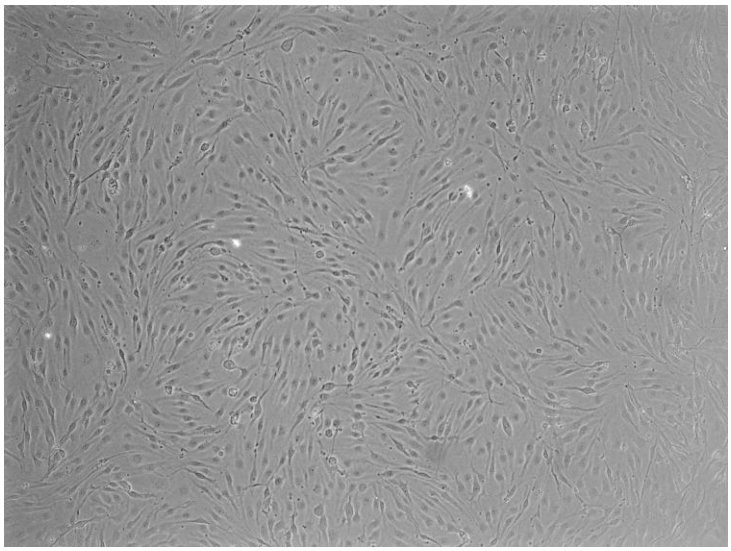
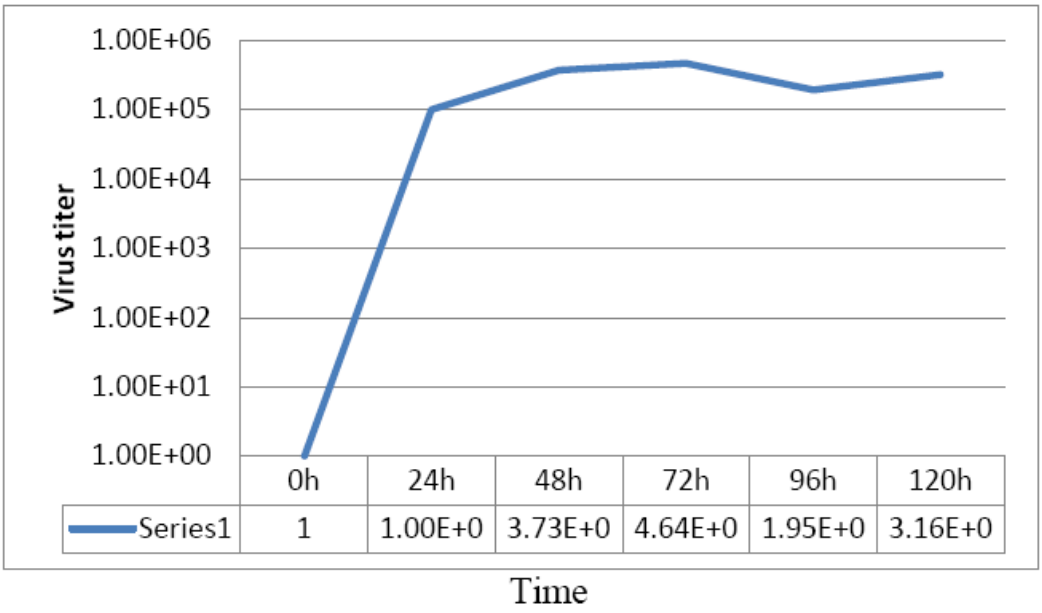


Figure 11: Virus titer of VEEV (TC-83) in mouse primary brain microvascular endothelial cells. A. Primary mouse BMECs were cultured in the *in vitro* conditions up to confluency. Cells were infected with VEEV (TC-83) at an MOI of 5 and supernatant were harvested at the time points indicated. Virus titer was estimated using $TCID_{50}$ in vero cells. B. BMECs infected with TC-83 at 24h p.i. No apparent CPE was observed in these cells.

a.



b.

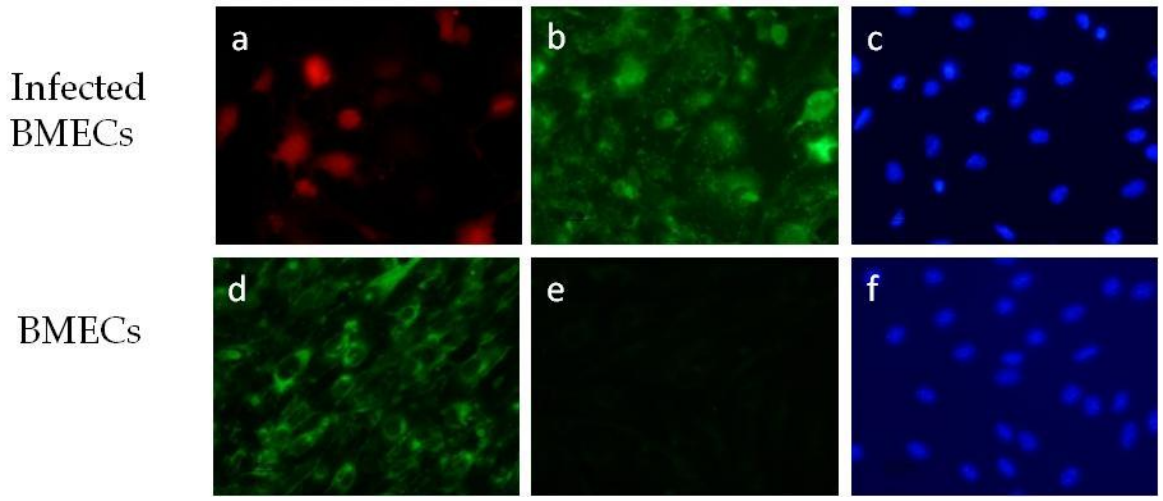


Figure 12: Infection of BMECs with TC-83 cherry red and immunofluorscence for VE cadherin in endothelial cells infected with TC-83. (A) Virus titer of infected BMECs with TC_83 with cherry red protein. (B) a)TC-83 infected cells, b) VE cadherin staining in TC-83 infected cells, c)DAPI stain , d) positive staining for VE cadherin in uninfected cells, e) Negative control for VE cadherin. f) DAPI control.

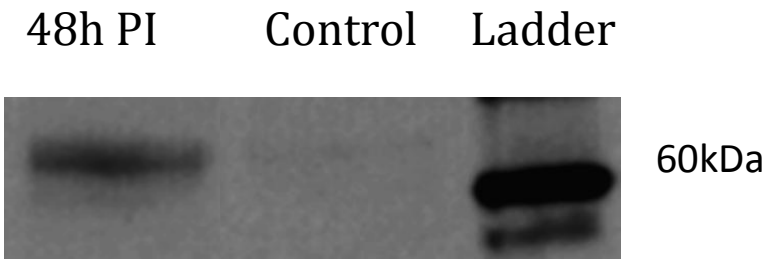


Figure 13: Presence of E2 glycoprotein of VEEV in cell lysates of infected BMECs**.** BMECs were infected with TC-83 at an MOI of 5. Cell lysates were used to probe for E2 glycoprotein. Lane 1: Cells infected for 48h 2. Cells infected for 24h, 3. Uninfected control, 4, Ladder.

VEEV infection modulates gene expression in BMECs: *In vitro* and *in vivo* infection experiments suggested the infection of endothelial cells which indicates that VEEV might modulate the expression of endothelial cells gene expression. Schaefer et al 2012 also suggested an increase in mmp-9 expression immediately after peripheral infection with VEEV. We hypothesized that this increased expression of MMP-9 may be because of the infection of endothelial cells by VEEV. To test this hypothesis, we analyzed the infected endothelial cells for endothelial cell specific genes expression modulation using focused gene array. This panel of gene array contains primers for 83 endothelial cells specific gene which belongs to the category of adhesion molecules, cell migration, apoptosis and endothelial cell activation. Cells were infected with an MOI of 5 and RNA was harvested for analysis at 12h p.i. Gene expression analysis showed a significant up regulation of three genes which include Agt, MMP-9, and Casp-6 and down regulation of Adam 17 was observed. Modulation of these genes so at 12h p.i confirmed the VEEV infection in these cells (Figure 14). Further we validated the data of the gene expression using western blot. Western blot analysis using an antibody for MMP-9 indicated a significant upregulation of MMP-9 protein in infected cells at 24 and 48h p.i. No significant difference in protein levels was observed between control and infected cells at 12h p.i. This difference may arise due to the time taken for protein synthesis after mRNA transcription and hence delayed appearance of protein than mRNA (Figure 15).

Genes Upregulated

S. No	Gene	Fold Change
1	Agt	1876
2	Casp6	314
3	Mmp9	18849

Genes Downregulated

S. No	Gene	Fold Change
1	Adam17	-5.28

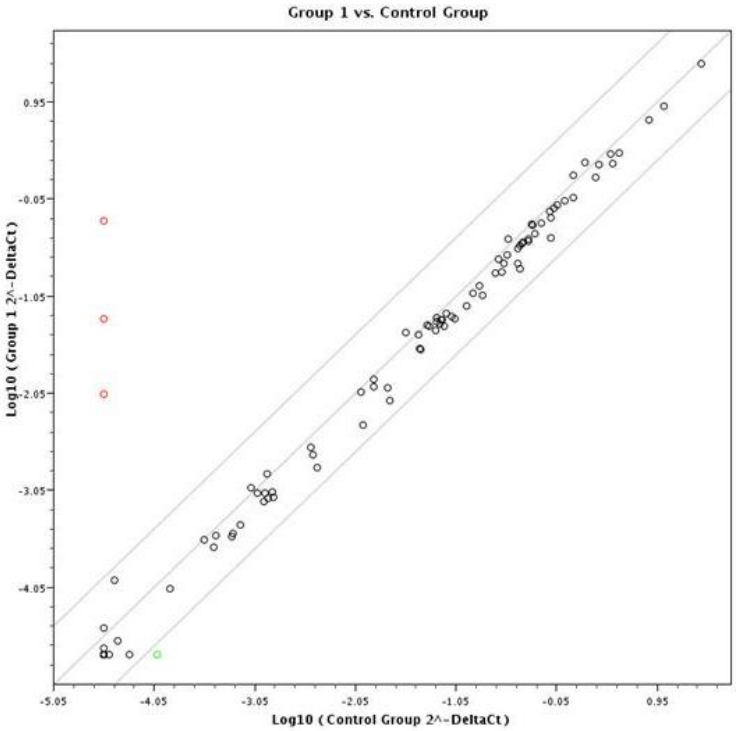
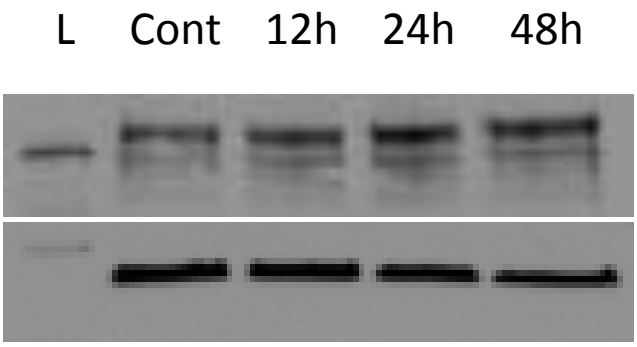


Figure 14: Modulation of gene expression of BMECs *in vitro* infected with VEEV (TC-83) at 12h p.i. RNA from infected BMECs was isolated and endothelial gene specific pcr array was used to profile endothelium specific genes.

A.



B.

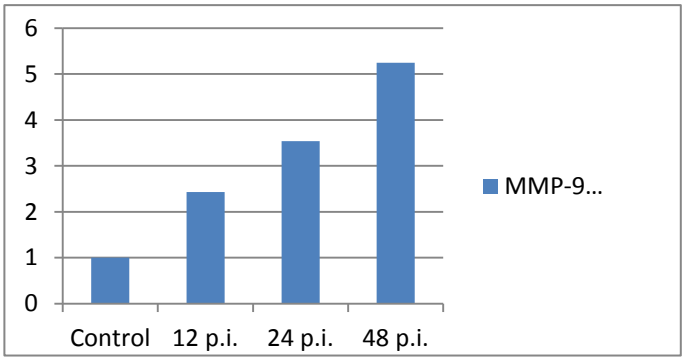


Figure 15: MMP-9 expression in BMECs infected with TC-83. A) Cells were infected at MOI of 5 and cells were harvested at 12h, 24h and 48h post infection. Western blot was performed using a monoclonal antibody against mmp-9. Lanes: 1: Cell lysates 48h p.i, 2: Cell lysates 24h p.i, 3:Cell lysates 12h p.i, 4: Control cells, 5: Ladder. B) Densitometric analysis of the western blot image.

Discussion

Entry into the CNS is a critical step in determining the fate of VEEV infections. In most cases the virus replicates to high titers in the peripheral system and is soon cleared off by host immune system. The end fate is determined whether the virus gains entry into the CNS or not. BBB is known to play a dynamic role in restricting the harmful pathogens to gain access to the brain but in many cases various pathogens have evolved ways to disrupt the BBB (McGavern and Kang 2011). One way of entering into the CNS through BBB is by infecting the cells and damaging the barrier properties. Several viruses which include JC virus, poliovirus, Epstein–Barr virus (EBV), mouse adenovirus 1 (MAV-1), human T-lymphotropic virus type 1 (HTLV1) and West Nile virus (WNV) have the capability to infect the micro vascular endothelial cells of BBB *in vitro* (McGavern and Kang 2011). Other mechanism to enter CNS includes trojan horse mechanism in which the virus enters CNS along with an infected monocyte and infection of peripheral neurons like sensory neurons in olfactory tract (McGavern and Kang 2011).

In case of VEEV, the initial virus entry into CNS occurs as early as 36h p.i in mouse where the virus first appears in the olfactory bulb (Charles et al 1995). The initial viral entry leads to a pro-inflammatory cytokine storm in the brain which is considered as a reason for the disruption of the BBB. This breach of blood brain leads to a secondary invasion of virus from the peripheral system which ultimately leads to high viral load in brain resulting in neuronal and the death of the host (Charles et al 1995, Steele et al 2010). In a recent study with VLPs of VEEV, it was found that mmp-9 expression was highly up regulated in mouse BMECs after VLP infection which contributes to the BBB damage. Treatment with an mmp-9 inhibitor largely preserves the BBB integrity after infection. This results in a delayed virus appearance in the brain which is also reflected in reduced morbidity and increased mean survival time in mice (Schäfer et al

2011). This observation raises one critical question related to viral entry into the CNS, that, if the primary route of entry into the CNS is via olfactory neuroepithelium then treatment with an mmp-9 inhibitor theoretically should not delay the virus appearance in CNS. Further, along with the delay in virus appearance the inflammation in the brain was also reduced thereby delaying the onset of BBB breach due to inflammatory molecules. This suggests that virus entry into the brain may not be solely dependent on olfactory tract but damaged BBB may also contribute to the virus dissemination in the CNS. We hypothesized that VEEV infects endothelial cells of the BBB and changes its permeability and hence gains access to the brain along with olfactory route.

Since there are no conclusive reports of the infection of endothelial cells of the BBB by VEEV, we carried out detailed experiments to test our hypothesis. First, we wanted to check VEEV infection of BMECs *in vivo*. On a gross observation, infection of endothelial cells did not appear very prominent, but careful observation of the revealed VEEV antigen staining in endothelial cells of the blood microvessels. However, not all the vessels appeared positive for VEEV infection early in the infection but their numbers increased with the disease progression. These findings are important as for the first time we showed infection of endothelial cells with both the attenuated as well as the virulent strain of VEEV.VEEV infection did not cause cell death, however, a widespread BBB alteration was observed *in vivo*. Therefore, a possibility may be that VEEV might change the permeability of the BBB with a low level infection in these cells.

Next, we wanted to test whether VEEV can infect BMECs *in vitro*. Our data indicates that VEEV infect BMECs under *in vitro* conditions with peak virus titers reaching at 24 p.i. Moreover, virus induced CPE which is a common phenomenon with VEEV infections appears very late in VEEV infection of BMECs. We verified our results with both virus titer and fluorescence microscopy. Fluorescence microscopy indicated that approximately 50% of the

total cells showed a productive infection of BMECs. These observations suggest that VEEV causes persistent infection of BMECs, Virus replication unlike fibroblasts is lower and exhibit no or delayed CPE. .

We further analyzed the modulation of genes which play an important role in maintaining the homeostasis in endothelial cells and BBB. PCR array data indicated modulation of genes which includes mmp-9 and adam-17. VEEV therefore, not only infected BMECs but also modulated the genes which help in maintaining an intact BBB. Among these genes, mmp-9 is of particular importance since upregulation of mmp-9 in endothelial cells have been reported with other viruses as well as with VEEV VLPs which lead to a leaky BBB (Agarwal et al 2008, Candelario-Jalil et al 2009). We further confirmed the protein expression level of mmp-9 in infected BMECs using western blot which confirmed the results of pcr array. This increase in mmp-9 may explain the localization of mmp-9 in endothelial cells during early phase of peripheral VEEV infection as reported by schäfer et al 2011.

With these findings, we conclude that though the initial virus appearance in the CNS may occur through olfactory tract but at the same time, a low level infection of BMECs by virus also allows passage of virus across the BBB which is independent of inflammation caused by initial infection. Further studies in non-human primate which closely mimic the pathology of VEEV in human cases can improve our understanding about the role of endothelial cells in VEEV infection.

CHAPTER: 3

Role of phosphoinositidyl-3-kinase and Akt pathway in Venezuelan equine encephalitis virus infection.

Abstract: Phosphoinositidyl-3-kinase (Pi3) pathway play an important role in cellular signaling pathways in response to external stimuli to drive cellular growth and development. Several downstream mediators of the pathways are phosphorylated subsequent to the activation of pi3-kinase pathway. Few of the important downstream regulators are AKT or protein kinase b and mTORC. Pi3-akt pathway modulation has been implicated in various pathogen infection including RNA viruses. Pi3-akt pathway has been shown to be utilized by virus to suppress the cellular machinery to support their own replication. In case of VEEV, the role of this pathway is unknown. In this study we have analyzed the modulation of Pi3-AKT pathway in response to the viral infection. We have found that VEEV infection transiently activates the pathway which is followed by reduction in the phosphorylated forms of AKT. A second upregulation phase of the Pi3-akt pathway was also observed. Treatment with inhibitor of the pathway, LY294002, resulted in a significant decrease in the yield of the virus indicating the importance of the pathway for virus replication. Lastly, we treated the cells with inhibitor of mTORC, rapamycin, to evaluate the role of mTORC pathway in VEEV replication. No significant decrease in virus titer was observed with rapamycin treatment, indeed a marginal increase in virus was observed. These results indicate a role of Pi3-akt pathway in virus lifecycle which can be utilized to develop novel antiviral therapy.

Introduction

Phosphatidylinositol 3-kinases (Pi3-kinases) are a group of enzymes which catalyzes phosphorylation of plasma membrane phosphoinositides at the 3'-OH of the inositol ring to produce lipid second messengers. Pi3-kinases are categorized as three different classes of enzymes, Class-I, II and III. Class I Pi3-kinase are heterodimeric proteins which consists of an 110kDa catalytic domain and a regulatory domain. The regulatory domains are further classified as class Ia and Ib. In class Ia, the regulatory subunit consists of seven proteins which are coded by alternative splicing of three genes p85α, p85β and p55γ. In class Ib, the regulatory subunit consists of 101kDa adaptor molecule (Vanhaesebroeck et al, 2001).

Class Ia Pi3-kinases are activated when the Src homology domain (SH2) bind to the adapter subunit to auto phosphorylate tyrosine kinase receptors. Upon activation, Pi3 kinase phosphorylate phosphatidylinositol-4,5-diphosphate (PiP2) *in vivo,* to produce phosphatidylinositol- 3,4,5-triphosphate (PipP3) (Cantrell 2001). PiP3 then can phosphorylate a wide variety of downstream signaling molecules. One of the important downstream signaling molecules is AKT also termed as protein kinase B (PKC B) (Coffer & Woodgett, 1991). Three different types of AKT isoforms (AKT-1,2and3) are found in mammals and they have a wide tissue distribution (Cooray 2004). All the AKT isoforms have three domains, an N terminal PH domain, a catalytic domain and a C-terminal hydrophobic domain. The PH domain of AKT interacts with PiP3 followed by its localization to plasma membrane. Once on the plasma membrane, AKT is phosphorylated at Thr308 of the catalytic domain by phosphoinositide dependent kinase 1 (PDK-1) and Ser473 of the C-terminal hydrophobic domain by an uncharacterized protein kinase (Scheid, M. P. & Woodgett, J. R. 2003).

Phosphorylated AKT activates downstream signaling molecule by phosphorylating those molecules at Thr/Sr residues which then regulate cell growth, proliferation apoptosis and cell survival. Among the various signaling molecules, AKT regulates phosphorylation of many pro-apoptotic genes like BAD, member of bcl-2 family, caspase-9, glycogen synthase kinase-3 β (GSK-3β). These apoptosis inducer genes when phosphorylated are inactivated and no longer can induce apoptosis (Cardone et al, 1998).

Another important downstream effector molecule of AKT is mTOR kinase (mammalian target of rapamycin). Two functionally different mTOR complexes are found in mammalian cells mTORC1 and mTORC2 which differ in the major protein binding partner. Raptor protein is associated with mTORC1 whereas rictor is found with mTORC2 complexes. These two complexes differ in their sensitivity to rapamycin, mTORC1 is sensitive but mTORC2 is insensitive to rapamycin (Buchkovich et al 2008).

mTORC 1 protein complexes regulate the cap dependent translation initiation by phosphorylating p70S6 kinase (S6K) and the eIF4e binding protein (4E-BP) (Mamane et al 2006). When mTORC is active it phosphorylates 4E-BP protein and prevents it binding to eIF4e and hence the translation complex bids to the 5' cap and initiate translation. When mTORC1 is inactive, 4E-BP binds to eIF4e and dissociates the initiation complex thereby inhibiting translation and cell growth and differentiation. Together with all the interacting proteins, Pi3-kinase and AKT pathway regulate the cell growth survival and apoptosis.

It is increasing becoming clear that virus and other host parasites modulate Pi3-kinase pathway as a mechanism to inhibit host apoptotic machinery during an acute infection, chronic infection and virus induced cellular transformation (Cooray et al 2004, Ehrhardt et al 2009).

Many studies have reported the modulation of Pi3-kinase pathway by DNA viruses such as DNA tumor viruses. Suppression of Pi3-kinase pathway by these viruses was essentially to inhibit apoptosis and cellular growth. Few of the viruses and the protein responsible for modulation of Pi3-kinase pathway includes, LMP1 protein of Epstein-Barr virus, the E5 protein of Papillomavirus and HBx protein of Hepatitis B viruses (Cooray, 2004).

Like DNA viruses, few RNA viruses also cause latent infection of the cells which result in carcinomas. One such example is the hepatitis C virus which chronically infects and transforms hepatocytes of the liver by modulating the Pi3-kinase pathway (Cooray et al 2005). The non-structural protein 5-A (NS5A) of the Hepatitis C virus bind to the SH-3 domain of p-85 subunit of Pi3-kinase and phosphorylates it and hence activating the pathway and suppress apoptosis (Street et al 2004).

Most RNA viruses are known to cause lytic infection resulting in cell death. Role of pi3-kinase pathway have been studied in many different RNA viruses. Respiratory syncytial virus (RSV), suppresses apoptosis by activation of NFkB complex through activation of Pi3-kinase pathway (Ehrhardt et al 2009). Inhibition of Pi3-kinase pathway by specific inhibitor leads to extensive apoptosis by RSV (Thomas et al 2002). Other RNA viruses which modulates pi3-kinase pathway to suppress cellular apoptosis include, coxsackie B virus, SARS coronavirus , rubella virus , dengue virus, Japanese encephalitis virus and poliovirus (Zhang et al, 2003, Mizutani et al, 2004, Cooray et al, 2005, Lee et al, 2005, Autret et al, 2008). In addition to suppression of apoptosis, Pi3-kinase has been shown to lay an important role in the entry of some RNA viruses. Ebola virus activates pi3-kinase pathway very early in the infection which is necessary for the viral entry. Inhibition of the pathway leads to the impaired entry of the virus (Saeed et al 2008).

In alphavirus, role of Pi3-kinase pathway has been studied in drosophila cells where infection of sindbis virus leads to the activation of the pi3-kinase pathway and its activation was shown to be a necessary event in a productive sindbis virus infection (Patel et al 2012). In contrast to drosophila cells, sindbis virus replication was shown to be independent of pi3-kinase pathway in human embryonic kidney cells and virus efficiently replicates in presence of inhibitors of Pi3-kinase, akt and mTOR (Mohankumar et al 2011).

In VEEV, role of Pi3-AKT-mTOR pathway has not been studied yet. Our study on miRNA in mouse fibroblasts (Chapter 1) post VEEV infections indicated Pi3-kinase pathway molecules as a target for the modulated miRNAs. We therefore hypothesize that VEEV infection modulated Pi3-kiase pathway and utilize it for virus survival and replication. In this report we have shown the effect of VEEV replication on Pi3-AKT-mTOR pathway.

Material and Methods

Cell culture: Mouse L-11 cells were cultured as previously described previously in chapter 1. Virus infection of mouse L-11 cells was done with a laboratory strain TC-83 of VEEV which is a live attenuated vaccine strain of VEEV. Cells were infected with an MOI of 1 in 1 XPBS and incubated for 1h at 37 ºC and 5% CO2. Virus was washed after 1h and cells were supplemented with fresh medium.

Inhibitor Treatment: Rapamycin and Pi3-kinase inhibitor LY294002 was acquired from Cell signalling Inc. Rapamycin was resuspened in dimethyl sulphoxide (DMSO) (Sigma Inc). Cells were serum starved for 24h before treatment with Pi3-kinase inhibitor LY294002 or rapamycin. Dose used for rapamycin treat are 10, 20 and 30nM whereas does for LY204002 used were 5, 10 and 20µM.

Antibodies: Primary rabbit antibodies for AKT, p-AKT, pi3-kinase, p-pi3-kinase, beta actin were acquired from Cell signaling Inc. Goat anti-rabbit alkaline phosphatase conjugated secondary antibodies were obtained from Bio Rad Inc.

TCID 50: Virus titer estimation was performed as described previously in chapter 2.

Cell survival assay: Cell survival assay was done using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. MTT assay kit was acquired from Life Technologies Inc. Briefly, 5000 vero cells were seeded in 96 well plates and incubated overnight. Cell was treated with either rapamycin or LY294002 at various concentrations under serum starved conditions for 24h. After incubation, 10µl of MTT reagent as was added to each well and was incubated for 6h to allow for the development of the formazone crystals. 100ul of the solublization buffer, as provided with the kit, was added to each well and incubated at 37□C

overnight. Absorbance reading was taken using an ELISA plate reader a 570nm. The absorbance from the blank well was deducted from all the cells and relative cell survival was calculated.

Western Blot: Cell lysates were harvested using 1X RIPA buffer (G-Biosciences, Inc) supplemented with EDTA free protease inhibitor and phosphatase inhibitors. The lysates were then briefly sonicated to remove clumps due to the presence of genomic DNA. Total protein in the lysates was quantitated using protein assay kit (Biorad Inc). Western blot analysis was done as described in materials methods section in chapter 2.

Results

VEEV infection of L-cells modulates Pi3-kinase signaling by phosphorylating Pi3-kinase and AKT early in the infection: Mouse L-11 fibroblasts cells were grown till confluency followed by 24h serum starvation. Cells were serum starved to study the pathway modulation due to virus infection since serum growth factors are known to activate the cellular pathways and hence can introduce bias in the findings. Cells were infected with a laboratory vaccine strain of VEEV, TC-83 at an MOI of 1. Cells were harvested at various time intervals starting at 15m post infection till 24h p.i. Virus titration was done by estimating $TCID_{50}$ to determine positive infection. All the cells were positive for infection as indicated by the virus titer (Figure 16). To determine whether VEEV infection modulates the Pi3-kinase pathway we used the cell lysates and probed it with antibody with for phosphorylated and non-phosphorylated forms of pi3-kinase. Endogenous forms of phosphorylated pi3-kinase are present at a very low levels in the cells and hence the signal on the western blot was very weak (data not shown). AKT which is a downstream signaling molecule gets phosphorylated upon activation of pi3-kinase. Hence, we assumed the phosphorylation of AKT as a direct measurement of pi3-kinase activation. Phosphorylated forms for AKT increased immediately after the VEEV infection with high levels of phosphorylated AKT were present at 15m and 30m after the infection which gradually decreased till 12h and increased thereafter. This is consistent with the observation with other RNA viruses where the virus transiently increases the Pi3-kinase activity to support its replication immediately after the virus entry. Levels of total AKT protein were used as baseline measurement (Figure 17).

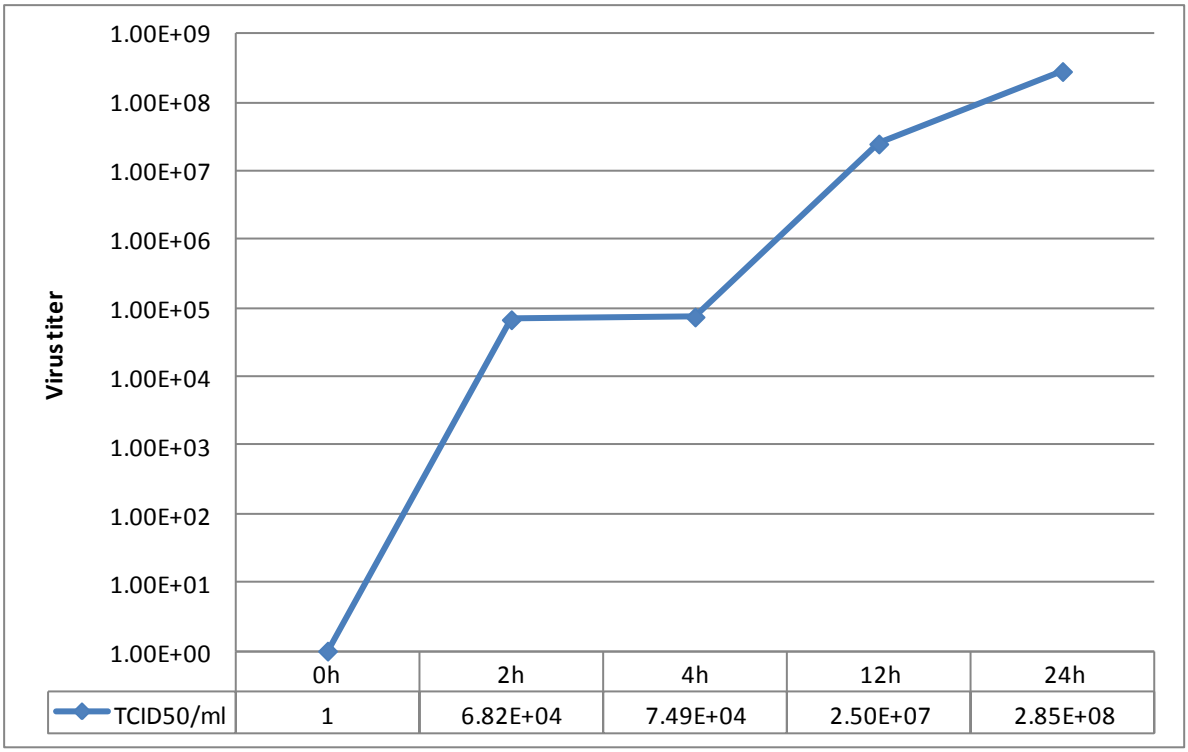


Figure 16: Virus titer in L11 cells infected with TC-83. Cells were infected at an MOI of 1 and supernatant was collected at 2h, 4h, 12h and 24h p.i. TCID50 was performed using vero cells. Virus titer increased with time and peak titers were observed at 24h p.i.

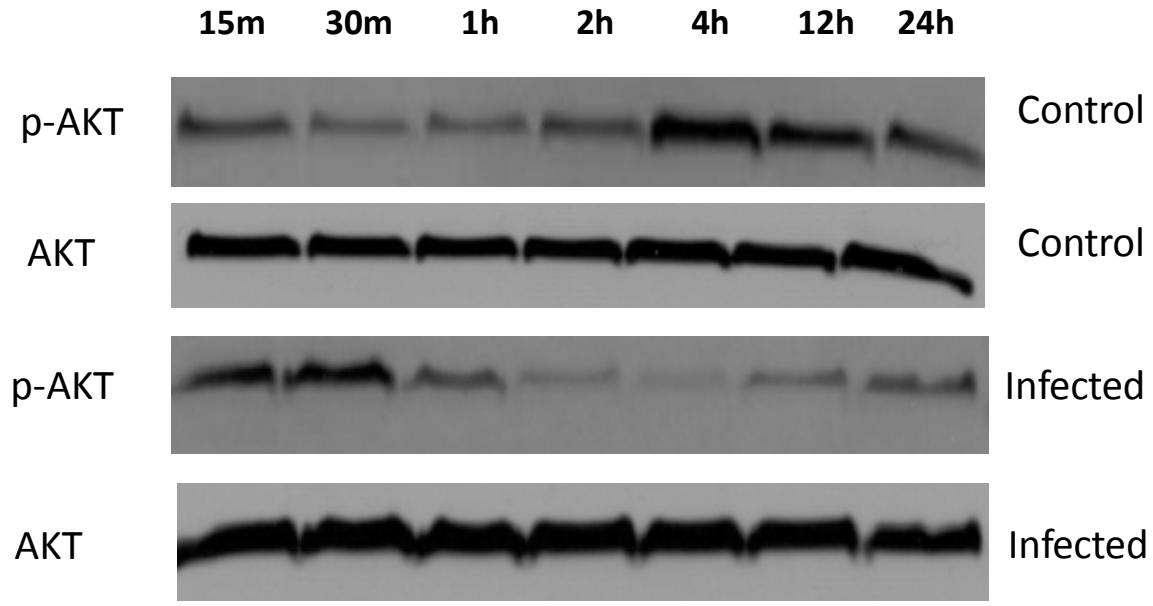
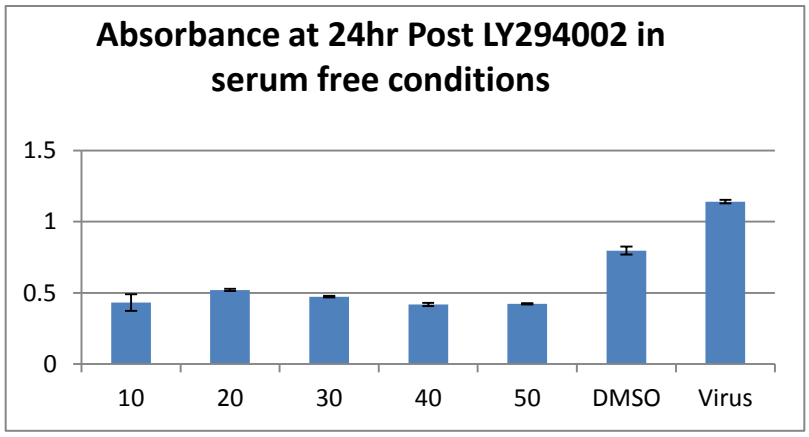


Figure 17: Western blot showing modulation of phosphorylated AKT post VEEV infection in L11 fibroblast cells. The top two panels show the levels of phosphorylated AKT (p-AKT) in control cells which suggest an almost uniform expression. The total akt protein was used as a baseline control. The bottom two panels show the levels of p-AKT in l cells infected with VEEV. It was observed that at 15 and 30m p.i there appears a transient increase in phosphorylation of AKT due to VEEV infection. The p-AKT levels thereafter decreased and again increased at 12 and 24h p.i.

Decrease in virus load after LY294002 treatment of L cells: Since it was clear that VEEV increases the phosphorylation events in the Pi3-kinase pathway, we hypothesized that blocking the phosphorylation may decrease the virus replication. To test our hypothesis, we used a chemical inhibitor of Pi3-kinase, LY294002, which specifically blocks the activity of Pi3-kinase. To determine whether treatment with the inhibitor is toxic to the cells, a MTT assay was performed to determine the cell proliferation after virus infection and LY294002 treatment. Cells were serum starved followed by treatment with increasing doses of LY204002 from 10µM to 50µM. Absorbance reading from MTT assay indicated a reduction in the overall metabolic activity of the cells after treatment with LY294002, which was almost consistent among all the doses (Figure 18). This reduction is expected since blocking of the Pi3-pathway will arrest cell growth. To confirm that the treatment with LY294002 did not cause cell death we observed the cells under microscope. Images taken by microscope showed a reduction in cell division but no apparent toxicity (Figure 19). To test the effect of inhibitor on virus replication, cells were serum starved and treated with LY294002 for 24h before infection. Cells were infected with TC-83 at an MOI of 1 and the cell supernatant was collected at 24h p.i. Western blot was performed on the cell lysates to confirm the inhibition of phosphorylation AKT and Pi3-kinase which indicated a complete inhibition of phosphorylation of both AKT and Pi3-kinase (Figure 20). To estimate the virus replication, cell supernatant were collected and virus titers were determined by $TCID_{50}$. Virus titer results indicated that treatment with LY294002 reduced the virus replication at 20uM doses, virus replication was inhibited by more than 85% compared to control cells (Figure 21).

A.



B.

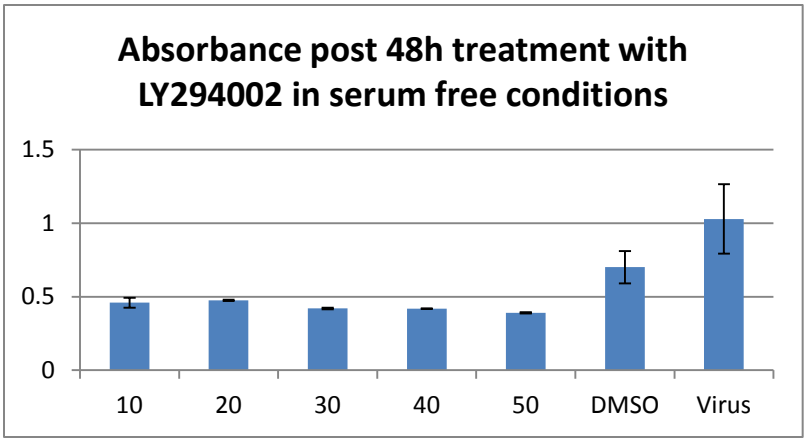


Figure 18: Cell proliferation assay for determining the cytotoxicity of the treatment with LY294002 in L-11 cells. (A) Cell proliferation at 24h post treatment with LY294002. (B) Cell proliferation at 48h post treatment with LY294002.

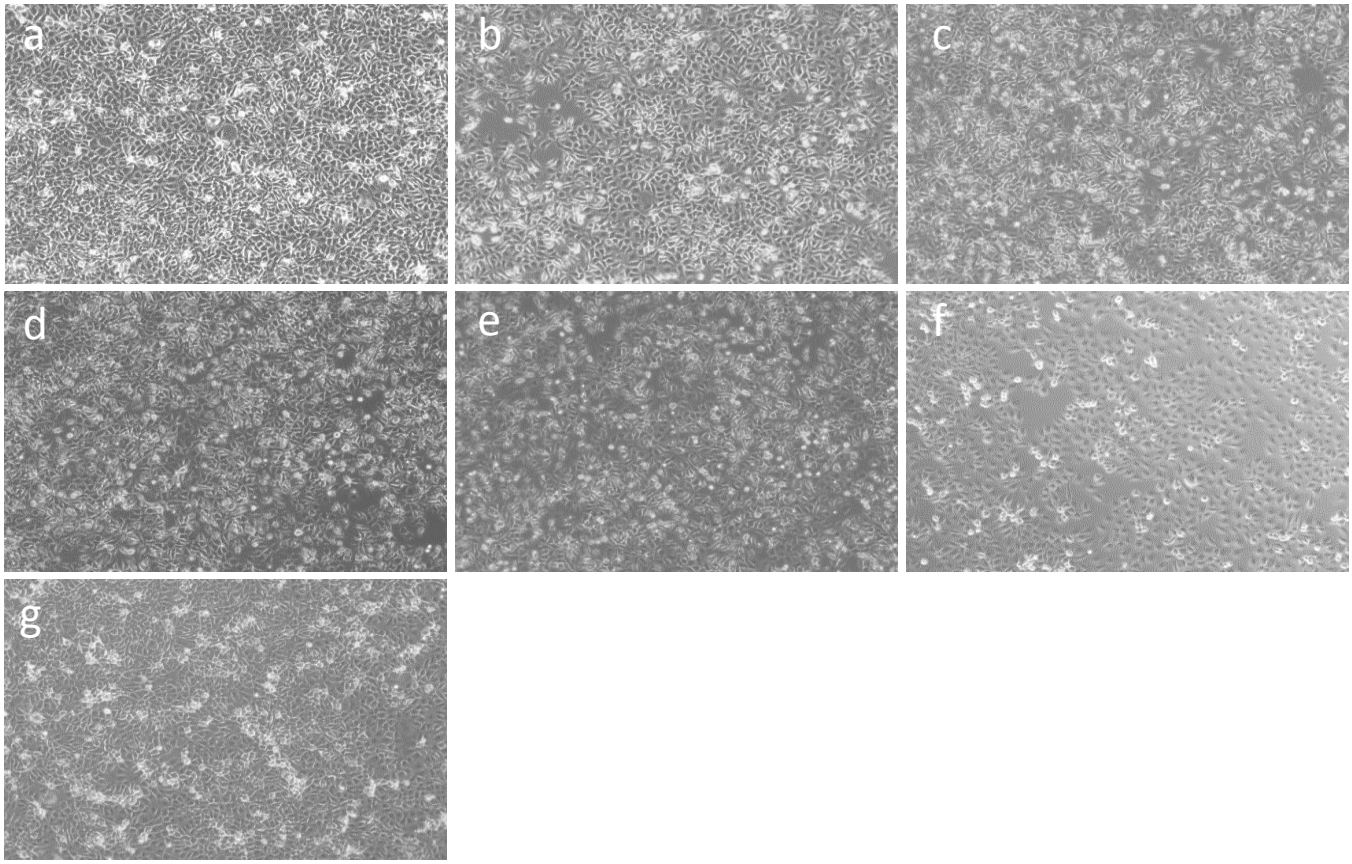


Figure 19: Cell morphology of L-11 cells after treatment with different doses of LY294002 in serum free conditions 24h post treatment. A) 10uM, b) 20um, c) 30uM, d) 40uM, e) 50uM, f) DMSO treated, g) Control. Magnification used is 20X.

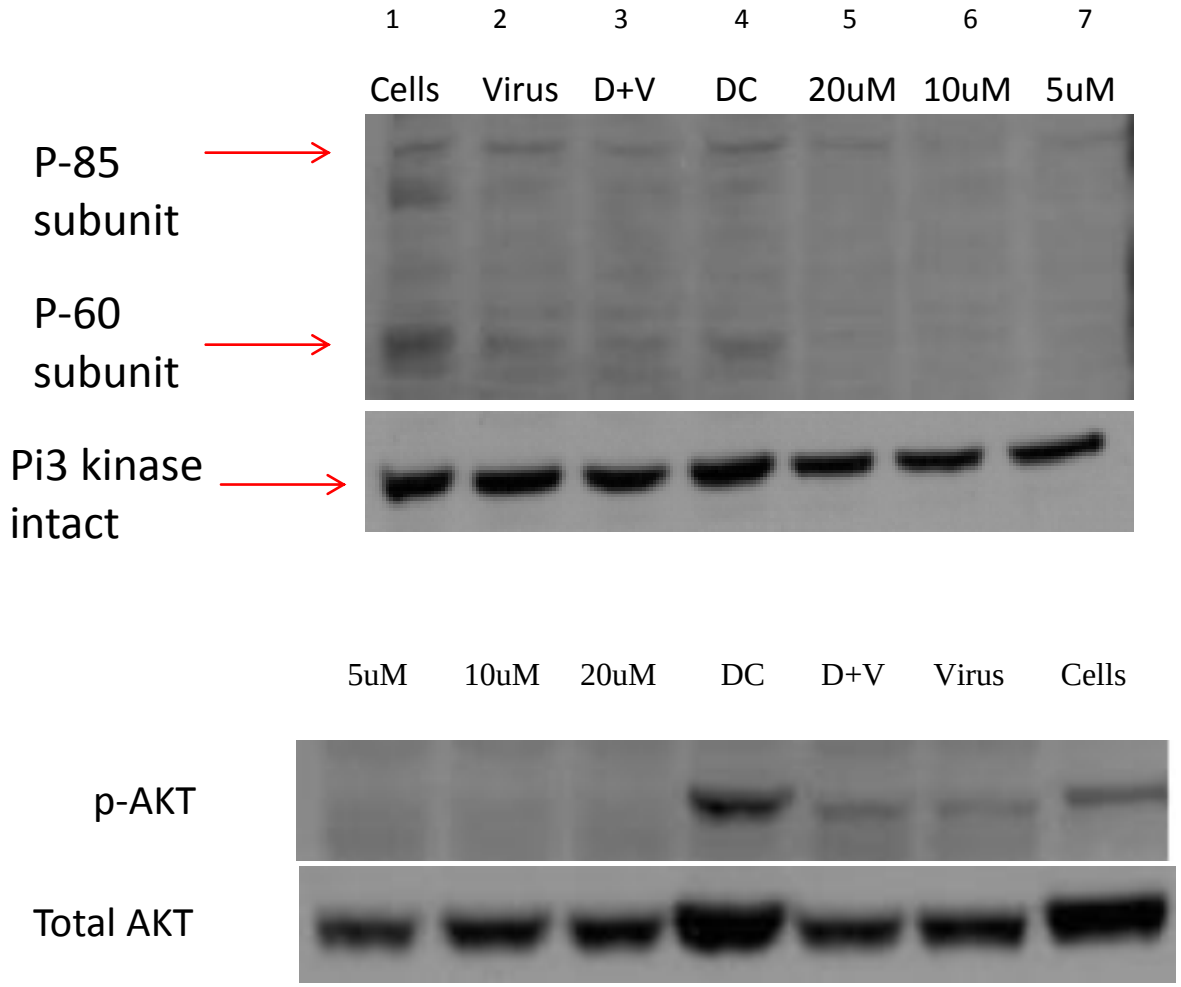


Figure 20: Inhibition of activation of Pi3-Akt pathway by treatment with LY294002. (A) Western blot analysis on cell treated with LY294002 and serums starved for 24h. Western blot was performed using antibody against Pi3-kinase which detects the two subunits p-85 and -60 of the intact kinase. Western blot bands indicated complete inhibition of phosphorylation lane 5, 6, 7. Levels of p-Pi3-kinase remain unchanged in control and DMSO treated cells (lane 1&4) whereas reduced levels were observed in virus cells and DMSO treated (virus infected cells (Lane 2&3). (B) Levels of p–AKT were completely suppressed after LY204002 treatment (lane 1-3) whereas p-AKT levels remained constant in control groups (Lane 4-7).

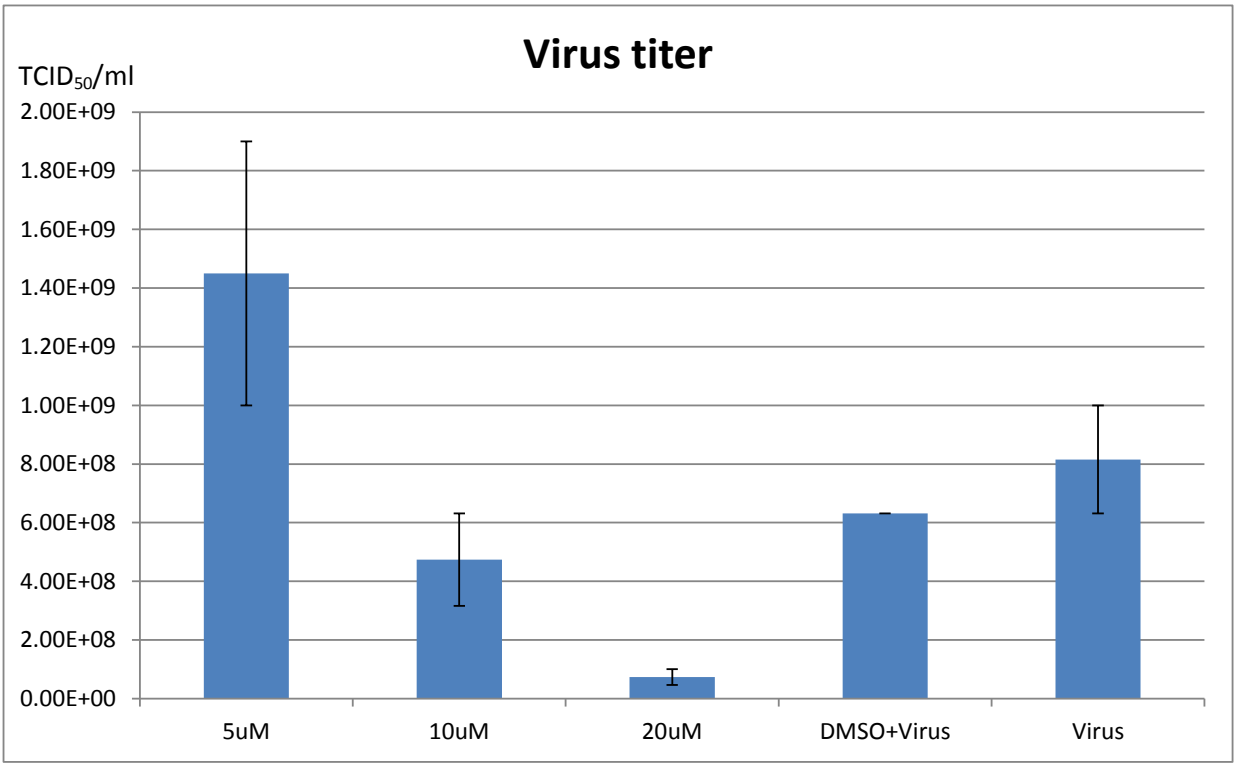
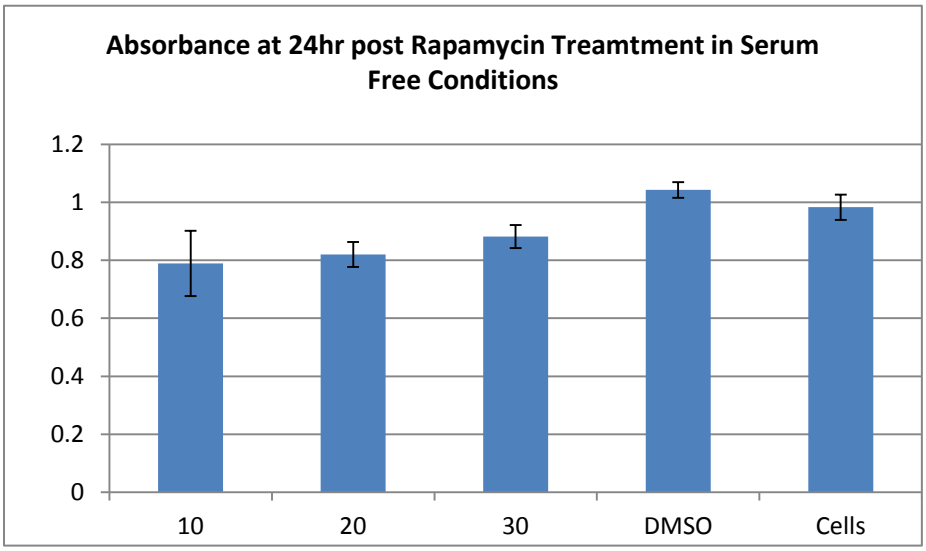


Figure 21: Effect of LY294002 treatment on VEEV titer in serum free conditions. L11 cells were serum starved and treated with LY294002 inhibitor. Cells were infected the following day with an MOI of 1. Cell supernatant was collected at 24h p.i. and $TCID_{50}$ was performed to estimate the virus titer. A dose dependent decrease in virus release from the infected cells was observed with a 90 % reduction in virus titer at a dose of 20µM of LY294002.

VEEV infection is insensitive to rapamycin treatment: As mentioned before, mTOR is one of the several downstream effector molecules of Pi3-kinase pathway. Activation of mTOR induces cell growth and differentiation whereas its inhibition leads to cell death or autophagy. Since VEEV transiently increases the phosphorylation of both pi3-kinase and AKT, we wanted to know whether VEEV replication is dependent or independent of mTOR pathway. Rapamycin is a specific inhibitor of mTOR protein complex in eukaryotes. Before testing the effect of rapamycin on VEEV replication, we evaluated the toxicity of rapamycin on L-cells using MTT assay as mentioned previously. MTT assay showed no apparent toxicity by rapamycin at the 10nM, 20nM and 30nM dose (Figure 22). Cell imaging also confirmed the MTT assay observations, which did not show any increased level of cell death after rapamycin treatment (Figure 23). Cells were serum starved and treated with rapamycin for 24h before infections, and supernatant was collected at 24h p.i. No reduction in virus titer from was observed between the cells treated with rapamycin and control cells instead a marginal increase in virus titer was observed in rapamycin groups (Figure 24).

A.



B.

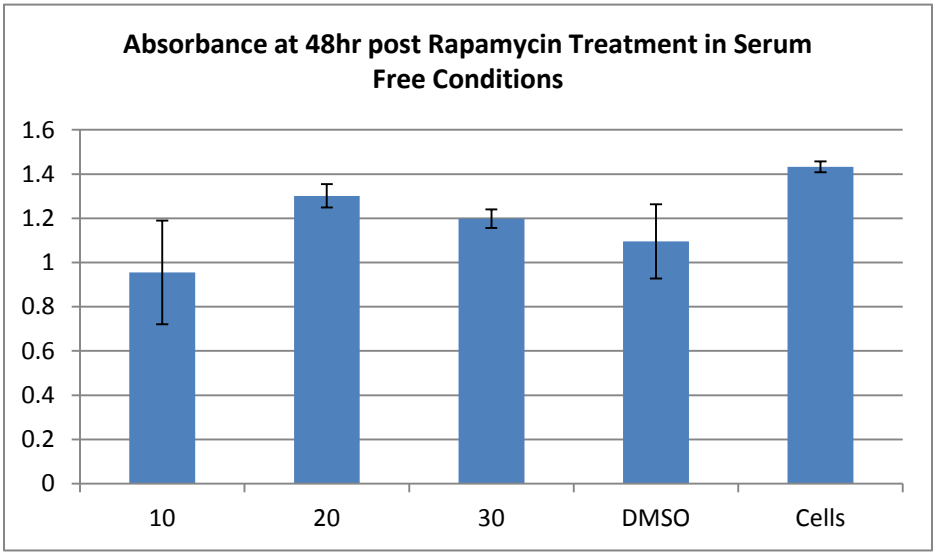


Figure 22: Cell proliferation assay for determining the cytotoxicity of the treatment with Rapamycin in L-11 cells. (A) Cell proliferation at 24h post treatment with rapamycin. (B) Cell proliferation at 48h post treatment with rapamycin. No significant cytotoxicity was observed due to rapamycin treatment.

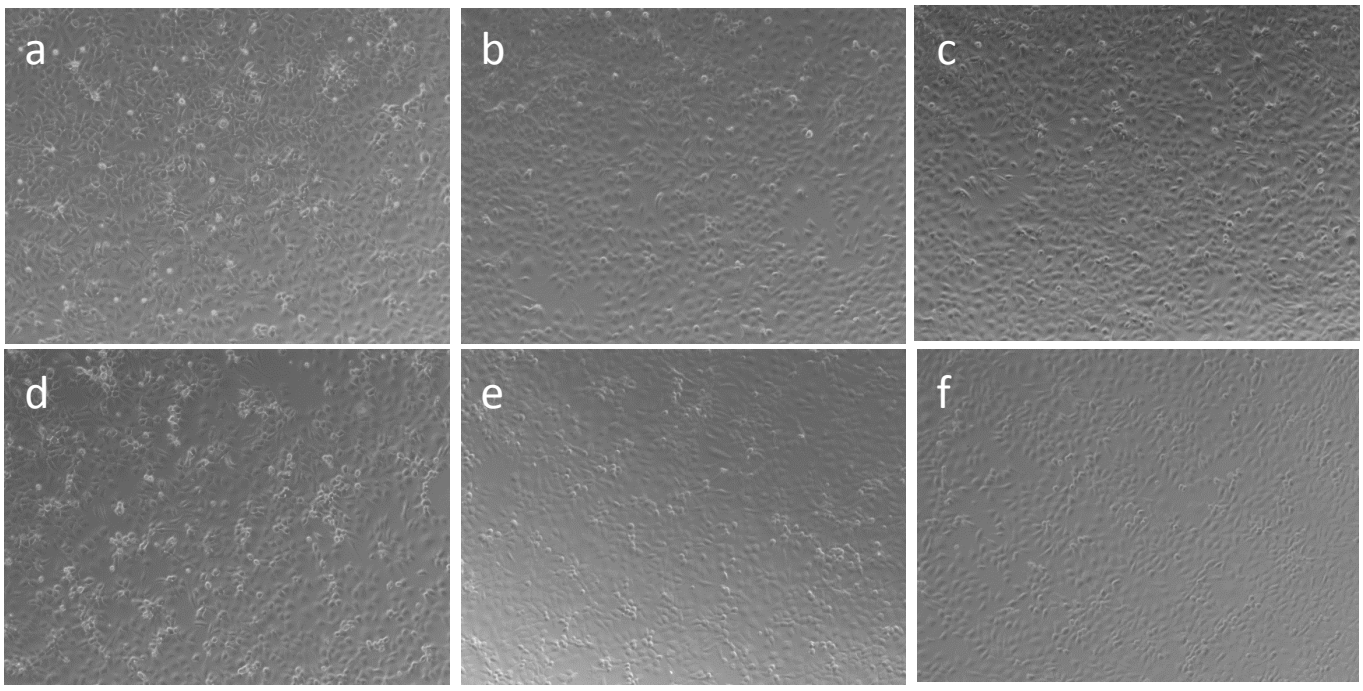


Figure 23: Cell morphology after infection with MOI 5 of TC-83 with pretreatment of Rapamycin for 24h in serum free conditions. a) 10nM, b) 20nM, c) 30nM d) DMSO+Virus, e) Virus control, f) Control cells. No significant visual difference was observed in the overall health of the cells.

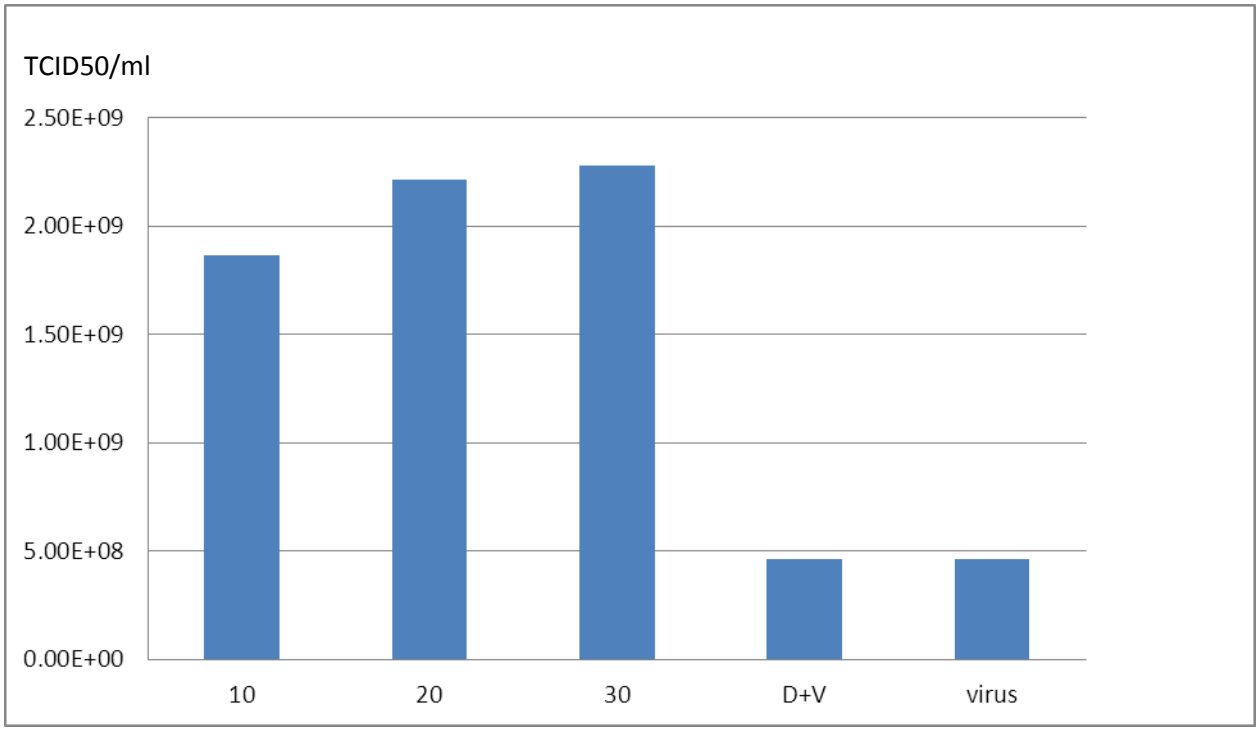


Figure 24: Effect of treatment on virus titer in L-11 cells with rapamycin treatment. L cells pretreated with rapamycin in serum free conditions. Cells were infected with an MOI of 1. Doses of rapamycin represented are in nanomolar concentration. No change is virus release was observed upon treatment with rapamycin.

Discussion

Alphaviruses infects a wide variety of host cells including vertebrate and invertebrate cells but the replication and infection mechanism in these two types of cells is different. In vertebrate cells the virus replicates by efficiently shutting down the host transcriptional and translational machinery ultimately leading to cell death. In case of invertebrate cells like that of arthropods, virus replicates in these cells equally efficiently causing a chronic infection without causing a translational arrest or cytopathic effect in these cells (Patel et al 2012). Regulation of Pi3-kinase pathway by virus plays a central role in the determining whether it will cause cell survival or apoptosis. Most RNA viruses causes acute infection in the cells where virus replicates its genome followed by cell death but some RNA viruses like hepatitis C also causes latent infection which is to alphavirus infection of the invertebrates (Erhardt et al 2009).

In this study, we have investigated the role of Pi3-kinase pathway in VEEV replication. For this we have used a mouse fibroblast cell line, since VEEV productively replicates in fibroblasts cells. Infection with VEEV in L-11 fibroblasts transiently increased the phosphorylation of AKT at 15m and 30m followed by a gradual decrease in phosphorylation. A second round of activation in phosphorylation was also observed at later in the infection cycle which occurred at 12h and 24h post infection. An early activation of Pi3-kinase pathway can occur just by virus binding or internalization which may not need viral protein translation or RNA replication to occur. Since Pi3-kinase activation will increase the cellular metabolic activity, the virus may use this initial activation for translation of its own non-structural proteins needed to facilitate virus replication. This pattern of initial activation of Pi3-kinase pathway has been shown in other RNA viruses (Cooray 2004). In case of alphaviruses, sindbis virus was shown to down regulate the Pi3-kinase activation post virus infection but in that study the Pi3-

kinase activation at very early time points were not studied (Mohankumar et al 2011). In contrast, a sustained increase in pi3-kinase activation was observed in mosquito cells upon infection with sindbis virus (Patel et al 2012). We further investigated whether the initial activation of Pi3-kinase in important for virus replication. Treatment with specific inhibitor of Pi3-kinase LY294002 prior to virus infection resulted in significant decrease in virus replication. This indicates that activation of this pathway is necessary for the survival of virus. In absence of Pi3-kinase activation other host pathways may compensate for its activity but still virus yield indicates that Pi3-kinase is primarily required for establishment of a productive infection. Interestingly, treatment with LY294002 also reduced the yield of viral RNA copies but no apparent difference in virus yield was observed in sindbis virus. This suggests that though the two viruses VEEV and sindbis, belongs to the same family, their mechanism of infection remains different. Similar reductions in virus titer upon pi3kinase inhibitor have been shown in influenza virus and other RNA virus including arena virus and hepatitis c virus (Shin et al 2007, Urata et al 2012, Mannova and berretta 2005).

Pi3-kinase and AKT transduce signals to a downstream effector molecule mTOR. Activation of AKT leads to the activation of the mTOR complex which leads to the translational activation. To study, whether inhibition of mTOR with specific inhibitors will also reduce the viral load similar to that of LY294002, we treated L-11 cells with rapamycin which is a specific inhibitor of mTOR. Treatment with rapamycin did not affect the virus replication. Similar observation were found in sindbis virus indicating that other host compensatory pathways may be responsible for the insensitivity of VEEV replication in response to rapamycin treatment.

In summary, our studies have identified the role of pi3-kinase pathway during VEEV replication. VEEV replication enhances pi3-kinase pathway flux in a biphasic manner. Also the

early activation of the pathway is critical for an efficient virus replication in the host cells. These findings are critical since many pi3-kinases and AKT pathway inhibitors are currently under clinical trials for their utility in treating different types of cancer and initial results have indicated their safety in humans (Nakabyashi et al 2012, yap et al 2011). Further studies are required in animal models to validate the utility of these inhibitors as antiviral drugs for treatment of VEEV infection.

Chapter 4

Antiviral countermeasure strategies for VEEV using siRNAs, artificial microRNAs and antiviral compounds.

Abstract:

At present, there are no FDA approved antiviral treatment or vaccine for the treatment of VEEV. Many different approaches for the development of novel antiviral have been reported in literature. VEEV RNA dependent RNA polymerase (RdRp) is central to VEEV replication. Therefore, we hypothesize that targeted inhibition of VEEV RdRp using siRNA and/or artificial microRNAs may efficiently inhibit VEEV replication. We tested several four siRNAs for their efficacy against VEEV infection in cell culture. One of the four siRNAs was found be effective in suppressing the viral infection. We also tested the efficacy of siRNAs delivered by an artificial miRNA vector. In this vector the siRNAs are cloned under a promoter of an endogenous miRNA making the expression of siRNA endogenous in nature. Five artificial miRNAs were tested out of which two showed very effective inhibition of VEEV replication. In conclusion, we have shown that RNAi effectively inhibits VEEV replication and can be used to develop targeted therapies. Also, siRNAs expressed under the artificial miRNA vectors show enhanced and longer protection against VEEV infection.

During VEEV infection inflammation plays a major role in the disease severity. Inflammation is attributed as one of the causes of the neuronal cell damage. Therefore, reducing the inflammation with non-steroidal anti-inflammatory drugs (NSAIDs) in conjunction with anti-viral compounds could result in an effective anti-viral therapy. In this study we have studied the effect of antivirals (ribavirin, amantadine and rimantadine) and anti-inflammatory (aspirin, indomethacin, oxaprozin and naproxen) drugs singly as well as in combination in reducing the

severity of the VEEV disease. Based on some preliminary findings, we selected ribavrin and naproxen for our further studies. Mice were treated with these drugs followed by V3000 challenge. Mortality and clinical symptoms were monitored and blood, brains and spleens will be collected. Total RNA was isolated using Trizol reagent and the amount of virus present was estimated by RT-PCR. Our studies suggests that antiviral and anti-inflammatory drugs alone does not offer any protection against VEEV but a combination therapy of carefully targeted anti-inflammatory and anti-viral drug should be tested for effective viral suppression in the host.

Introduction

Due to the non-availability of effective FDA approved antiviral drugs for Venezuelan encephalitis virus, various antiviral approaches have been tested. Antiviral approaches can be either host directed or pathogen directed. Most of the antiviral studies on RNA viruses have focused on targeting the pathogen using either known antiviral drug or other small molecule inhibitors of virus replication. Several antiviral approaches have been used for VEEV which are discussed in the earlier chapter.

Among these methodologies targeting viral genome using small interfering RNAs have received special attention. Post transcriptional gene silencing (PTGS) was first discovered in plants and later successfully tested in Drosophila, C. elegans, plants, mammalian cells and against viruses (Banan et al 2004). PTGS is a phenomenon where introduction of double stranded RNA (dsRNA) in the cell system results in a target specific degradation of mRNA resulting in gene silencing. Synthetic short 21-nt RNA duplexes (siRNA) are introduced into the cell. Upon introduction, dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. Then, the siRNAs assemble into endo ribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA. Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand. (Forte et al 2005, Xia et al 2002). Recent studies with siRNA have suggested that they have immense potential to be used as a therapeutic for various human viral diseases. Human immunodeficiency virus, polio virus, hepatitis B virus, West Nile virus, severe acute respiratory syndrome associated coronavirus, foot and mouth disease virus and Influenza virus have been inhibited both in vitro and in vivo using virus

specific siRNAs (Jaque et al 2002, Ge et al 2003, Hui et al 2004, Tompkins et al 2004, Wang et al 2004, Giladi et al 2003, Chen et al 2004).

SiRNAs are expressed through RNA polymerase III which leads to the overexpression which may cause cellular cytotoxicity (Xia et al 2012). Recent studies have shown that siRNAs which are expressed under the promoter regions of miRNAs termed as artificial miRNAs use RNA polymerase II promoters and does not induce cellular cytotoxicity due to low expression levels inside the cells (Zhou et al 2011). Artificial miRNAs have been used to develop antiviral for many viruses including HIV, HSV and rabies viruses (Xia et al 2012, Israsena et al 2012, Xang et al 2012). In the first part of the study, we have evaluated the efficacy of siRNAs, shRNAs and artificial miRNAs against VEEV infection.

Another pathogen directed antiviral countermeasure is to test the efficacy of known antiviral compounds and small molecules against viral infection. Currently, very few effective antiviral drugs are available for RNA virus infection. Some of the well-known antiviral which has been shown good a result against HCV is ribavirin. Other antiviral includes amantidine and rimantidine which have been shown to be effective against influenza infection and are currently used in clinic to treat patients. In the second part of the study we have tested the efficacy of these drugs in animal model against lethal VEEV challenge.

Inflammation causes the secondary neuronal damage in VEEV infection. Non-steroidal anti-inflammatory drugs (NSAIDs), aspirin, indomethacin, oxaprozin and naproxen were tested in animal models for reduction of inflammation and its effect on infection outcome.

Materials and Methods

For siRNA, shRNA and artificial miRNA invitro experiments.

Cell Culture: BHK cells and Vero cells were purchased from ATCC and were cultured as per recommended protocol. Briefly, BHK and VERO cells were taken out from liquid nitrogen and thawed at 37C waterbath. Both the cells were cultured in 1X MEM media with 10% new born calf serum along with 1% penicillin and streptomycin solution. Cells were incubated at 37˚C at 5%CO_2 concentration.

Virus Infection: *In vitro* virus infection experiments were carried out with either wild type strain of VEEV, V3000 or the attenuated vaccine strain TC-83. Viruses were diluted in 1 X Dulbecco's modified Earle's medium (DMEM) were incubated with cells for 1h at 37˚C in 5% CO_2. Virus suspension was then washed with saline and cells were replenished with fresh culture medium.

Sequence design and synthesis of siRNAs: The sequence of siRNA targeting VEEV replicase mRNA was designed by using Dharmacon Inc. software. Blast searches showed no significant identity with other known human or viral sequences. The siRNA duplexes, along with the positive and negative controls were acquired from Ambion Inc in ready to use form.

Sequence design and cloning of shRNAs: ShRNA sequences were designed using Dharmacon Inc software. In addition to the targeting sequence overhang sequences were added on 5' and 3' end of the oligos to facilitate directional cloning in the expression vector. The sequences were synthesized at the bioinstrumentation center, USUHS and were supplied in a lyophilized form.

Sequence design and synthesis of artificial miRNAs: Artificial miRNA sequences were also designed against the viral polymerase. The viral polymerase sequence was used to design the artificial miRNAs using Invitrogen's RNAi webdesigner tool. The sequences were synthesized by invitrogen and were supplied in ready to use form. The artificial miRNAs were cloned into pcDNA™6.2-GW/EmGFP-miR vector (Invitrogen Inc) as per the manufacturer's protocol.

Cloning of shRNAs: ShRNA sequences were resuspended in nuclease free water and annealed to form duplex oligos. These duplex oligos were digested with BamH1 and EcoR1 restriction enzymes. Expression vector pSIREN DSRED express which express a red fluorescent protein downstream of the shRNA promoter was a kind gift from the laboratory of Dr Christopher Broder, USUHS. The vector was linearized using BamH1 and EcoR1 and then ligated with the shRNA duplex oligos using T4 DNA ligase (New England Biolabs, Inc). The ligated plasmid was transformed into DH5-α competent cell and selected on ampicillin plates. Selected colonies were grown and plasmid was harvested.

Transfection: siRNA were diluted at the desired concentration in PBS buffer and were transfected with lipid based transfection reagent. SiPORT amine reagent was acquired from Ambion Inc and transfection was performed according to the manufacturer's protocol. Transfection of shRNAs and artificial miRNAs were performed using Fugene 6 (Roche Inc) transfection reagent as per manufacturer's protocol. Briefly, Fugene 6 was diluted in OptiMEM medium (Cellgrow Inc) at a concentration of 1:3 (DNA: transfection reagent). Oligos and transfection reagent were incubated at RT for 20 and then added to the cells. Cells were allowed to get transfected for 6h and then the cells were replenished with fresh medium.

MTT assay: Vero cells were cultured in 96 well tissue culture plates and grown till 80% confluency. Cells were transfected with siRNAs against virla polymerase 12h before then virus infection. Cells were infected with either TC-83 or V3000 at an MOI of 1 followed by an incubation of 12 and 24h. 100µl of MTT dye (Roche Inc) was added to each well and incubated for 4h. After incubation, 100ul of solublizing agent was added and again incubated for 4-6h. Optical density readings were taken in spectrophotometer at 570nm.

Virus Titer: Virus titer estimation was performed from the cell supernatant either by plaque assay or TCID50 as described in previous chapters.

Immunofluroscense: For detecting the surface antigen expression of VEEV in infected cells, immunofluorescent staining was used. Cells were grown in chamber slides and infected with VEEV with MOI of 1. Formalin or Acetone: Methanol (1:1) solution fixed cells were washed in water and pretreated in citrate buffer (2.1g/ l, pH 6.0) at 970C for 20 min for antigen retrieval. The VEEV polyclonal antibody, diluted 1:1000 was applied and incubated for 1 h at room temperature and then followed by incubation with fluorescence labeled goat anti rabbit immunoglobulin (Alexa 288 or 597-labbled, Molecular probes,) for 1h at room temperature. Cells were then washed and mounted using mounting media containing DAPI of propidium iodide (Vectasheild, Vector Labs, Burlingame, CA) for nuclear counterstaining.

Antiviral and anti-inflammatory efficacy studies

Animals: 5-6 week old male CD-1 mice (Charles River Laboratories, Wilmington, MA) were obtained. Ten animals per group were used for mortality and 5 animals per time point were used in kinetic experiments in all in vivo experiments. Mice were housed in microisolator cages and provided with food and water ad libitum, except as noted. For the portions of the study

involving live VEEV, mice were housed in a biosafety level 3 (BSL-3) facility accredited by the American Association for Accreditation of Laboratory Animal Care. In conducting research with mice, the investigators adhered to the Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, NIH Publication No. 86-23, revised 1996).

Virus and challenge procedure: Molecularly cloned, virulent strain of VEEV, V3000 was used in the animal studies. Stock virus solution was diluted in 1 X Dulbecco's Phosphate Buffered Saline (GIBCO BRL, Invitrogen Corporation Carlsbad, CA) supplemented with 0.1% fetal bovine serum to 1000pfu/25ul. Mice were anesthetized lightly using inhalation anesthesia, isofurane and 100-1000 plaque forming units (lethal dose) of V3000 in 25µl volume was injected in the left rear footpad. Control animals were injected with 25ul of 1 X Dulbecco's Phosphate Buffered Saline supplemented with 0.1% fetal bovine serum.

Treatment with anti-viral and anti-inflammatory drugs: Antiviral drugs that were used in this study are ribavirin, amantadine and rimantadine (Invitrogen Corporation, Carlsbad, CA.). Anti-inflammatory drugs that were used are non steroidal anti inflammatory drugs (NSAIDs), aspirin, indomethacin, oxaprozin and naproxen (Invitrogen Corporation, Carlsbad, CA.). Ribavirin is a guanosine analog that inhibits the replication of RNA and DNA viruses by inhibiting messenger RNA formation. Amantadine and rimantadine are primarily used for Influenza A prophylaxis and treatment. These inhibit penetration or uncoating of the virus (MERK Manual, sec. 13, Ch. 154, Antiviral Drugs). Non-steroidal anti inflammatory drugs (NSAIDs) are used in medicine for their analgesic and anti-inflammatory properties. NSAIDs work in two ways (1) reducing the sensation of pain, and (2) reducing the inflammation that often accompanies and worsens pain. NSAIDs produce these effects by reducing the production

prostaglandins. Other mechanisms of action are unknown (The Merck Manual--Second home edition-online, Non-steroidal Anti-Inflammatory Drugs).

Histopathology and immunohistochemistry evaluations: For the kinetic studies, at each time point 5 mice were anesthetized using isofurane and euthanized by cervical dislocation. Brains and spleen were collected and right hemisphere was fixed in 10% buffered neutral formalin (BNF) for 3-4 weeks and left hemisphere was snap frozen at -800C. Tissues were then transferred into fresh BNF and routinely processed and paraffin embedded. 5μm histology sections were prepared, mounted on glass slides and stained with hematoxylin and eosin (HE). Duplicate sections of selected tissues were mounted on silane-coated slides (Sigma Diagnostics, St. Louis, MO) and were stained for VEE virus antigen by immunohistochemistry. To detect VEE viral antigen, an immunoperoxidase method (Vector ABC System, Vector labs) was used according to the manufacturer's recommendations. Briefly, sections will be deparaffinized and rehydrated to dH20, then placed in citrate buffer (2.1 g/L, pH 6.0) at 97°C for 20 min for antigen retrieval. Sections were then washed in dH20 and blocked for endogenous peroxidase using 3-5%H_2O_2. Sections were incubated with the primary antibody, a rabbit polyclonal antiserum raised against VEE virus, eastern equine encephalitis virus, western equine encephalitis virus and Sindbis virus (provided by Dr. Grieder, USUHS) diluted 1:10,000, for overnight at 40C. After washing with 3 changes of PBS, sections were incubated with peroxidase-labeled antibody against rabbit immunoglobulin (Vector Kit) for 10 min at RT. Color was developed by incubating in a solution containing 3,3'-diaminobenzidine and H2O2 (Vector kit) for 7 min at RT. Additional sections were treated with nonimmune rabbit serum as negative controls. HE-stained and immunohistochemically-stained brain sections were observed by light microscopy and subjective determinations of the amounts of virus antigen, inflammatory cell infiltrates and

neuronal death were made using graded criteria for 9 different regions of the brain: olfactory bulbs, olfactory portion of the brain proper, pyriform cortex, basal nuclei, diencephalon, hippocampus, neocortex, brainstem and cerebellum.

Semi Quantitative PCR for detection of the viral antigen: Expression of different regions of VEEV genome specifically nsP4 were detected. Total mRNA from VEEV infected cells were isolated using TriZOL (Invitrogen Corporation, Carlsbad, CA.). Reverse transcription was carried out using first strand synthesis kit (Invitrogen Corporation, Carlsbad, CA.) as per the manufacture's protocol. PCR amplification of VEEV specific genes was done using VEEV genome specific primers as described by Brightwell et al., 1998. Briefly, PCR was carried out in Perkin Elmer 9600 thermal cycler in a total volume of 49µl (2ulcDNA, 45µl PCR mix (Invitrogen Corporation, Carlsbad, CA.), 1µl each of sense and antisense primer). Specific amplification was detected by electrophoresis on agarose gel and was visualized using ethidium bromide in a gel documentation system.

Results

Evaluate the RNA interference with 21-nt siRNA duplexes for degrading the VEE nsP4 in vitro: Viral RNA polymerase is critical for replication and therefore present an ideal candidate for developing antiviral strategies. In this experiment, VEEV viral polymerase gene i.e. nsp -4 was targeted utilizing siRNAs specific for four different regions of the nsp-4 gene (Table 7). In the initial screening no significant cytotoxicity of the siRNAs was observed *in vitro* (Figure 25). The protective effect of siRNA against VEEV infection was demonstrated by cell proliferation assay. Cells were transfected with 100ng of siRNA using SiPORT Amine Master Mix (Ambion Inc.). Cell proliferation was measured by MTT assay (Roche Inc) as per manufacturers' protocol. A clear protection of cells was observed by siRNAs against VEEV infection (Figure 26).

Protection from VEEV infection was measured by VEEV specific immunofluorescence. A clear reduction in the VEEV specific immunofluorescence was observed in the cells that were transfected with anti-VEEV siRNAs. VEEV infection in the cells transfected with the negative-control siRNA was similar to that of the infected control cells. In contrast to cell proliferation assay, siRNA1 and 2 were relatively less efficient in inhibiting VEEV replication as compared to the siRNA3 and 4. SiRNA3 was considerably more effective in inhibiting siRNA though small pockets of VEEV specific immunofluorescence were still observed in these cells. siRNA4, however, completely blocked VEEV infection. Pooled siRNAs also completely blocked the VEEV infection which may be more due to siRNA4 and less of a synergistic block of VEEV replication. These results are illustrated in Figure 27 which shows a complete image of each well with respective siRNA transfection. Image was developed using in-house software (BIC, USUHS, Bethesda, MD) which merged individual sequential images of a well to form a full well composite. In a separate experiment with BHK cells 1-3 log fold reduction in the virus titers was

observed in the supernatants of the cells transfected with siRNAs (Figure 28).

Inhibition of VEEV replication in vitro by shRNAs: SiRNAs duplexes suffer from a limitation of short half-life and thus the inhibitory effect reverses at later time points such as 48 hour post infection. To overcome this limitation siRNA sequences were cloned in eukaryotic expression vector pSIREN DS RED (A kind gift from Dr. C. Broder, Dept of Microbiology, USUHS, Bethesda, MD). In addition to the four siRNAs that were tested in the above experiment four additional siRNA sequences were cloned. Following sites were inserted in the clone sequences at 5' and 3' respectively: BAM H1 restriction site sequence= 5'-gatcc-3'; EcoR1 end restriction site sequence= 3'-cttaa-5'; Mlu1 restriction site sequence= 5'-acgcgt-3'; Hairpin loop= 5'-TTCAAGAGA-3'; Terminator sequence= 5'-TTTTTT-3' (Figure 29).

Following cloning of the siRNAs in the vector, these shRNA were transfected. BHK cells were transfected with pSIREN DS RED expression plasmid containing shRNA (1-7) (Table 8) of interest and red fluorescence protein (RFP) gene. Cells appearing red have active expression of RFP and also are expressing the shRNA of interest. ShRNA were designed to target nsp4 region of the virus genome. Cells were infected with VEEV and expression of virus antigen was visualized by using VEEV specific primary antibody and FITC conjugated secondary antibody. ShRNA 1, 4, 5 and 7 specifically inhibited the virus replication as indicated by the absence of co-localization of green and red fluorescence (Figure 30). It is clear from figure 30 that not all the cells in the culture were constitutively expressing the shRNA; however the cells that does express the shRNA (red florescence) showed almost complete absence of VEEV infection clearly indicating the shRNAs were efficiently inhibiting the VEEV replication. To overcome this problem of poor transfection efficiency we tried to enrich the shRNA transfected cells by FACS. However this approach was not successful after repeated trials (data not shown) as high

number of cell death was observed in the FACS purified cells that inhibited the establishment of shRNA transfected cells colony.

Table 7: Sequences of siRNA designed against nsp-4 region of VEEV. Negative siRNA: Scrambled RNA sequences 4G 7T 4C 6A (21 nt): 5' TAGAGCGTAT TACGTATACC T 3' Only 76% identical to 2 oryza sativa (rice) genes AP006523 AND AP008208 was used.

SiRNA	Sense Strand	Antisense strand
siRNA1	5'-GACUUUGACGCUAUUAUUGUU-3'	5'-CAAUAAUAGCGUCAAAGUCUU-3'
siRNA2	5'-GCUGCGCAGCUUUCCAAAGUU-3'	5-CUUUGGAAAGCUGCGCAGCUU-3
siRNA3	5'-AUAUCAUCAAUACAUUUGCUU-3'	5'-GCAAAUGUAUUGAUGAUAUUU-3'
siRNA4	5'-AAAGAAAUUGCAACGUCACUU-3'	5'-GUGACGUUGCAAUUUCUUUUU-3'

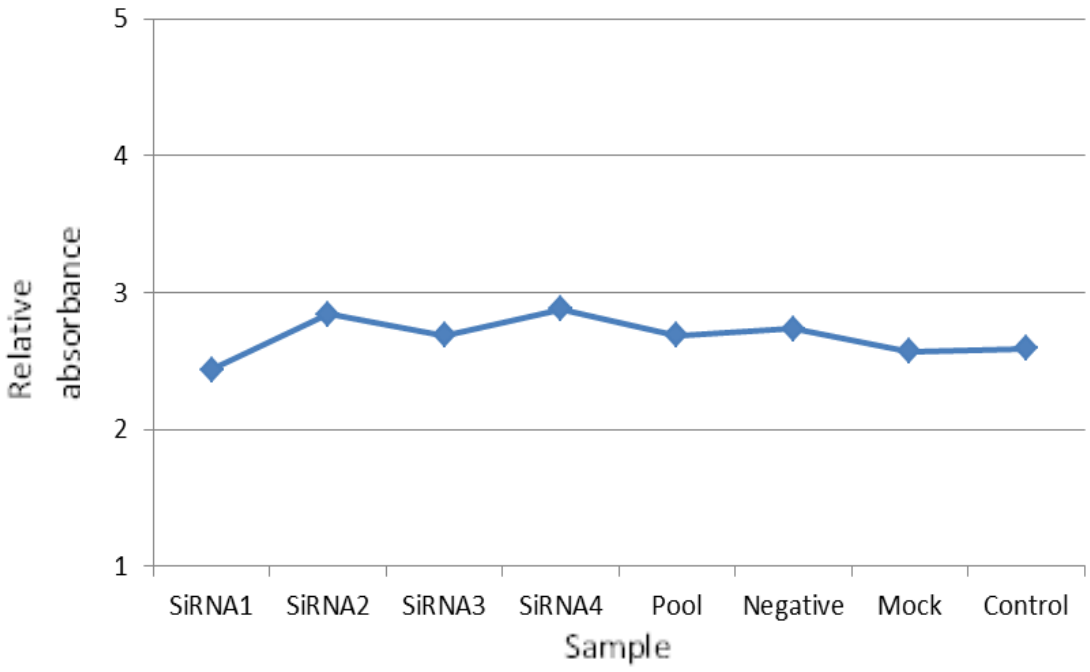


Figure 25: Cell proliferation assay for evaluating cytotoxicity of siRNAs: BHK cells were grown in 96 well plates and transfected with 100ng of siRNA duplex. After 12 h of transfection MTT reagent was added to determine siRNA toxicity. No significant cellular cytotoxicity

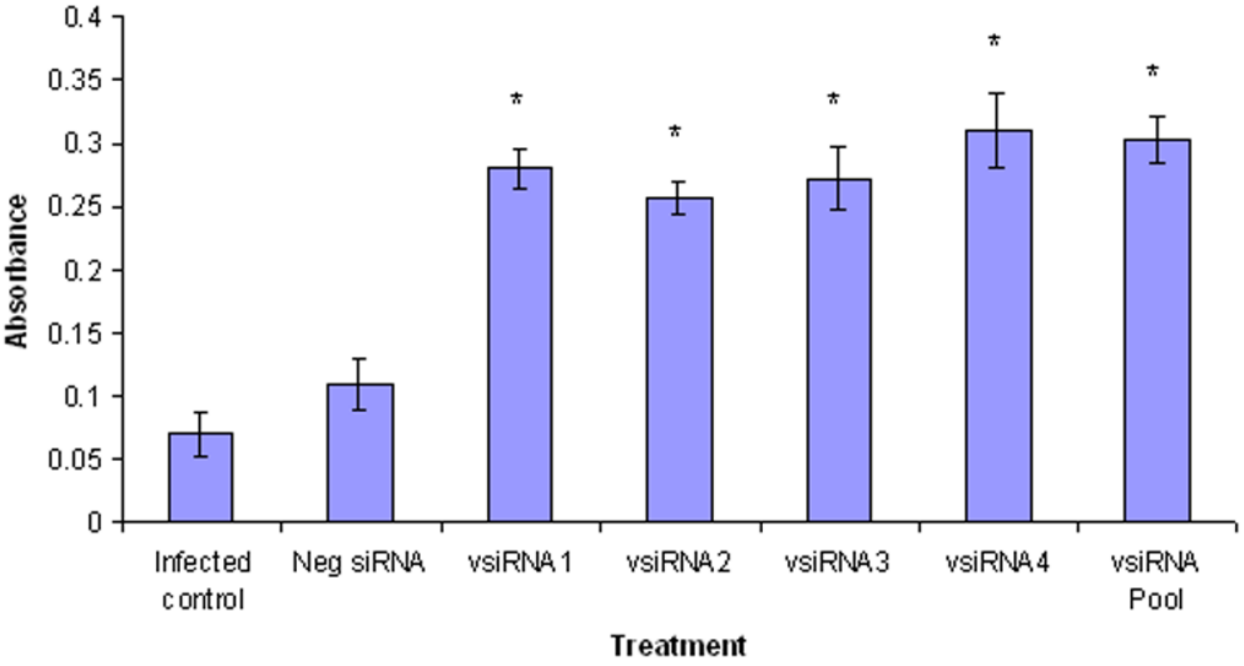


Figure 26: Cell proliferation assay in cells transfected with siRNA duplex and infected with VEEV: Vero cells were grown in 96 well plates and transfected with 100ng of siRNA duplex targeted against nsp4 region of the virus genome. After 12 h of transfection cells were infected with VEEV (MOI=0.1). MTT assay was done to quantify the cell proliferation. Values ± SEM, *$P \leq 0.05$.

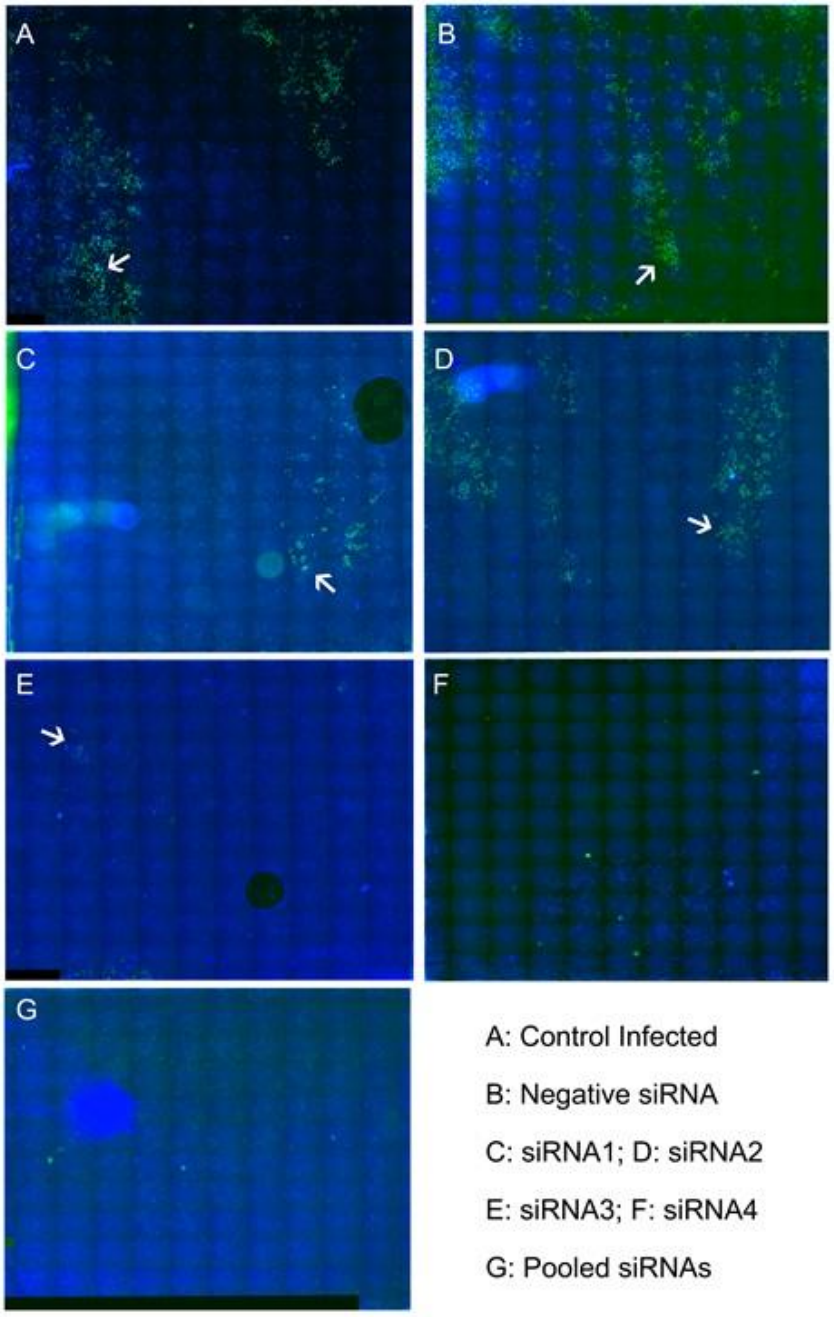


Figure 27: Localization of virus antigen in VERO cells transfected with siRNA duplex and infected with VEEV using immunofluorscence microscopy. Each panel represent a collage of indicidual microscopic field and then combined as one image.

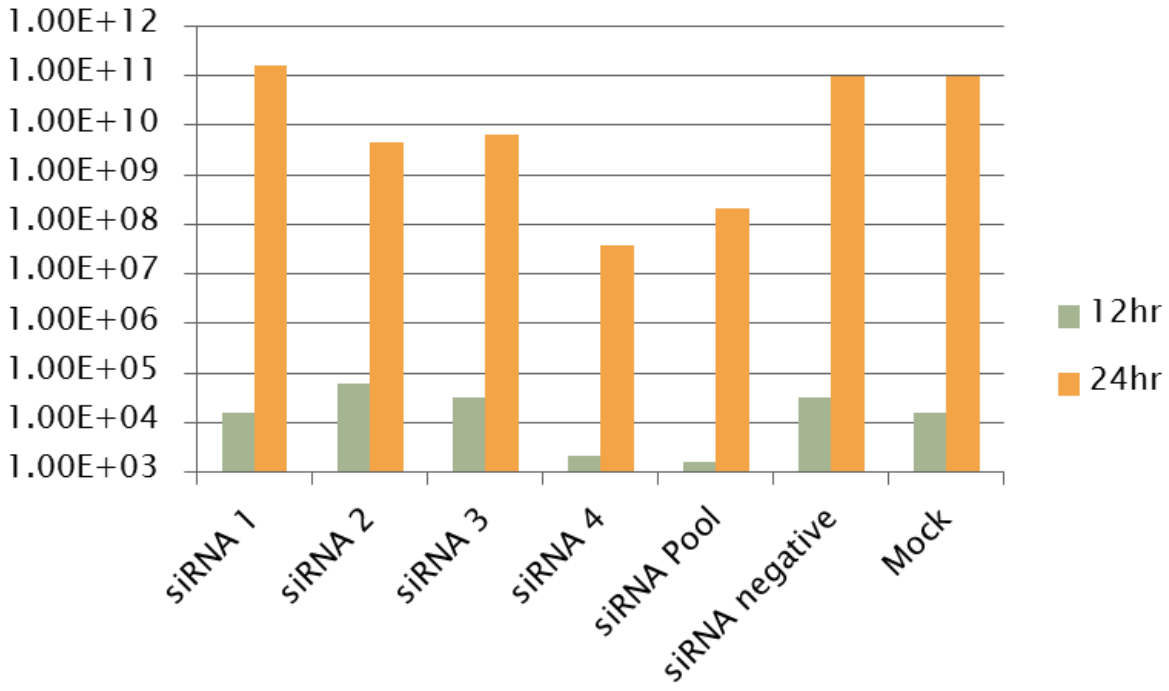


Figure 28: Effect of siRNA on replication of VEEV in BHK cells. BHK cells were transfected with 100ng siRNA. After overnight transfection, cells were infected with 0.1MOI of TC-83. Cell supernatant was collected at 12 and 24h post infection. Virus titer was done using TCID50

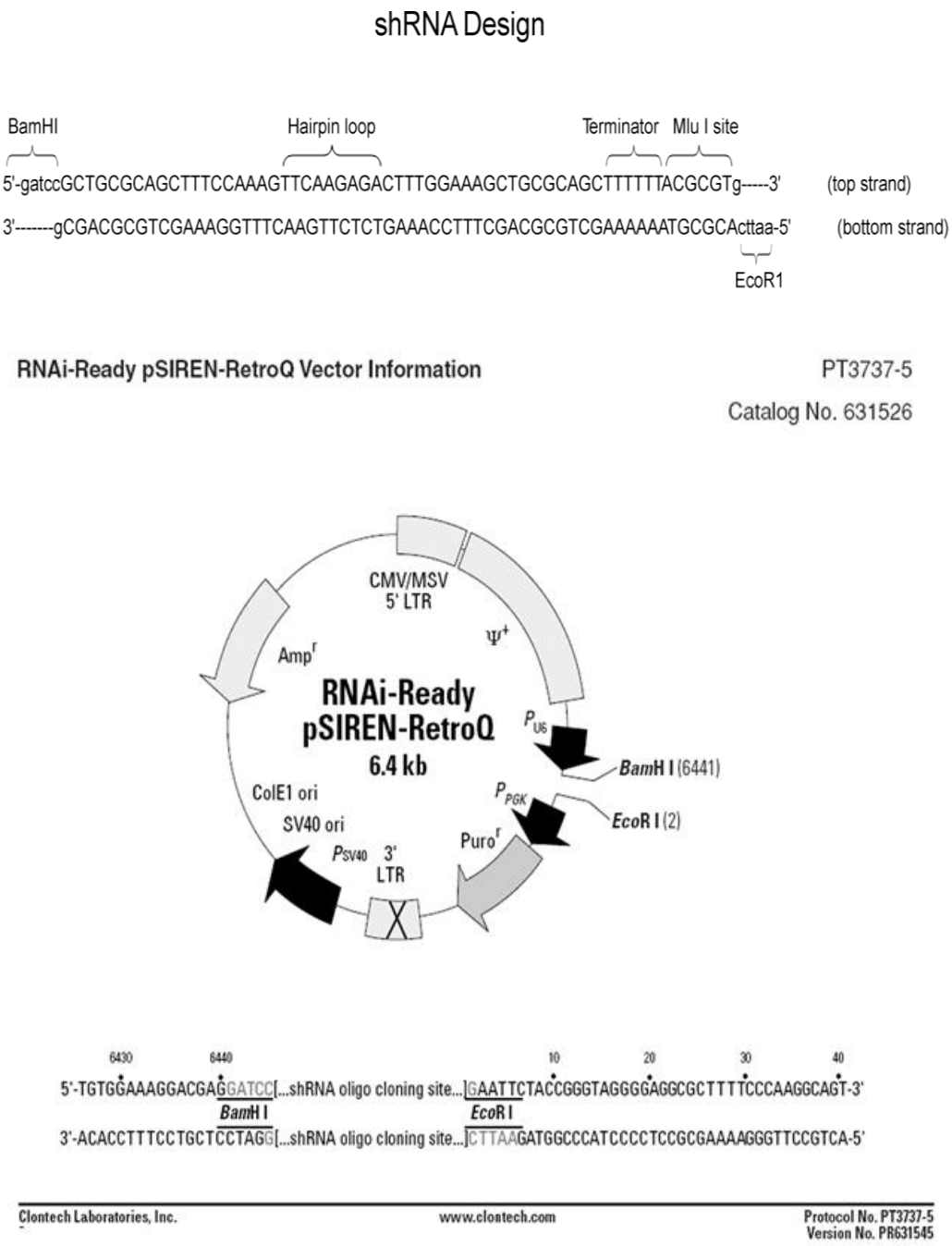


Figure 29: ShRNA design and map of RNAi-ready-pSIREN-RetroQ plasmid

Table 8: Oligo sequences for shRNAs directed against nsp-4 region of VEEV.

vshRNA1

Top strand: 5-gatccGACTTTGACGCTATTATTCTTCAAGAGAGAATAATAGCGTCAAAGTCTTTTTTACGCGTg-3

Bottom strand: 5' - aattcACGCGTAAAAAAGACTTTGACGCTATTATTCTCTCTTGAAGAATAATAGCGTCAAAGTCg - 3'

vsiRNA2

Top strand: 5' gatccGCTGCGCAGCTTTCCAAAGTTCAAGAGACTTTGGAAAGCTGCGCAGCTTTTTTACGCGTg 3'

Bottom strand: 5' aattcACGCGTAAAAAAGCTGCGCAGCTTTCCAAAGTCTCTTGAACTTTGGAAAGCTGCGCAGCg 3'

vsiRNA3

Top strand: 5'-GATCCGATATCATCAATACATTTGCTTCAAGAGAGCAAATGTATTGATGATATCTTTTTTACGCGTG-3'

Bottom Strand: 5'-AATTCACGCGTAAAAAAGATATCATCAATACATTTGCTCTCTTGAAGCAAATGTATTGATGATATCG-3

vsiRNA4

Top strand: 5'gatccGAAAGAAATTGCAACGTCACTTCAAGAGAGTGACGTTGCAATTTCTTTCTTTTTTACGCGTg 3'

Bottom strand: 5'aattcACGCGTAAAAAAGAAAGAAATTGCAACGTCACTCTCTTGAAGTGACGTTGCAATTTCTTTCg 3'

vshRNA5

Top strand: 5-gatccGCTGAAGACTTTGACGCTATTAttcaagagaTAATAGCGTCAAAGTCTTCAGttttttacgcgtg-3

Bottom Strand: 5'-aattcacgcgtaaaaaaCTGAAGACTTTGACGCTATTAtctcttgaaTAATAGCGTCAAAGTCTTCAGCg - 3'

vsiRNA6

Top strand: 5-gatccGCGGCGCTTCTTGTTGCTTAGAttcaagagaTCTAAGCAACAAGAAGCGCCGttttttacgcgtg-3

Bottom Strand: 5'-aattcacgcgtaaaaaaCGGCGCTTCTTGTTGCTTAGAtctcttgaaTCTAAGCAACAAGAAGCGCCGCg - 3'

vsiRNA7

Top strand: 5-gatccGAGGCGGCTTTCGGCGAAATAttcaagagaTATTTCGCCGAAAGCCGCCTCttttttacgcgtg-3

Bottom Strand: 5'-aattcacgcgtaaaaaaGAGGCGGCTTTCGGCGAAATAtctcttgaaTATTTCGCCGAAAGCCGCCTCg - 3'

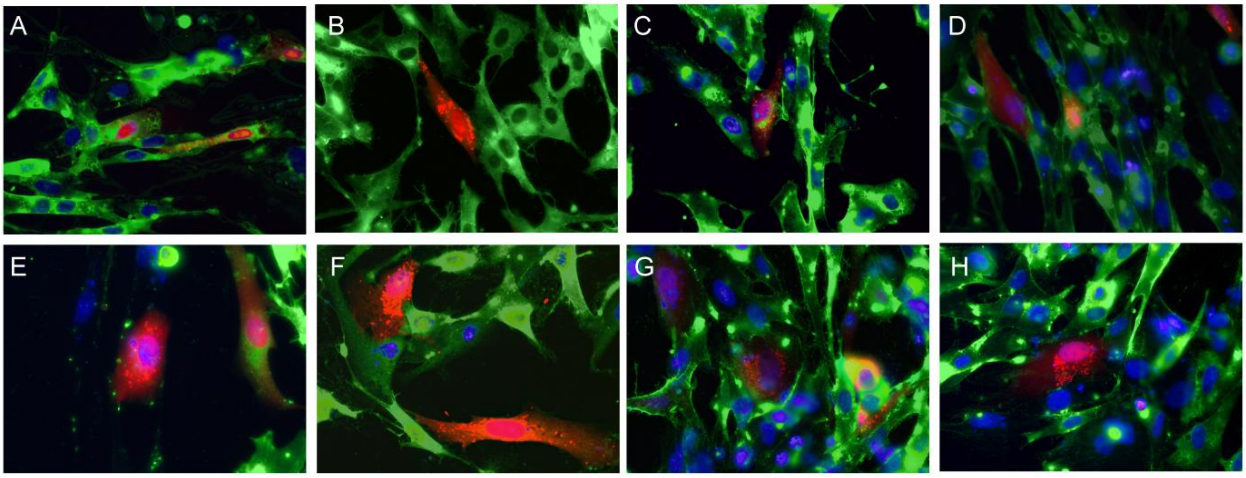


Figure 30: Immunofluorscence staining for VEEV antigen in BHK cells transfected with shRNAs for evaluation of protective efficacy of shRNAs. Co-localization of shRNA transfected cells and VEEV antigen in the BHK cells transfected with shRNA expressing plasmid and VEEV infected. A: negative control; B: shRNA1; C: shRNA2; D: shRNA3; E: shRNA4; F: shRNA5; G: shRNA6 and H: shRNA7.

Inhibition of VEEV replication by artificial microRNAs: Artificial miRNAs are expressed though RNA polymerase II and are argued to be less cytotoxic than siRNAs due to their relatively low concentration inside the cells (Boudreau et al., 2009). Artificial miRNAs have been studied as an antiviral approach for many viruses including adenoviruses, rabies virus, dengue virus and porcine reproductive and respiratory virus and has been shown to be an effective inhibitor of virus replication with minimal or no cytotoxicity (Ibrišimović et al., 2013, Xia et al., 2013, Xie et al., 2013 and Israsena et al., 2009).

Artificial miRNA sequences were designed and three high scoring artificial miRNAs were selected and the oligos were synthesized along with a negative control sequence which is not specific against nsp-4 sequence (Invitrogen Inc.) (Table 9). To determine the toxicity of artificial miRNAs in *in vitro* experiments, we measured cell proliferation experiment using MTT assay. 100ng of plasmid vector harboring artificial miRNAs were transfected in BHK-21 cells in each well of 96 well plates and incubated overnight. MTT assay was performed to evaluate the percentage of metabolically active cells. In our data, no significant toxic effect was observed due to the presence of artificial miRNAs in cells (Figure 31a). Antiviral efficacy against VEEV was tested *in vitro* in BHK-21 cells using TC-83 which is a vaccine strain of VEEV and approved to be used in BSL-2 laboratory. Moreover, the sequence of nsp-4 in TC-83 strain is identical to that of virulent VEEV strain. To assess the antiviral activity of artificial miRNAs against VEEV infection, BHK-21 cells were transfected with artificial miRNAs using lipofectamine 2000 transfection reagent (Life Technologies Inc.) and then infected with 0.1 MOI of TC-83. Cell supernatant and total cell lysates were harvested at 12 and 24h post infection (p.i.). To estimate the amount of virus inhibition by artificial miRNAs, $TCID_{50}$/ml (50% tissue culture infectious dose) was calculated from the cellular supernatant. $TCID_{50}$ data suggested a significant inhibition

of VEEV replication in presence of artificial miRNAs. Specifically Mir-5 showed the maximum inhibitory effect against VEEV *in vitro* (Figure 31b). Further, we performed real time PCR (RT-PCR) to estimate the reduction in the viral RNA load in the cells transfected with artificial miRNAs and infected with VEEV 12h p.i.. In cells treated with artificial the total viral load was significantly reduced with Mir-5 showing the maximum inhibition in viral RNA replication (Figure 32a). We then quantitated the total viral protein to understand whether the inhibition of viral RNA transcription is also reflected at the translational level. Western blot was performed with a monoclonal antibody specific to E2 glycoprotein of VEEV. Mir-5 treated BHK-21 cells again showed the least amount of virus presence at 12h p.i. in comparison to negative miRNA control cells. Inhibition of viral protein was also observed in Mir-1 and Mir-3(Figure 32 b). We also observed the presence of artificial miRNAs and virus using fluorescent microscopy. Artificial miRNAs vectors express a green fluorescent protein (EmGFP) which indicates the presence of artificial miRNAs. BHK-21 cells were transfected with artificial miRNAs and then infected with 0.1 MOI of TC-83 which express a cherry red protein. We found that cells which are positive (green) for Mir-1, Mir-3 or Mir-5 did not show the presence of TC-83 virus (red). Cells which were not transfected with artificial miRNAs were all positive for VEEV infection which is shown in red color (Figure 32c).

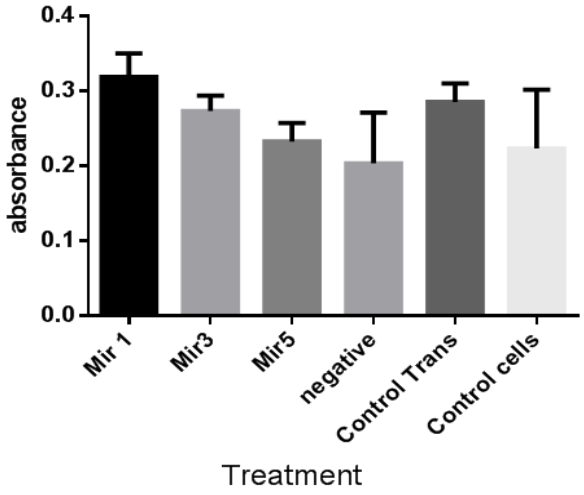
It has been previously shown that expressing more than one miRNA in one single artificial miRNA vector construct may provide better inhibition of virus (Israsena et al 2009). We therefore cloned two artificial miRNAs together in the same expression vector. Two recombinant plasmids vectors were generated, Mir15 with Mir-1 and Mir-5, and Mir-35 which contains single miRNA sequence of Mir-3 and Mir-5. We then investigated the synergistic effect of expressing two miRNAs through a single expression vector against VEEV infection in BHK-

21 cells. Cells were transfected as previously described and cell lysates and supernatant were collected. Virus titer from cell supernatant 12h p.i. showed protection by both Mir-15 and Mir-35 in comparison to the negative control but the percent inhibition of virus titer was less in comparison to treatment with individual miRNAs (Figure 33a). We further quantified the total viral protein from the cell lysates using western blot. In accordance with the virus titer data, reduction in total viral protein was observed in the cells treated with Mir-15 and Mir-35 but the combination treatment lead to a slight increase in the total viral protein in comparison to Mir-1, 3 and 5 treatment alone (Figure 33b).

Table 9: MicroRNA sequences for cloning into artificial miRNA vector. Five sequences were designed against nsp-4 region of VEEV genome as target using Block-iT RNAi Designer, (Invitrogen).

Mir 1:	TOP STRAND	5′-TGC TGT GTA AAT GCC CTT GAC CGG TGG TTT TGG CCA CTG ACT GAC CAC CGG TCG GGC ATT TAC A-3′
	BOTOM STRAND	5′- CCT GTG TAA ATG CCC GAC CGG TGG TCA GTC AGT GGC CAA AAC CAC CGG TCA AGG GCA TTT ACA C-3′
MIR 3:	TOP STRAND	5′-TGC TGA ATA CGG GCA ATT CTC TCA TTG TTT TGG CCA CTG ACT GAC AAT GAG AGT TGC CCG TAT T -3′
	BOTTOM STRAND	5′-CCT GAA TAC GGG CAA CTC TCA TTG TCA GTC AGT GGC CAA AAC AAT GAG AGA ATT GCC CGT ATT C -3′
MIR5:	TOP STRAND	5′-TGC TGT TGA CTT CCA TAT TCA ACC AGG TTT TGG CCA CTG ACT GAC CTG GTT GAA TGG AAG TCA A -3′
	BOTTOM STRAND	5′-CCT GTT GAC TTC CAT TCA ACC AGG TCA GTC AGT GGC CAA AAC CTG GTT GAA TAT GGA AGT CAA C -3′

A.



B.

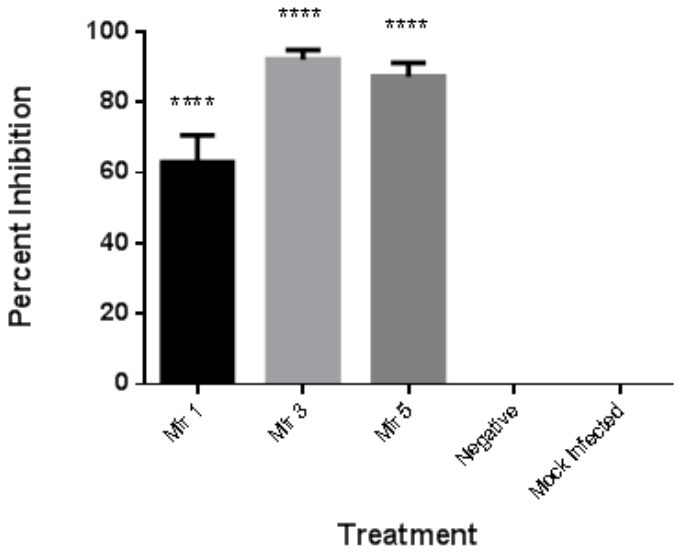
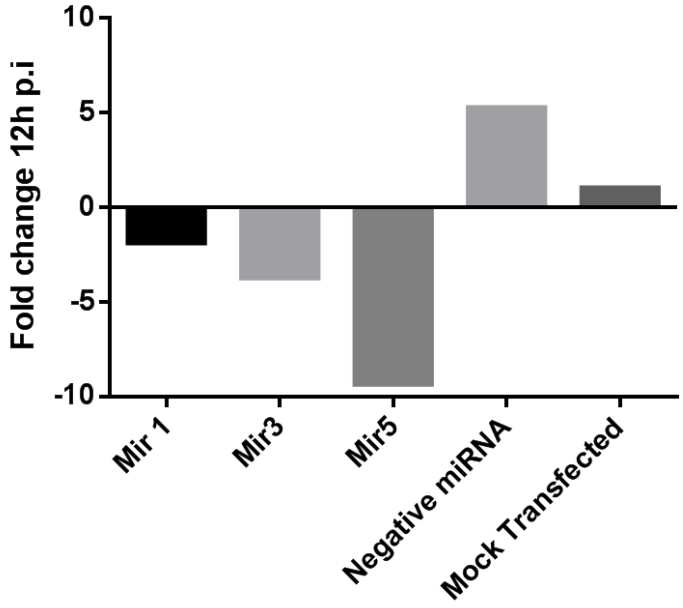
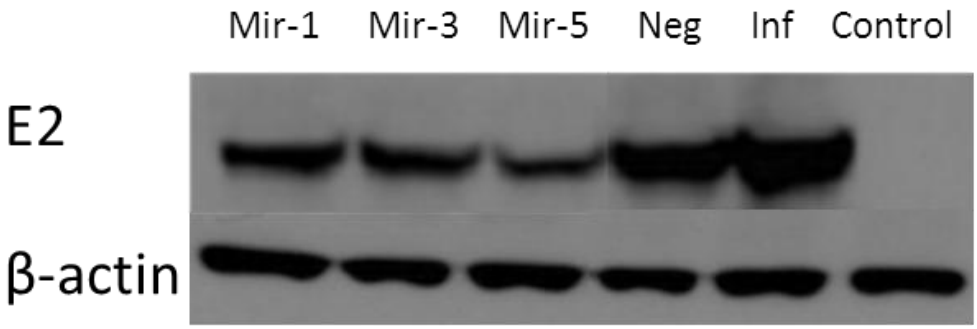


Figure 31: Toxicity and antiviral activity of artificial miRNAs in BHK-21 cells. A: Cell proliferation assay to evaluate toxicity of artificial miRNAs in BHK cells. BHK cells were grown in 96 well plates and transfected with 100ng of artificial miRNA. After 24 h of transfection MTT reagent was added to determine miRNA toxicity. No significant cellular cytotoxicity was seen. B: Safety efficacy of artificial miRNAs in BHK-21 cells against VEEV infection. BHK cells were transfected with 2µg miRNA per well of 12 well plates. After overnight transfection, cells were infected with 0.1MOI of TC-83. Cell supernatant was collected at 12 h post infection. Virus titer estimation was performed using $TCID_{50}$. Statistical significance was calculated using 1 way multiple comparison ANOVA. $P < 0.0001$. Data is shown here is representative of atleast three experimental repeats.

A.


Fold change 12h p.i
10
5
0
-5
-10
Mir 1
Mir3
Mir5
Negative miRNA
Mock Transfected

B.


Mir-1
Mir-3
Mir-5
Neg
Inf
Control
E2
β-actin

C.

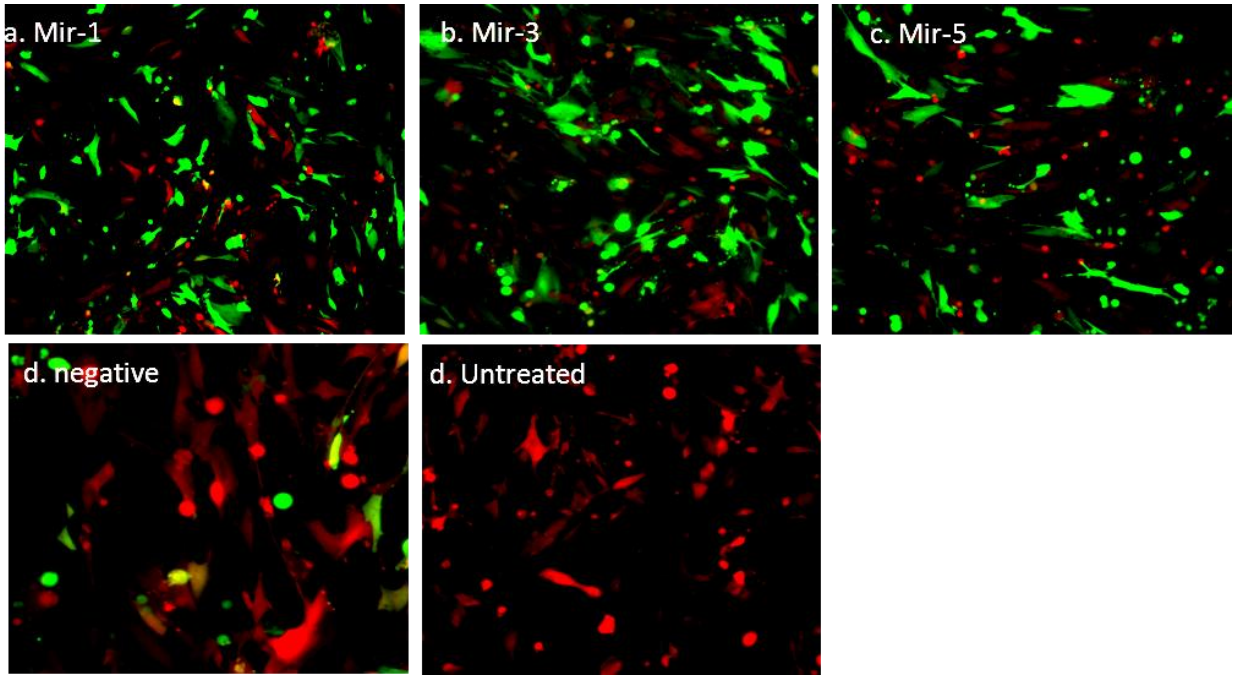
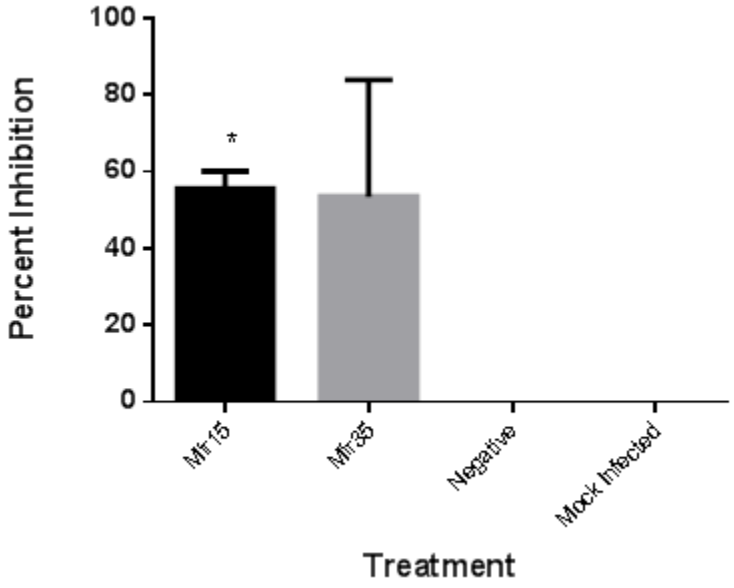


Figure 32: Antiviral efficacy of artificial miRNAs in BHK-21 cells. A: Viral RNA copy number by RT-PCR. BHK cells were treated with artificial miRNAs and infected with 0.1 MOI of TC-83. Total cellular RNA was isolated at 12h p.i and RT-PCR was performed with specific primers for VEEV nsp-4 region. Fold changes were calculated by normalizing the values to GAPDH expression as endogenous control. B: Western blot for VEEV E2 glycoprotein in BHK-21 cells treated with artificial miRNAs. Cells lysates was collected at 24h p.i and probed for presence of VEEV E2 glycoprotein. A marked reduction in viral glycoprotein was observed in cells treated with Mir 1, 3 and 5 whereas an intense signal was observed in untreated cells. Beta –actin was used as loading control C: Fluorescence microscopy images of BHK-21 cells transfected with artificial miRNA and green fluorescence indicates the expression of EmGFP along with artificial miRNAs. Cells were infected with TC-83 strain which express cherry red protein. In cells treated with Mir-1, 3 and 5 does not show any overlap in the presence of artificial miRNAs (green) and virus (red). Cells treated with non-specific artificial miRNA shows overlapping green and red color indicated by white arrows.

A.



B.

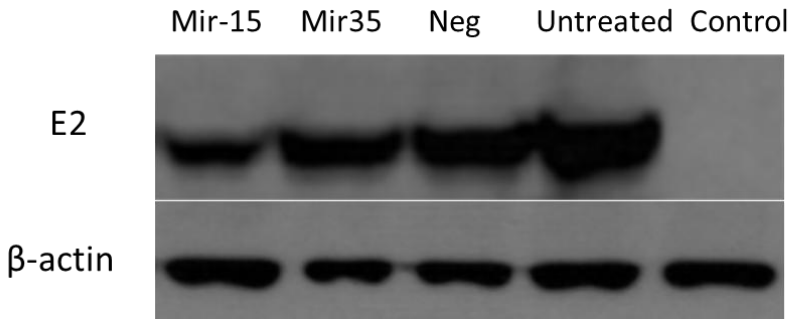


Figure 33: Antiviral effect of chained artificial miRNAs. A: Inhibition of virus replication after treatment with combination of two artificial miRNAs. Mir 15 and Mir35 were generated by combining individual miRNAs in under one promoter. BHK-21 cells were transfected and supernatant was assayed for inhibition of virus replication by $TCID_{50}$. Virus titer indicated a modest inhibition by Mir 15 in virus replication in comparison to individual miRNAs whereas no significant inhibition was observed in case of Mir-35. Statistical significance was calculated using 1 way multiple comparison ANOVA, $p < 0.01$. Data is shown here is representative of atleast three individual experimental repeats. B: Western blot showing amount of viral envelop glycoprotein in the BHK-21 cell lysate upon after treatment of combined miRNAs. Western blot data corroborated with the virus titer data with increased presence of envelope glycoprotein in the cells indicating minimal inhibition of virus by combined artificial miRNAs.

Efficacy of antiviral and anti-inflammatory drugs on VEEV infection: VEEV infection of brain and subsequent CNS disease development is being increasingly recognized due to host inflammatory response and secondary neuronal damage due to unregulated inflammation. Therefore we tested several FDA approved anti-viral and anti-inflammatory drugs for their efficacy in treating the VEEV infection. These drugs were selected based on their mechanism of action on other RNA viruses and their ability to suppress excessive inflammation during viral infection. Three anti-inflammatory drugs were tested which were, Naproxen, indomethacin and aspirin. The antiviral drugs which were tested are ribavirin, amantadine and rimantidine. Other than these drugs, curcumin was also tested for its efficacy against VEEV infection. Curcumin is an anti-inflammatory compound. Curcumin is a known inhibitor of nuclear factor- kappa beta (NF-kB) (Freudlsperger et al 2008, Bachmeier et al 2008, Dhandapani et al 2008) and this property of curcumin finds it application in disease where reduction in inflammation hold key to the amelioration of disease condition.

Mice were treated with both antiviral and anti-inflammatory compounds at various doses regime as indicated in figure 34. The initial doses were selected based on the data available in the literature. From our first study in animals, indomethacin 50mg/kg, 25mg/kg and 4mg/kg dose were found to be toxic and therefore, indomethacin treatment was no longer pursued. Animals were treated with both anti-viral and anti-inflammatory for seven days prior to virus infection followed by infection with virulent VEEV in the right footpad. The drug treatment was continued for another seven days and the animals were monitored for up to two weeks after VEEV infection. Results of this study indicated little to no protection against VEEV infection, hence another study with higher doses of the drugs was performed in the similar way as described above. The combined results of both these studies suggested that antiviral and anti-

inflammatory drugs individually have limited to poor beneficial effect against VEEV infection (Figure 34, 35). When a combined dose of anti-inflammatory and antiviral drug was given ribavirin and naproxen combination showed some beneficial effect early in the course of infection though a significant difference in survival post infection was not observed (Figure 36). However, clinical appearance of the disease symptoms were significantly delayed in the animals treated with naproxen, an anti-inflammatory drug.

The initial experiments with the drugs indicated that neither antiviral nor anti-inflammatory drugs are useful in combating VEEV infection. However, a marginal effect was onserved in the clinical symptoms of animals treated with ribavirin and naproxen. Hence we conducting another experiment in which animals received combined treatment anti-viral and anti-inflammatory drugs. The virus load was estimated by performing semi quantitative real time pcr for a virus gene in the brain of the animals.

Naproxen treated VEEV infected CD-1 mice early in the infection (0-5 days) showed lesser degree of ruffled fur, lethargy, hunched back, and delayed appearance of shivering and paralysis as compared to the untreated mice thereafter, these mice quickly became sick and there was no difference in the mean survival time of naproxen treated and untreated VEEV infected mice. A marked decrease but not total ablation in the vascular inflammation was observed in the naproxen treated VEEV-infected mice at 96 and 120 h pi (n = 3 each group) (Figure 37). Viral load in the brain was evaluated by RT-PCR at 48, 72, 96 and 120 h p.i. (n = 5 each group). There was no significant difference in the viral load in the brain of the naproxen treated and untreated VEEV infected mice groups. However, when taken as individual sets of mice, more viral load was observed in the brain of some of the naproxen treated mice as compared to the untreated ones (Figure 38). Treatment with ribavirin (80 mg/kg, once a day), a known anti-viral drug,

alone or in combination with naproxen also did not affect the mortality or the viral load in VEEV infected mice (Figure 39). However, a combined treatment of ribavirin and naproxen showed reduction in virus levels in majority of the animals though no increase in mean survival times was observed (Figure 40).

These results show a complex role of inflammation in VEEV pathology, initial better health of naproxen treated mice may be due to reduced inflammation in the brain. However, increase in brain viral load in naproxen treated animals may be due to unchecked viral replication due to reduced inflammation. Thus a synergistic effect of combined treatment with antiviral and anti-inflammatory compounds is suggested for future studies.

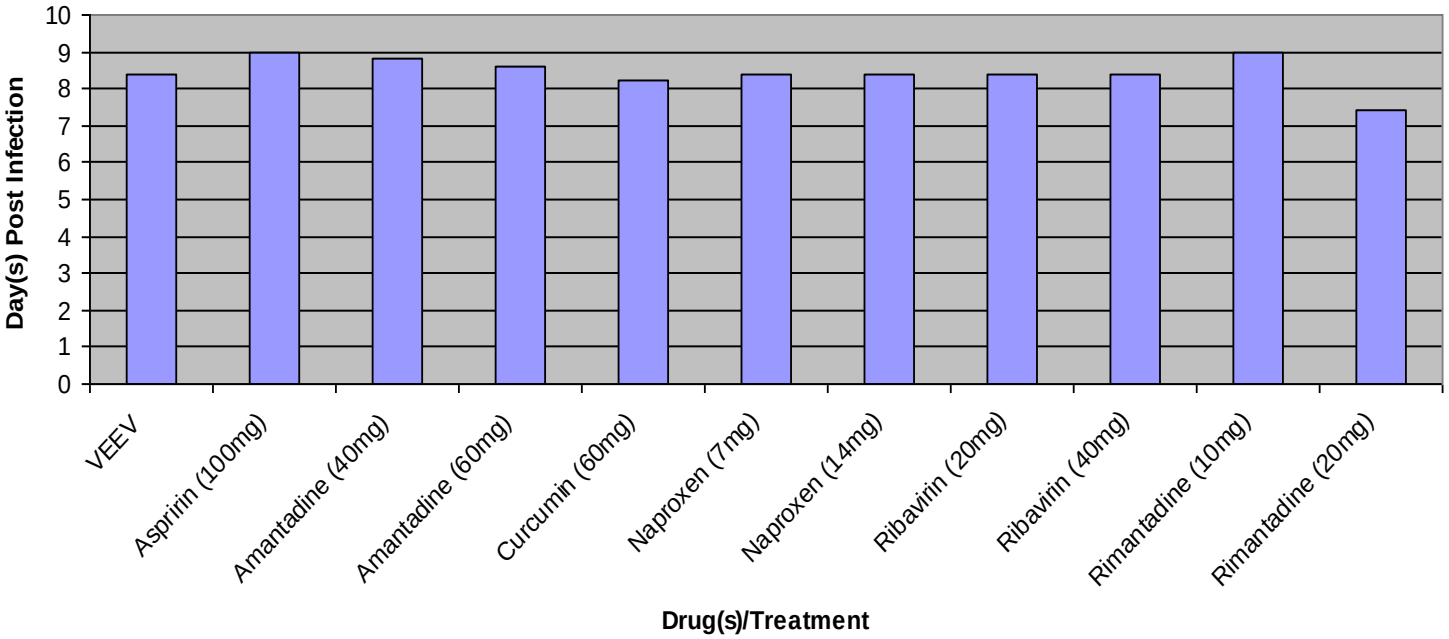


Figure 34: Effect of antiviral and anti-inflammatory compound on mean survival time (MST) post VEEV infection in mice: Group of 5 mice each was pre-treated daily with single oral dose of antiviral or ant inflammatory drugs for seven days. On 7th day mice were inoculated with 1000 pfu of V3000 in the left rear foot pad under inhalation anesthesia of isoflurane. Treatment was continued for seven more days and mice were observed for clinical signs of disease for a period of two weeks post infection.

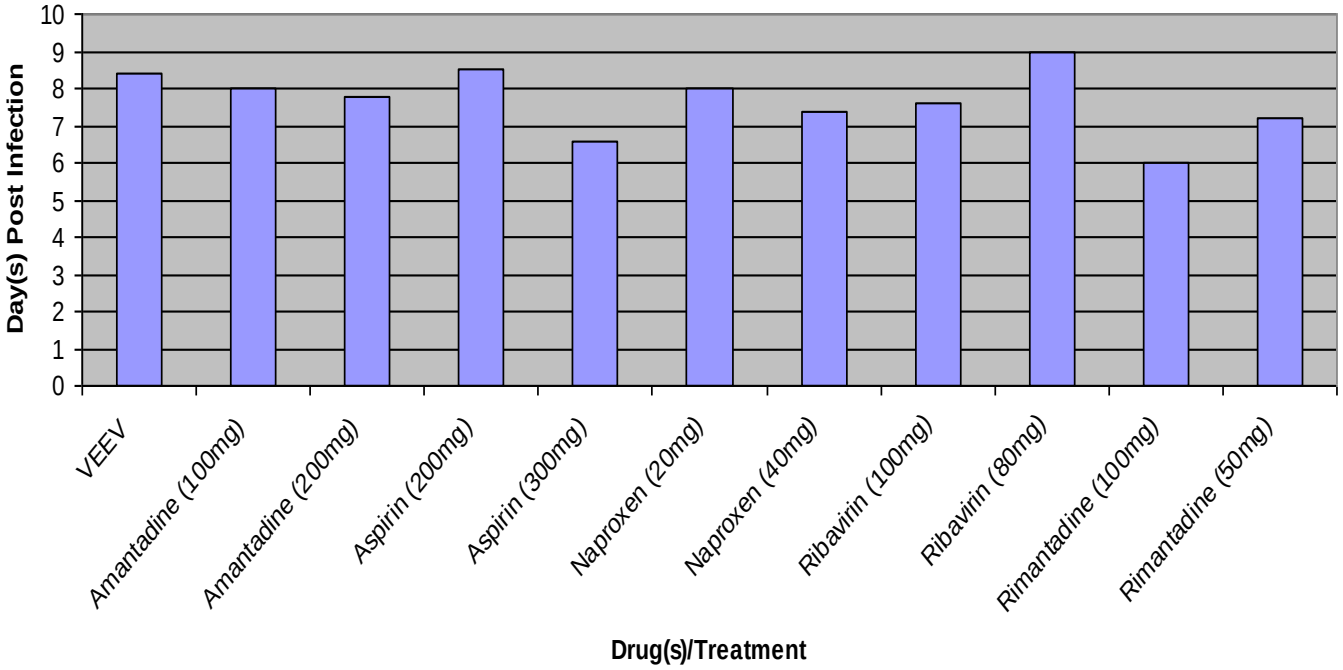


Figure 35: Effect of antiviral and anti-inflammatory compound on mean survival time (MST) VEEV infection in mice: No significant elongation of MST was observed in the animals that were treated with the anti-inflammatory or anti-viral drugs. However, animals that were surviving in the Naproxen treatment appeared clinically better (smoother fur, less hunched, less lethargic, reduced shivering and degree of paralysis) on day by day basis as compared to the VEEV alone group.

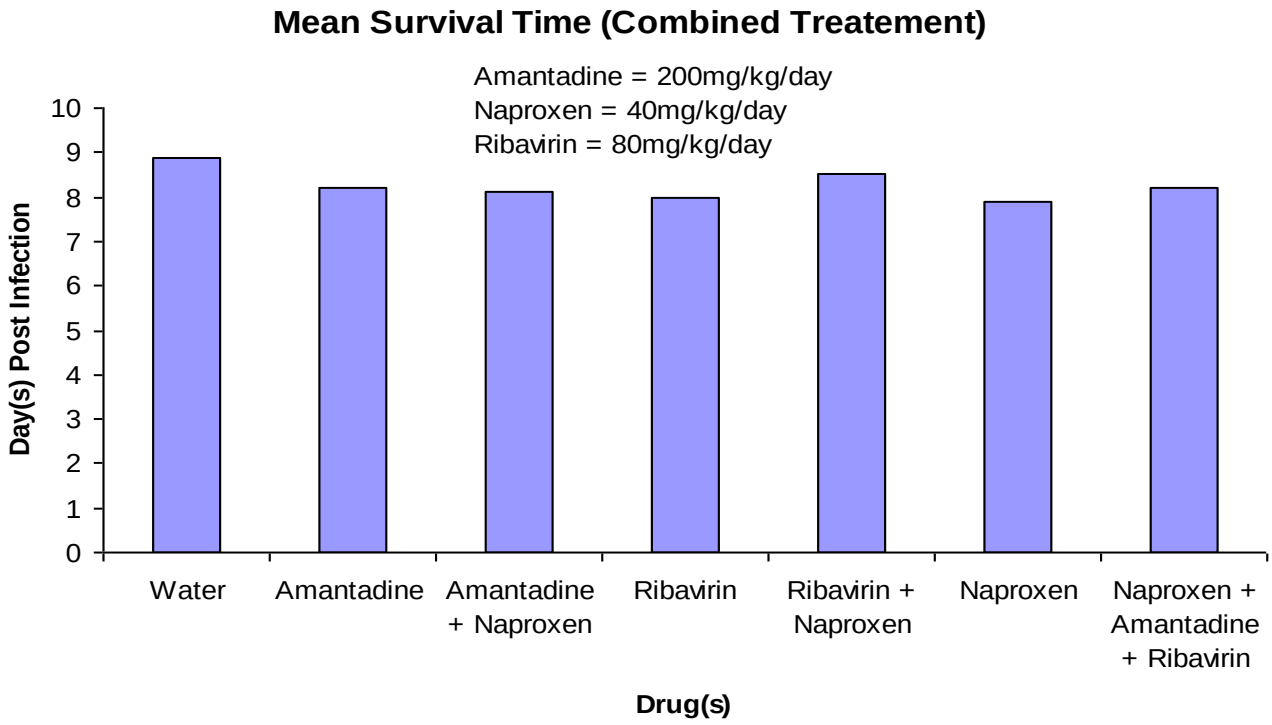


Figure 36: Effect of combined treatment of antiviral and anti-inflammatory compound on VEEV infection in mice. Mice were treated daily single oral combined dose of antiviral and anti-inflammatory drugs. Animals were treated 7 day pre infection and 7 day post infection. Animals were observed for the clinical sign of disease development such as excitability, ruffled fur, lethargic, paralysis and final end point at death.

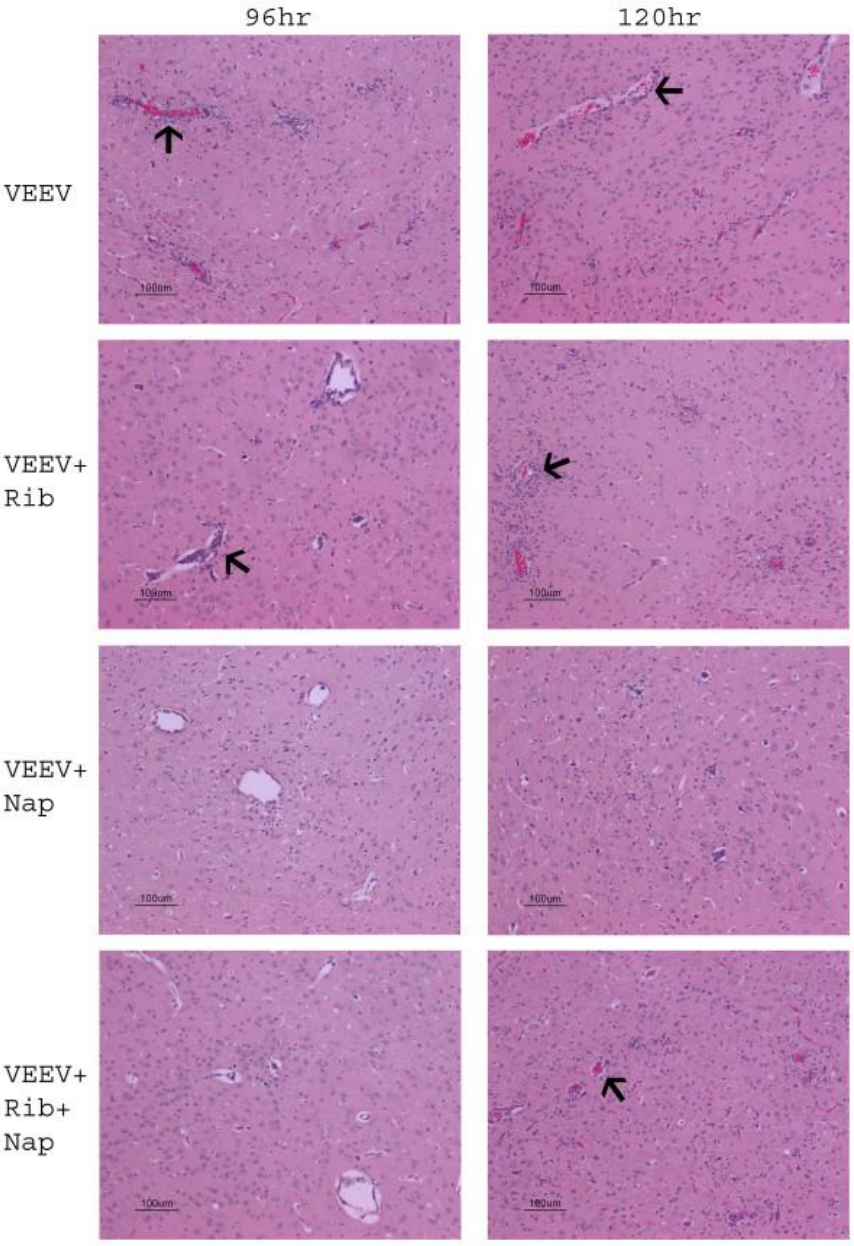


Figure 37: Inflammation was reduced in VEEV infected mice treated with naproxen or naproxen plus ribavirin. Animals were treated with naproxen (40 mg/kg/once a day) only or naproxen plus ribavirin (80 mg/kg/once a day) at the time of infection (1000 pfu of VEEV inoculated in left rear foot pad). Animals were monitored twice a day and clinical symptoms of disease were monitored for two weeks.

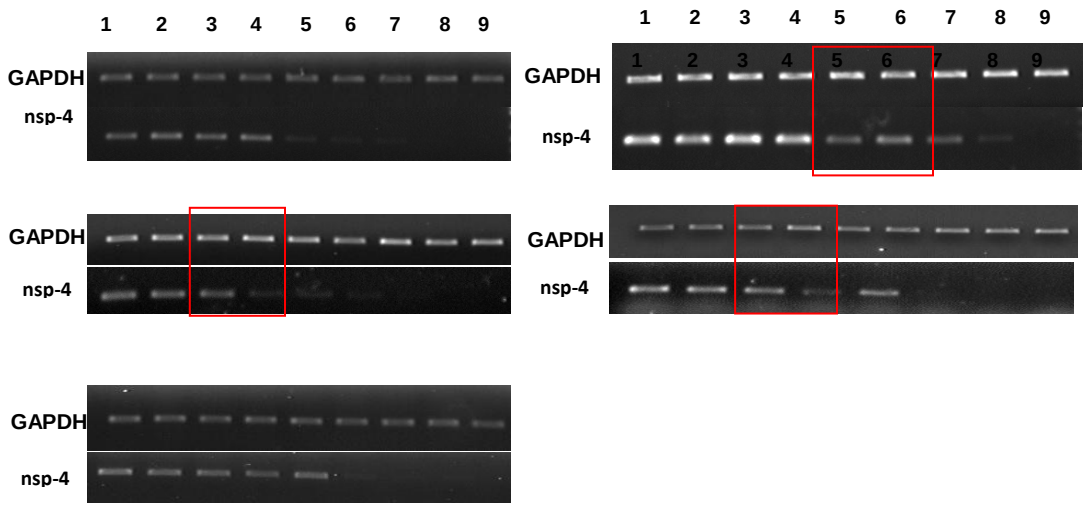


Figure 38: Presence of VEEV in brain of animals treated with Naproxen: VEEV presence was detected in the brain by RT-PCR specific nsp4 gene of VEEV. RNA was isolated from the whole brain and cDNA was made using First Strand Synthesis Kit (Invitrogen Inc, Carlsbad, CA). nsp4 specific PCR was done using PCR supermix (Invitrogen Inc. Carlsbad, CA). Results for 5 individual mice of the group are represented above. Two out of five mice showed increase in the virus levels at 96 h post infection and one mouse showed increase in the virus presence at 72 h post infection. One mouse showed slight reduction in the virus presence at 72 h post infection. In remaining one set of mice there was no difference in virus presence in drug treated group as compared to the control mice. Lane 1: Test 120hr p.i.; Lane 2: Control 120 p.i.; Lane 3: Test 96 h p.i.; Lane 4: Control 96h p.i.; Lane 5: Test 72h; Lane 6: Control 72 h p.i.; Lane 7: Test 48 h p.i.; Lane 8: Control 48 h p.i.; Lane 9: Uninfected control.

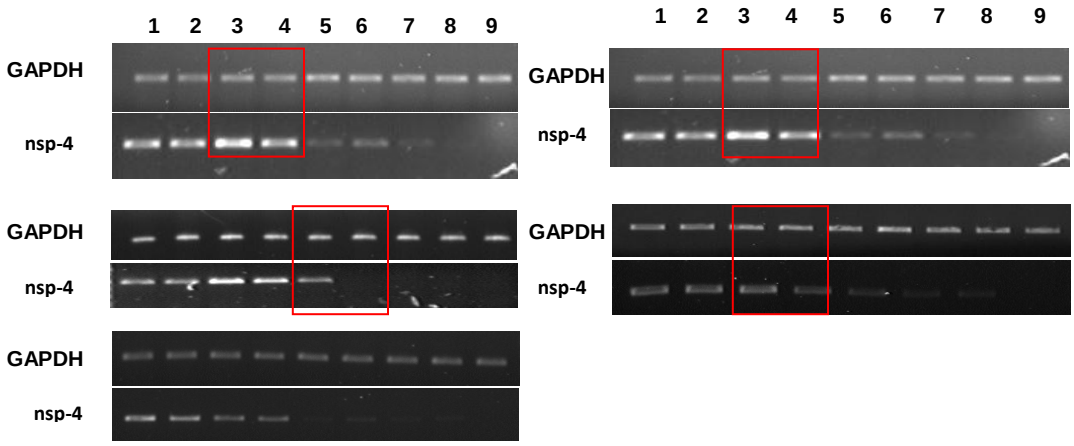


Figure 39: Presence of VEEV in brain of animals treated with Ribavirin: VEEV presence was detected in the brain by RT-PCR specific nsp4 gene of VEEV. RNA was isolated from the whole brain and cDNA was made using First Strand Synthesis Kit (Invitrogen Inc, Carlsbad, CA). nsp4 specific PCR was done using PCR supermix (Invitrogen Inc. Carlsbad, CA). Results for 5 individual mice of the group are represented above. Four out of five mice showed increase in the virus levels at 96 h post infection and one mouse showed slight reduction in the virus presence at 72 h post infection. In remaining one set of mice there was no difference in the virus levels at any time post infection. Lane 1: Test 120h p.i.; Lane 2: Control 120 p.i.; Lane 3: Test 96 h p.i.; Lane 4: Control 96h p.i.; Lane 5: Test 72h; Lane 6: Control 72 h p.i.; Lane 7: Test 48 h p.i.; Lane 8: Control 48 h p.i.; Lane 9: Uninfected control.

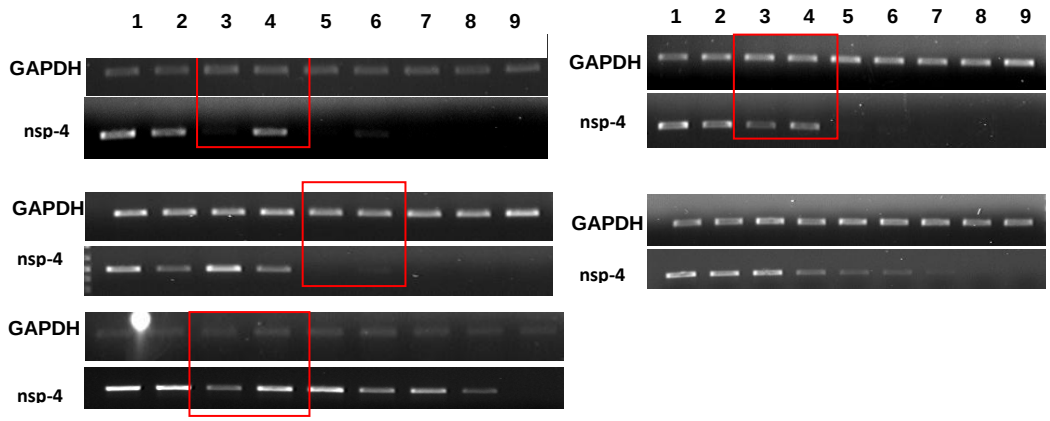


Figure 40: Presence of VEEV in brain of animals treated with Ribavirin and Naproxen: VEEV presence was detected in the brain by RT-PCR specific nsp4 gene of VEEV. RNA was isolated from the whole brain and cDNA was made using First Strand Synthesis Kit (Invitrogen Inc, Carlsbad, CA). nsp4 specific PCR was done using PCR supermix (Invitrogen Inc. Carlsbad, CA). Results for 5 individual mice of the group are represented above. Three out of five mice showed reduction in the virus levels at 96 h post infection and one mouse showed slight reduction in the virus presence at 72 h post infection. In remaining one set of mice at 96 and 72 h post infection virus presence was more in drug treated group as compared to the control mice. Lane 1: Test 120h p.i.; Lane 2: Control 120 p.i.; Lane 3: Test 96 h p.i.; Lane 4: Control 96h p.i.; Lane 5: Test 72h p.i.; Lane 6: Control 72 h p.i.; Lane 7: Test 48 h p.i.; Lane 8: Control 48 h p.i.; Lane 9: Uninfected control.

Discussion

Alphavirus nonstructural proteins are primary mediators of viral replication inside the host cell. In this study we have compared the antiviral viral potential of RNA interference against VEEV in cell culture model. We targeted viral polymerase which is encoded by nsp-4 gene in the viral genome. Nsp-4 was selected as a target because it encodes for the viral RdRP which is the primary mediator of viral genomic replication and also the N-terminal region of nsp-4 is highly conserved not only among the species but also across the viral species with similar RdRP.

In our first experiment, we tested whether siRNA duplexes which are to be tested are toxic to the cells since double stranded RNA molecules can elicit a TLR mediated immune response (Hornung et al 2005). As expected, we did not observe any significant cytotoxicity due to the presence of double stranded RNA molecules in the cell since the siRNA design parameters eliminated the sequences which are recognized by TLRs. We then tested the efficacy of four different siRNA sequences against the virulent VEEV infection by immunofluorescence and cell protection assays. The data suggested a strong inhibition of virus replication which was evident from immunofluorescence staining against the VEEV antigen and cell protection assay by siRNA 4 and the pooled siRNAs. Further experiments revealed that the efficacy of the pooled siRNA was due to the presence of siRNA 4 since no synergistic effect was observed by the presence of any other siRNA (Data not shown). This was validated by a reduction in the virus titer by more than 95% by siRNA4 at both 12 and 24h post infection. These data suggest that siRNA 4 is specifically inhibit the replication of virulent VEEV and increases cellular survival.

SiRNAs have a limitation of short half-life inside the cells which makes their practical application questionable. Recently, siRNAs were cloned into the plasmid expression vector called shRNAs. In this vector, the siRNAs are expressed under the U6 promoter. The expression

of siRNAs from U6 promoter alone is low; hence CMV enhancer elements are cloned in the vector to enhance the expression of siRNAs. Results from efficacy testing against VEEV from shRNA experiments were almost similar to that of siRNAs duplexes.

Artificial miRNAs are small RNA molecules like siRNAs which are expressed under the promoter of enogeneous miRNA like Mir-155. Once inside the cells, these vectors are processed by RNA pol II like endogenoeus miRNA which results in a better and efficient processing and RNAi mediated target inhibition. Further, since these molecules are expressed by RNA pol II, only a limited number of RNA copies which are necessary are expressed where in shRNAs, RNA pol III over expresses the siRNA thereby saturating the RNAi machinery of the cells. A comparative study was performed to evaluate the efficacy of artificial miRNAs to siRNAs. It was found that artificial miRNAs tend to inhibit VEEV replication in cell culture more efficiently without causing any cellular cytotoxicity. In particular mir-3 was very efficient in inhibiting VEEV replication with no detectable virus until 24h post infection.

These studies show that RNAi is a very powerful technique to develop an antiviral therapy against VEEV infection. Designing of the oligos is very critical which otherwise can have0 off target effect on other genes and can elicit interferon response (Mali Ketzinel-Gilad et al 2006). Further, RNA viruses are notorious for undergoing adaptive mutations and hence making RNAi mediated therapy ineffective. In order to overcome this problem, the selection of genomic region for RNAi should be highly conserved among the other member of the species (O'Brien 2007). Finally, the delivery vehicle has to be highly specific and efficient for sufficient delivery to the required tissue. Many novel delivery vehicles have now been under clinical investigations for RNAi based therapeutic for other viruses (Burnett et al 2011), which can be utilized with the tested siRNAs in animal models for VEEV infection.

In another approach we have tested the efficacy of various antiviral drugs which are currently used for treatment against other viral infections. Ribavirin, rimantidine and amantidine are used to treat influenza, respiratory synctial virus, HCV and other RNA viruses (Leyssen et al 2008, Clark and Nelson 2012). Ribavirin is an inhibitor of viral replication by lowering the intracellular pool of GTPase whereas amantidine and rimantidine interfere with the viral protein uncoating which is required for replication (Leyssen et al 2008). Treatment with these drugs in animals against VEEV infection did not confer any significant protection against VEEV challenge which indicated that none of these drugs were very effective antiviral activity against VEEV.

After the appearance of virus in the brain, a strong inflammatory response causes a irreversible damage to the neurons and supportive neuronal cell infrastructure. Inflammation leads to secondary neuronal injury and which results in neurodegenration in brain (Schoneboom et al 2000). Reactive gliosis is associated with the apoptotic neurons in the areas of the brain that are free from the viral antigen (Schoneboom et al 1999). NSAIDs are known for their analgesic and anti-inflammatory properties. There are two mechanism of action for NSAIDs (1) reducing the sensation of pain, and (2) reducing the inflammation that often accompanies and worsens pain by reducing the production prostaglandins (The Merck Manual--Second home edition-online, Non-steroidal Anti-Inflammatory Drugs).

Treatment with anti-inflammatory drugs naproxen showed delayed appearance of the clinical signs of the disease. The animals looked healthy for a longer time but surprisingly all of them succumb to infection along with untreated animals. RT-PCR data indicated presence of higher load of virus in few of the naproxen treated animals along with a reduced inflammation in the brains of these mice. This suggest that the delayed appearance of clinical symptoms is due to

the reduced inflammation in the brain but a less immune activated brain subsequently allowed more virus vigorous virus replication resulting in the death of the host. A combined treatment of ribavirin and naproxen resulted in a decreased load of virus but failed to increase the mean survival time of animals. This shows that inflammation is a critical part of VEEV which needs to be carefully regulated.

The conclusion from these studies is that a combination of a specific antiviral to reduce the virus replication and an anti-inflammatory to reduce the inflammation associated with the infections has the best chances to control the viral replication and damage to the host.

Chapter: 5

MicroRNA based attenuation of V3526 to generate a live attenuated vaccine candidate for VEEV infection- A proof of concept pilot study.

Abstract: Currently there are no FDA approved vaccine for protection against VEEV infection for both animals and humans. Various strategies have been used to develop both inactivated and live attenuated vaccine strategies. Currently, TC-83 which is a live attenuated vaccine candidate is used for vaccination for at risk laboratory personnel. Another live attenuated vaccine candidate V3526 showed a great promise in preclinical studies but recently failed in human trials. Recently, miRNAs have been used develop live attenuated vaccine candidates by restricting the replication of virus in the cells where it can cause disease and allowing replication elsewhere which can elicit a good systemic immune response. VEEV is lethal only if the virus reaches the brain otherwise it presents severe flu like symptom. V3526 showed residual toxicity of virulent VEEV, hence a restricted replication of V3526 in CNS may make it a safe vaccine candidate. In this study, we have engineered complementary copies of brain enriched miRNAs into V3526 without altering its replication kinetics. Presence of brain specific miRNA sequences will lead to bnding of endogenous miRNA resulting in inhibition of virus replication. We have shown in our invitro study that molecular clones carrying miRNA complementary sequences fails to reach high virus titers in neuroblastoma cells expressing endogenous miRNAs. These data suggest that miRNA bases vaccine strategies can be used to develop novel vaccine candidates for VEEV.

Introduction

There have been many efforts to develop safe and efficacious vaccine candidate for VEEV. Various strategies have been used to develop both live attenuated as well as inactivated vaccine candidates (Passeler and Weaver, 2009). Despite significant advancements in the field of vaccine development, a safe and effective vaccine is not available for VEEV as well as other alphaviruses. Much of the earlier effort for vaccine development was focused towards live attenuated vaccines. Many attenuated strains of VEEV were developed among which the most promising candidates were TC-83 and V3526. TC-83 is currently been used as a vaccine for at risk personnel for laboratory but has 20% non-responders. On the other hand, V3536 which was developed using site directed mutagenesis is very safe and elicit very good immune response (Rao et al 2006). Despite being safe in animals, V3526 showed signs of residual toxicity in human clinical trials. Since V3526, has already been attenuated and elicits a high immune response, we hypothesized that further attenuation of V3536 without altering its structural proteins integrity will make it more safe along with good immunogenicity.

Recently, miRNAs have been used to develop vaccine strains and oncolytic viruses to limit their replication in specific tissue (Lauring et al 2010). Induction of this tissue tropism allows the virus to replicate in the tissues where it cannot cause a disease whereas concurrently it can replicate elsewhere to elicit immune response. VEEV is known to be lethal only if the disease reaches the neurological phase whereas a high replication in the peripheral system causes the clinical symptoms associated with the morbidity. We hypothesized that if the replication potential of V3526 can be completely eliminated in the central nervous system and the rate of replication in the peripheral system can be reduced then, V3526 will be safer without its immunogenic potential being altered. This can be achieved by cloning complementary sequences

of miRNAs in the cDNA clone of V3526. These miRNAs should be constitutively expressed or enriched in cells of the CNS along with low level expression in the peripheral system. Hence, a virus recused from such a cDNA clone will harbor these miRNA sequences. These sequences will be recognized by their commentary endogenous miRNAs in the CNS cells which will then lead to the degradation of the genome by RNAi pathway (Figure 4).

For this study we selected three miRNAs which are known to be expressed in high levels selectively in neurons which are mir-124a and mir-125b (Lagos-Quintana et al 2002). Among these, mir-124a have relatively more expression in the brain whereas mir-125b is mainly expressed in the cortical neurons. However, these miRNAs are also expressed in other tissues of the body at basal levels. In this preliminary study, we have cloned the complementary sequences of these miRNAs in the cDNA clone of V3526 to attenuate the replicative potential of V3526. For cloning we have used the nsp3 region of VEEV genome where insertion of these oligos does not have a deleterious effect on the replication of the virus (Beitzel et al 2010). We found that presence of these miRNAs in the genome of reduced the replication of the mutant viruses by many fold in comparison to the control and wild type strains particularly when grown in cells of neuronal origin. These studies indicate a potential of miRNA based attenuation of alphavirus vaccine candidates.

Materials and methods

Designing of oligos: The mature miRNA sequences of miR-124a and miR-125b were taken from miRbase database. Both the sense and anti-sense strands of these sequences containing two repeats and flanked by a Sal1 and BglII site at either ends were chemically synthesized and HPLC purified from Biomedical instrumentation center in USUHS. The oligo sequences used are:

MiR	Orientaion	Sequence
Mir 124a	Sense	5‘agtacatgtcgactaaggcacgcggtgaagccctaaggcacgcggtgaatgcccagatctagtacat3’
	Antisense	5’atgtactagatctgggcattcaccgcgtgccttagggcattcaccgcgtgccttagtcgacatgtact3’
Mir 125b	Sense	5’gatctcacaagttagggtctcagggatcacaagttagggtctcagggatcacaagttagggtctcagggatcacaag ttagggtctcaggga-3’
	Antisense	5’gatctccctgagaccctaacttgtgatccctgagaccctaacttgtgatccctgagaccctaacttgtgatccctgaga ccctaacttgtga-3’
Scramble	Sense	5’agtacatgtcgacaacgcaccagtcccaccatcccccac agcactcgctgcatcccccagatctagtacat3’
	Antisense	5’atgtactagatctgggggatgcagcgagtgctgtgggggatggtgggac tggtgcgttgtcgacatgtact3’

Cloning of oligos: Chemically synthesized single strand oligos were resuspended in nuclease free water(Invitrogen) to achieve a concentration of 1µg/µl. Equal volumes of the oligo solutions were heat denatured at 95°C for 15 minutes followed by snap chill prior to annealing. Annealing was carried out with Annealing buffer(Invitrogen) at 95°C for 10 minutes followed by gradual cooling to room temperature over approximately 30 minutes. Agarose gel purified(Qiagen) annealed oligos were digested with SalI and BglII for directional cloning into nsp3 region of V3526.

SalI restriction site was inserted at the 3’ end of the nsp3 gene cloned in pBlunt vector using site directed mutagenesis kit (Agilent technologies). Directional cloning of the oligos into nsp3 region was done using T4 DNA ligase (Invitrogen) according to the protocol and the ligated vector was transformed into One Shot Top 10 competent cells(Invitrogen). The transformed colonies were selected from LB agar-Kanamycin(50µg/ml) plates. Insertion of the oligos were checked by band shift on PCR amplification with nsp3 specific primers (5'-AGCTAGCGTGACCAGGTCGAC-3' and 5'-GCTGACGTTGCCCCGAGATCT-3') and the plasmids sequences were checked by sequencing. AvrII and BsiwI digested nsp3 gene containing the miR target sites were directionally cloned into V3526 cDNA as mentioned earlier. The transformants were selected and verified as mentioned above.

Viral RNA synthesis.: 5’ capped viral RNA was synthesized from agarose gel purified NotI linearized plasmids of the above clones by in vitro T7 RNA polymerase transcription kit(New England Biolabs) in presence of m7G(5')ppp(5')G RNA Cap Structure Analog (New England Biolabs) according to the manufacturer’s protocol. The RNA was purified from enzymes and unincorporated NTPs and template DNA using phenol –chloroform extraction method and RNeasy kit (Qiagen) respectively pior to in vitro transfection.

RNA transfection: 4µg of the purified capped viral RNA was transfected into BHK-21 cells seeded overnight in six well plate at 50% confluency using Lipofectamine transfection reagent (Invitrogen) according to the manufacturer’s protocol. The transfection medium was replaced with fresh medium at six hours post transfection. The cell supernatant was collected at 72 hours post transfection for viral titration.

Virus titration: Virus titration of the viral RNA transfection supernatant was determined by plaque assay on Vero cells. Vero cell monolayers seeded in (15mm X 60mm) tissue culture treated plates to 95% confluency overnight were inoculated in duplicates with 0.2 ml of serially diluted virus containing supernatant. Serial dilutions of viral suspensions were prepared in sterile 1X PBS (Calbiochem). Cells inoculated only in sterile 1X PBS served as the assay negative control. The viral suspensions were removed 1hour post incubation and a 1% agarose overlay was added to the cells. Composition of the agarose overlay is 1%(w/v) agarose(International Biotechnologies Inc), 6%(v/v) New born calf serum (Thermo Scientific), 50%(v/v) 2X Minimum essential medium (Invitrogen), 1.2%(v/v) Penicillin-Streptomycin solution (Mediatech Inc) and nuclease free water (Bioworld). On solidification of the agarose overlay at room temperature, the plates were incubated at 5% CO2 incubator for 72-96 hours for plaque formation. Prior to plaque counting, the plates were fixed with 2% formalin and stained with 0.1% crystal violet solution. The total number of plaques per plate was calculated and the virus titer expressed as plaque-forming units per milliliter (pfu/mL).

Results

Cloning and rescue of V3526 clones with complementary copies of miRNAs.

In this pilot experiment we have used complementary copies of miR-124a and 125b were cloned via directional cloning in the backbone of V3526. A scrambled copy of miR-124a was also cloned for negative control. Two consecutive repeats of the miRNA sequences were cloned to increase the chances of binding with the endogenous miRNA. The insertion position was chosen in nsp-3 region of the genome which is amenable to insertional mutations without causing any deleterious effect to virus replication. The location of insertional mutations was provided by Dr Brett Brietzel from their studies with VEEV genome (Brietzel et al 2011). Directional cloning was performed using BamH1 and Sal1 restriction enzyme sites in V3526. The cDNA clones were then used to generate positive sense RNA by *in vitro* transcription. Invitro transcribed RNA was then transfected into BHK-21 cells using lipofectamine 2000 (Life Technologies, Inc). At 72h post infection, cytopathic effect due to virus replication was observed and supernatant was harvested. To quantitate the virus, equal volume of supernatant was taken and plaque assay was performed using VERO cells. Plaque assay results indicated a productive virus replication in all the mutated clones along with wild type virus (Figure 41). Interestingly scrambled and wild type virus replicated at much higher titers in comparison to the mutated clones of miR-124a and miR-125b.

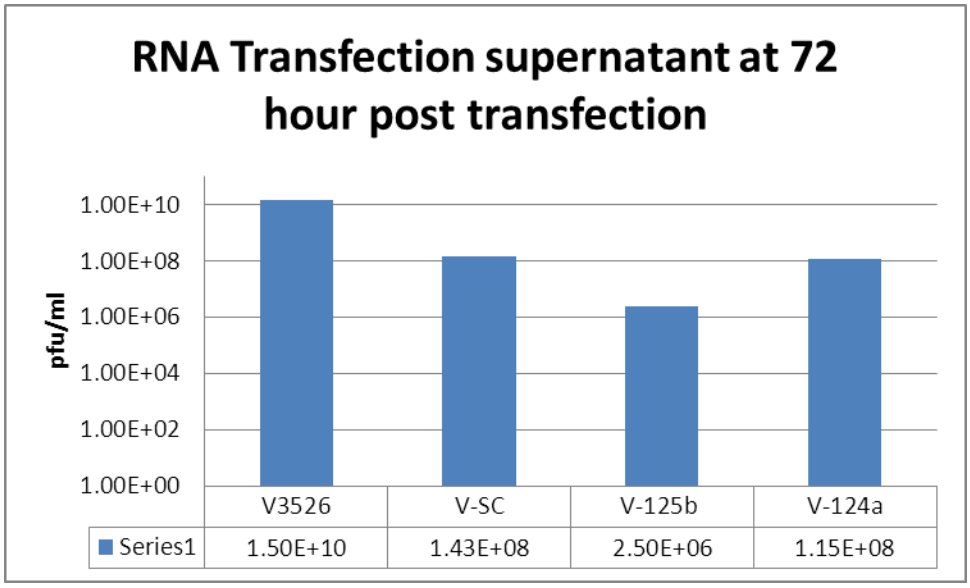


Figure 41: Virus recue from cDNA clone of V3526 wild type strain and miRNA engineered strains. 1ug of invitro transcribed RNA was transfected into BHK-21 cells and supernatant was harvested after 72h post infection. Virus titer was performed using VERO cells by standard plaque assay.

Multiplication kinetics of V3536 with miRNA complementary repeats.

After the successful rescue of virus from the cDNA clones, we wanted to test whether the presence of endogenous cellular miRNAs can bind to the complementary miRNA repeats in the viral genome and inhibit its replication. Though, most of the cell type express miR124a, 125b but their express is found to be abundant in cells of neuronal origin. Hence, we selected HTB-11 cell line which is a human neuroblastoma cell line and known to express the miRNAs of interest *i.e.* miR124 and miR125-b. HTB-11 cells were infected with MOI-1 with all the strains of the virus including the scrambled control. After the initial infection, cells were washed and new medium was added. Supernatant from these cells was collected at 6h, 12h, 24h and 48h post infection. Virus titer with this supernatant was estimated by standard plaque assay on VERO cells. Virus titer data indicated a robust replication of wild type V3526 along with the V-SC attaining high virus titers at 24h post infection. The viral clones of V-124a and V125b were also able to replicate in HTBI-11 cells but their virus titer was significantly less in comparison to the wild type V3526 and V-SC controls (Figure 42). This indicates that the clones which contain the miRNA repeats are selectively repressed in HTB-11 cells possibly due to the presence of miRNA 124a and 125b in these cells. Further, V-124a and V-125b grows to approximately same titer suggesting that the repression of their growth is similar i.e miRNA mediated inhibition.

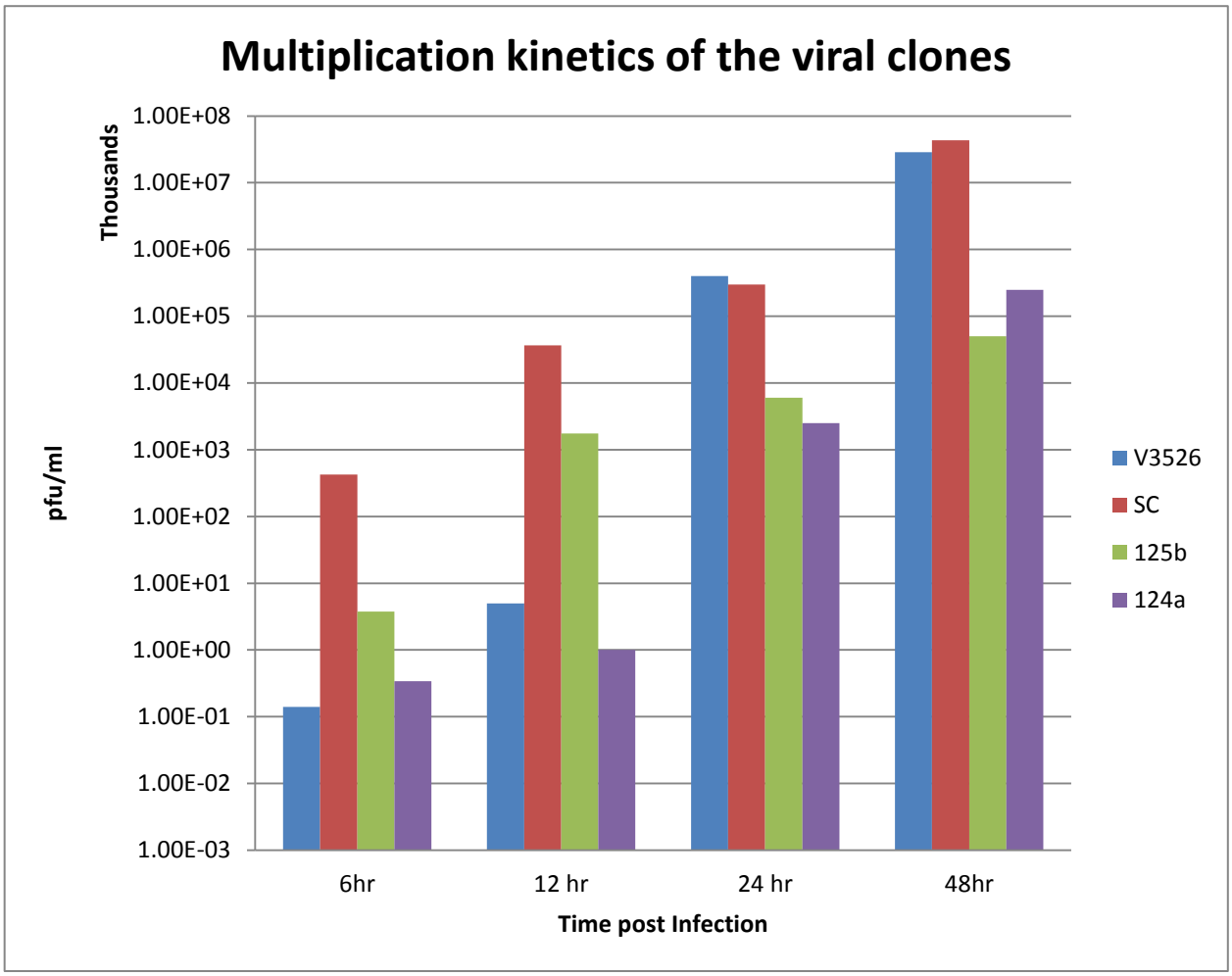


Figure 42: Multiplication kinetics of viral clones. HTB-11 cells were infected with MOI-1 with wild type V3526, V-SC, V-124a and V125b. Supernatant was harvested at 6, 12, 24 and 48h post infection and virus titer was performed on VERO cells by plaque assay. The virus titer values are representative of average of two repeats.

Discussion

In this proof of concept pilot study, we have demonstrated the use of miRNAs for the development of live attenuated vaccines for VEEV. V3526 which is currently considered as the best vaccine candidate has residual toxicity in humans. Other live attenuated vaccine candidates were also considered as unsafe for human trial. In case of inactivated vaccines, most of the inactivated vaccine candidates suffer from the problems of poor immunogenicity. To overcome this problem, we have incorporated miRNA elements in the viral genome to further attenuate it without interfering with its immunogenic ectodomains thus preserving the immunogenicity and increasing its safety.

VEEV is lethal only if the virus enters the CNS otherwise presents a flu like disease. Our working hypothesis is to inhibit residual replication of V3526 in CNS without severely impacting its replication in the peripheral system. We have cloned two complementary tandem repeats of miR-124a and miR-125b in the c-terminal end of the nsp-3 region of the VEEV genome. Nsp-3 region of the genome was chosen because it has been previously shown that this region of genome is not conserved and can allow insertion and deletion mutations without impacting the overall replication of virus. After incorporation of the miRNA complementary sequence repeats the virus was rescued by synthesizing the RNA using invitro transcription. The invitro transcribed RNA readily yielded infectious virus particles. Equal MOI infection these virus particles in BHK cells resulted in effective inhibition of virus growth of the mutant virus whereas the control and the wild type replicated to higher titers. Real-Time PCR using primers for mir-124a and mir-125b indicated presence of these miRNAs in BHK cells suggesting that the inhibition of mutant virus is due to the presence of miRNAs in the cells. Similar study with semliki forest virus has shown attenuation of virus replication in both invitro and in vivo

(Ylösmäki et al 2013). In this study, six tandem repeats of neuron specific mir-124a were cloned in the region between nsp-3 and nsp-4. This inclusion of miRNA repeat sequences lead to an effective inhibition of viral replication at both invitro and in vivo level. This study further strengthens our hypothesis that miRNA based virus attenuation can be used as a tool for new vaccine development.

A major concern for the use of live attenuated vaccine development for RNA viruses is the risk of reversion to wild type strains. RNA viruses are highly prone to mutations due to the inability of proofreading by the RNA dependent RNA polymerase. To identify if the mutants we generated have accumulated any mutations, we sequences the rescued virus after 3 successive passages and sequences the nsp-3 region containing the miRNA repeats. In our study, we did not observe any mutation in the region of interest atleast for the first three passages indicated that these new mutant strains are relatively stable. However, there is always a possibility that invivo, these strains may incorporate new mutations and hence will no longer be inhibited by the endogenous miRNAs but by then the immune system will be activated to stop the aggressive replication of the virus, Further, these medication in this case are done on a relatively safer vaccine candidate hence a risk to severe VEEV infection due to the strains reversal is also minimized.

According to our knowledge this is the first study to engineer novel live attenuated VEEV vaccine using miRNAs. Though the study is still in infancy but similar studies in other models suggest that these miRNA based live attenuated vaccines holds a great promise for development of successful vaccine candidates against VEEV.

Concluding Remarks

VEEV is an important human pathogen with both socioeconomic and biodefense impacts. The diseases which VEEV cause have a significant public health impact in the America's. Additionally, members of the same family of viruses have recently emerged as a leading public health concern in other parts of the world which include the recent outbreak of chikungunya virus in Asia.

The mechanisms of pathogenesis in among alphaviruses share a significant similarity and hence most of the studies have been done using the old world alphaviruses as a model system. However, there are specific differences between these Old World and New World viruses which results into a significantly different outcome of the disease. The molecular mechanisms driving the VEEV pathogenesis are not very well studied. In particular, the role of miRNAs in alphaviral pathogenesis has not been explored yet. MiRNAs are important host factors which regulates gene expression at both transcriptional and translational role. As mentioned before, miRNAs have been implicated as important host regulatory element which plays an important role in the outcome of a disease or disorder.

In this work, research efforts were directed to identify the important role of miRNAs in VEEV infection both *in vivo* and *in vitro* models. In the first study, we identified miRNA signatures in a VEEV infected mouse brain at early and late phase of infection. The results indicated the involvement of miRNAs which is a key regulator in the onset of inflammation in the brain during the early phase of infection. During the later phase of infection it appears the entire RNAi machinery is under the control of virus by down regulating miRNA expression except the miRNA which mediates inflammatory events. This suggests that miRNAs may play a

critical role in controlling the inflammation in the brain post VEEV infection. These results can be further explored to develop a new miRNA based therapeutic to target the inflammation which is a hallmark of VEEV as well as other alphavirus infections.

Further, the *in vivo* miRNA expression studies suggested a role of miRNAs related to brain microvascualar endothelium in alteration of BBB in VEEV infection. Experiments showed low but persistent infection of BMECs of BBB suggesting an alternative route of entry of VEEV into the brain. Entry via BBB has not been suggested as a possible initial viral entry route, but the data presented in this study suggest an active role of brain endothelium in VEEV in the brain (Figure 46).

Involvement of cellular survival pathways has not been studied or suggested to play a role in alphaviruses pathogenesis. *In vitro* miRNAs modulation indicated a role of these pathways in virus replication. Our results suggested the Pi3-AKT pathway is a key pathway which is necessary for the virus to enter and survive. Inhibition of this pathway reduced the virus replication and hence presents an attractive target for the development of novel host directed antiviral agents. Therefore, it becomes clear that understanding of miRNAs regulation in context of a viral infection may not only lead to direct understanding of the viral pathogenesis but also leads to identification of novels regulatory molecules which can be used for therapeutic interventions.

In the second part of the study, we have tested the utility of miRNAs as novels antivirals and vaccine development. From our results it is clear that artificial miRNAs provides a better protection against the viral infection *in vitro* along with being less toxic. These anti-viral RNAi based therapies may be better utilized to develop novel antiviral agents since the use of available

antiviral drugs have failed to provide significant protection. Moreover, providing vaccination against viral infection is the best way to prevent infection but currently there is no effective vaccine candidate for any alphavirus including VEEV. The miRNA based attenuation strategy discussed here strongly advocates the use of miRNA based attenuation of live attenuated vaccine candidates to provide safe and effective vaccines.

In conclusion, the present studies have increased our understanding of the molecular mechanisms of VEEV infection which also have led to identification of potential therapeutic targets and vaccine development.

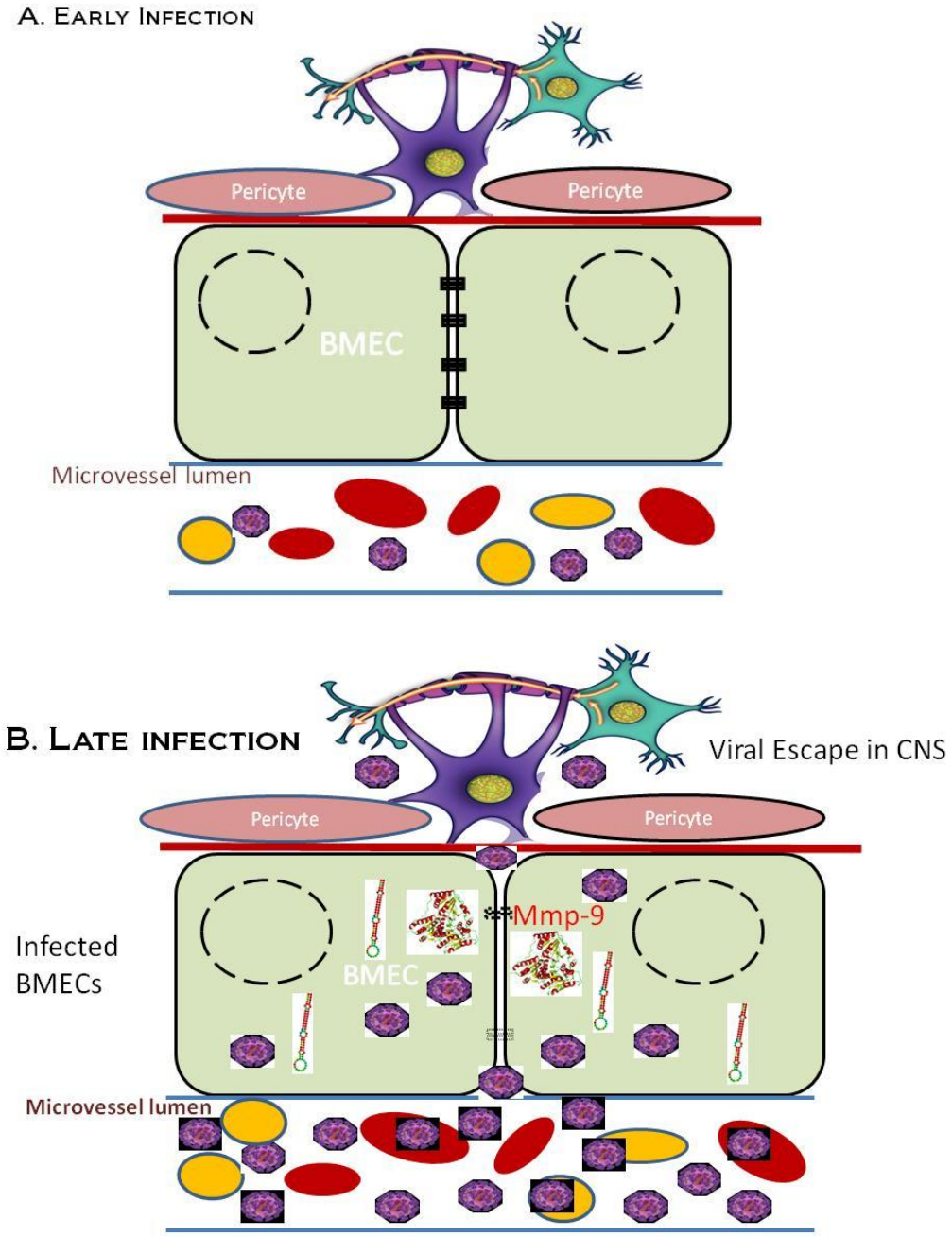


Figure 43: Proposed model showing alternative route of virus entry into CNS along with olfactory tract.A. Early in the infection few virus particles are present in the blood and it does not alter the BBB properties. B. During the later phase of infection, viremia in blood leads to low level infection of BMECs which increases the levels of MMP-9 and along with miRNA modulation which leads to the opening of BBB and virus escape into the CNS.

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List of Publications and Abstracts

1. Balakathiresan N, ***Bhomia M****, Chandran R, Chavko M, McCarron RM, Maheshwari RK. MicroRNA Let-7i Is a Promising Serum Biomarker for Blast-Induced Traumatic Brain Injury. J Neurotrauma. 2012 May 1;29(7):1379-87. (* Equal contribution)
2. Sharma A, **Bhomia M,** Honnold SP, Maheshwari RK. Role of adhesion molecules and inflammation in Venezuelan equine encephalitis virus infected mouse brain. Virol J. 2011 Apr 29;8:197.
3. **Bhomia M**, Balakathiresan N, Sharma A, Gupta P, Biswas R, Maheshwari R. Analysis of MicroRNAs induced by Venezuelan Equine Encephalitis virus infection in mouse brain. Biochem Biophys Res Commun. 2010 Apr 23;395(1):11-6. Epub 2010 Mar 19.
4. **Manish Bhomia**, Anuj Sharma Manoshi Gayen, Paridhi Gupta and Radha K Maheshwari. Artificial microRNA can effectively inhibit replication of Venezuelan equine encephalitis virus. (Paper under review at Antiviral Research).
5. Paridhi Gupta, Anuj Sharma, **Manish Bhomia**, Jing Han, Amy Yang, Raj K Puri and Radha K Maheshwari. Identification of novel host derived factors associated with neuroinvasion of Venezuelan equine encephalitis virus in mice. (Paper under review).
6. **Manish Bhomia**, Anuj Sharma and Radha k Maheshwari. Role of brain microvascular endothelial cells in Venezuelan equine encephalitis infection. (Manuscript under preparation).
7. **Manish Bhomia** and Radha K Maheshwari. Role of host Phosphatidylinositide 3-kinases-AKT pathway in Venezuelan equine encephalitis infection. (Manuscript under preparation).

Book Chapter

1. Degradation of Hexachlorocyclohexane (HCH) Degrading Bacterium by Dehalogenase Enzyme Activity and Characterization of *lin* Genes Involved in g-HCH Degradation. Natesan Manickam, **Manish Bhomia** and Ranjan Mishra. Biotechnological Approaches for Sustainable Development. M. Sudhakara Reddy 2004. Allied Publishers.
2. Molecular Mechanisms and Biomarker Perspectives of MicroRNAs in Traumatic Brain Injury. Nagaraja Balakathiresan, Anuj Sharma, **Manish Bhomia**, Raghavendar Chandran and Radha K Maheshwari. (Under Editing)

Abstract/Poster Presentation

1. Role of Brain Microvascular Endothelial cells in Venezuelan Equine Encephalitis Virus Infection. **Manish Bhomia**, Anuj Sharma and Radha K Maheshwari. USUHS Research Week poster Presentation, May, 2013
2. MicroRNA as Biomarkers for Traumatic Brain Injury. **Manish Bhomia** , Nagaraja S. Balakathiresan, Raghavendar Chandran, Anuj Sharma, Min Jia, He Li, Mikulas Chavko, Richard M. McCarron and Radha Maheshwari. Selected for Poster presentation in National Capital Area TBI symposia, May2012.
3. MicroRNA as biomarkers of mild traumatic brain injury. Sharma Anuj[1], Chandran Raghavendar[1], Hutchison Mary Anne, Barry Erin, Balakathiresan Nagaraja, **Bhomia Manish**, Gayen Manoshi, Jia Min, Fu Amanda, McCabe Joseph, Li He, Grunberg Neil, Maheshwari Radha K. Presented at 4th th Annual Trauma Spectrum conference at NIH 2011.

4. Analysis of MicroRNAs induced by Venezuelan Equine Encephalitis virus infection in mouse brain. **Bhomia M**, Balakathiresan N, Sharma A, Gupta P, Biswas R, Maheshwari R. Presented at annual meeting of microRNA in Human Disease and Development, 2010.
5. Expression profile of microRNAs in mouse brain upon VEEV infection. **Manish Bhomia**, Nagaraja Balakathiresan, Anuj Sharma, Paridhi Gupta, Roopa Biswas and Radha K Maheshwari. Presented at annual meeting of American Society of Microbiology Biodefense and emerging infectious diseases, 2010.
6. Novel inactivation method for enveloped and non-enveloped viruses: An approach for vaccine development. Anuj Sharma, Paridhi Gupta, **Manish Bhomia** and Radha K Maheshwari. 28th Annual Meeting of the American Society of Virology, The University of British Columbia, Vancouver, BC, Canada, 11-15 July 2009
7. Inhibition of Venezuelan equine encephalitis virus by artificial microRNA targeting virus polymerase. **Manish Bhomia**, Anuj Sharma, Paridhi Gupta and Radha K Maheshwari. Presented at Annual Meeting for American Society of Virology, 2009.
8. Venezuelan equine encephalitis virus replication is inhibited by artificial microRNAs targeted towards viral polymerase. **Manish Bhomia,** Anuj Sharma, Paridhi Gupta and Radha K Maheshwari. Presented at USUHS Research week 2009.
9. Targeted inactivation of virus protein and genome: Development of potential vaccine candidate for infectious diseases. Anuj Sharma, Paridhi Gupta, **Manish Bhomia** and Radha K Maheshwari. Research Week, USUHS, Bethesda, MD, May11-13, 2009.
10. Inhibition of VEEV Replication by siRNAs RNA directed against the Viral Polymerase. **Manish Bhomia,** Anuj Sharma, Paridhi Gupta and Radha Maheshwari. ASM biodefense, 2008

Appendix

1. Biosketch of Candidate

Manish Bhomia

2000 Baltimore Road, A21 ● Rockville, MD, 20851 ● Ph: 240-281-8476 ●

mkbhomia@gmail.com

EducationINSTITUTION AND LOCATION	**DEGREE**	**YEAR(S)**	**FIELD(S) OF STUDY**
Birla Institute of Technology and Science, Pilani, India.	M.Sc.	2005	Biological Sciences
Birla Institute of Technology and Science, Pilani, India	M.E.	2007	Biotechnology
Birla Institute of Technology and Science, Pilani, India	Ph D	Present	Biotecnolog/Virology

Advanced Courses / Training

1. Molecular Virology (credit course) conducted by Foundation for the Advanced Education in Sciences (FAES), National Institutes of Health. (2007)
2. Attended courses of Biostatistics, Scientific Ethics and Advanced Virology (audit course) at Uniformed Services University of Health Sciences (2007-2008).

Professional positions

2005-2007: Teaching assistant in Biological Sciences Department, Birla Institute of Technology and Science, Pilani, India

2007-Present: Research Assistant in Department of Pathology, Uniformed Services University of Health Sciences

Honors and Awards

1. Awarded **Certificate of Commendation** by Joint office of technology transfer (HJF) for contribution to filing a petent of the research titled "Altered microRNA expression in post traumatic stress disorder".
2. Awarded **travel award** for Oral presentation at 2012 Annual Neurotrauma Society meeting at Phoenix, Az.
3. Awarded **Superior Performance Award** by Henry M Jackson Foundation in 2010.
4. **Qualified CSIR-UGC NET** India-, National eligibility test for PhD programs, July 2007.
5. Awarded **Teaching Assistantship** in Biological Sciences Department, Birla Institute of Technology and Science, Pilani, India, 2005

Job Related Skills

- DNA: Cloning, DNA & RNA purification, Real Time PCR, Mutagenesis, Southern hybridization, mutational analysis, and transfection.
- RNA: Microarray techniques, Northern blotting, Reporter assays, RNAi.
- Protein: SDS PAGE, Western blots, 1-D & 2-D electrophoresis, ELISAs
- Cell Culture: Primary neuronal and astrocyte cultures, various murine and human cell lines.
- Microscopy: Immunohistochemisty, confocal, fluorescence and light microscopy.
- Animal Handling: Rodent (mouse) surgical procedures like intraperitoneal, tail vein injection, retro orbital bleeding, processing animal tissues.

Grant Writing

I have significantly contributed towards the recently funded grant titled" Role of MicroRNAs in Mild Traumatic Injury (TBI) and Posttraumatic Stress (PTSD): Identification of

Biomarkers and Therapeutic Targets."

Funding Agency: Defense Medical Research Development Program (DMRDP), PI: Radha K Maheshwari.

Computer Skills

Statistical software (SPSS), Scion Image Analysis software, Statminer, Ingenuity Pathway Analysis, MS Office. Proficient in use of Medline, Entrez, Blast, EndNote, Windows XP/Vista, MacOS X.

Research Experience

My research interests involve the understanding of role of microRNA in gene regulation. I have investigated the role of microRNAs in virus induced neurodegeneration in mouse model of infection. I have also been involved in evaluating the role of microRNAs as biomarkers in traumatic brain injury. My current research goal is to study the pathogenesis of traumatic brain injury to develop biomarkers and therapeutics.

2 Biosketch of supervisor

NAME **RADHA K. MAHESHWARI**	POSITION TITLE **Professor of Pathology**		
EDUCATION/TRAINING			
INSTITUTION AND LOCATION	DEGREE	YEAR(s)	FIELD OF STUDY
Lucknow University, India Birla Institute of Technology & Sciences, India Kanpur university, Kanpur, India (Ph.D Dissertations work was carried out at the Central Drug Research Institute, Lucknow)	B.S. M.S. Ph.D.	1968 1970 1974	Biological Sciences Biological Sciences Virology, Interferon and Immunology

A. *Personal Statement*

I have been extensively involved in biomarker identification and host response (mRNA as well as microRNA) studies over the past several years. My lab has been studying the blast overpressure injury in rat model and the weight drop model in mice for identification of serum biomarkers. Other than this, my lab has also been engaged in studying the molecular mechanisms of viral pathogenesis and neuro-degeneration specifically looking at identification of serum biomarkers, role of inflammation and innate immune response in alphavirus pathogenesis the host responses and during neurodegenerative viral infections. In past, I have also studied the protective efficacy of phytochemicals in prevention of hemorrhage, ischemia and reperfusion-induced injury; chemoprevention of cancer and enhancement of wound healing as evident through the peer reviewed publications. Over the years, my laboratory has been involved successfully and productively in interdisciplinary collaborative approach and based on the experience I am well suited for my role to serve as PI for the proposed study.

B. Positions and Employment

1977-1980: Post-Doctoral Fellow, National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, Bethesda.

1977-1981: Senior Research Associate, Department of Pathology, USUHS, Bethesda, Maryland.

1977-1982: Adjunct Faculty, Dept. of Microbiology, School of Medicine, Georgetown University, Washington, D.C.

1984-1989: Assistant Professor, Department of Pathology, USUHS, Bethesda, Maryland.

1990-1994: Associate Professor, Department of Pathology, USUHS, Bethesda, Maryland.

1990-present: Adjunct Professor, Birla Institute of Technology and Science, Pilani, India.

1995-Present Professor, Department of Pathology, Uniformed Services University of the Health Sciences, Bethesda

Other Experience and Honors

- Organized scientific meetings, symposia, and served as Chairman in scientific sessions;
- Invited speaker at many national and international meetings; and invited to participate in discussion groups
- Served on the editorial board and reviewed papers for journals
- Invited to write articles/chapters in books
- Appointed as the Coordinator of Indo-US Programs at USUHS
- Co-edited two books, "Interferon in Biomedical Research", and "Cell-Mediated Immunity in Tropical Diseases".
- Adjunct Faculty, Birla Institute of Technology and Science, Pilani, India.

- Served as a review member for Fogarty Center, NIH and for the United Nations.

C. *Selected Peer-reviewed Publications (Selected from 125 peer-reviewed publications)*

1. Balakathiresan N, Bhomia M, Chandran R, Chavko M, McCarron RM and **Maheshwari RK**. MicroRNA Let-7i is a promising serum biomarker for blast induced traumatic brain injury. J Neurotrauma (2012).

2. Sharma A, Bhomia M, Honnold SP, **Maheshwari RK**. Role of adhesion molecules and inflammation in Venezuelan equine encephalitis virus infected mouse brain. Virol J. 2011 Apr 29;8(1):197.

3. Bhomia M, Balakathiresan N, Sharma A, Gupta P, Biswas R, **Maheshwari RK**. Analysis of MicroRNAs induced by Venezuelan Equine Encephalitis virus infection in mouse brain. Biochem Biophys Res Commun. 2010 Mar 18.

4. Sharma A, **Maheshwari RK (2009)** . Oligonucleotide Array Analysis of Toll Like Receptors and Associated Signaling Genes in Venezuelan Equine Encephalitis Virus Infected Mouse Brain. *J Gen Virol.*

5. Sharma A, Bhattacharya B, Puri RK, **Maheshwari, RK (**2008). Venezuelan equine encephalitis virus infection causes modulation of inflammatory and immune response genes in mouse brain. *BMC Genomics.16;9:289.*

6. Thangapazham RL, Shaheduzzaman S, Kim KH, Passi N, Tadese A, Vahey M, Dobi A, Srivastava S, **Maheshwari R.K** (2008)Androgen responsive and refractory prostate cancer cells exhibit distinct curcumin regulated transcriptome.*Cancer Biol Ther.7(9):1436-40.*

7. Thangapazham, R.L, Sharma, A, and **Maheshwari, R.K** (2008). Protectve Effect of phytochemicals in cancer chemoprevention, wound healing and ischemia/reperfusion injury. p.135-144.

Ethnopharmacology- Recent Advances (edited by Puspangadan, George and Janardhan), Daya Publishing House, Delhi.

8. Singh, A.K., Warren, J., Madhavan, S., Kumar, R., Steele, K., Sharma, A., Sharma, S., Kulshreshtha, D., and **Maheshwari, R.K.** (2007) Picroliv accelerates epithelialization and angiogenesis in rat wounds. *Planta Medica* 73,251-256.

9. Rollwagen, F., S. Madhavan, A. Singh, Y. Li, K. Wolcott, and **R. Maheshwari**. (2006) IL-6 protects enterocytes from hypoxia-induced apoptosis by bcl-2 and fas mRNA regulation. *Biophysical Biochemical Research Communications* 347:1094-1098.

10. Steele, K, Seth, P., Catlin, K., Sconeboom, B., Husain, M., Grieder, F., and **Maheshwari, R.K.** (2006) Tunicamycin Enhances Neuroinvasion and neurodegeneration in mice with Venezuelan Equine Encephalitis virus. *Veterinerary Pathology* 43:904-913.

11. Thangapazham R. L, Sharma A, **Maheshwari R. K.** (2006). Biomarkers of Angiogenesis in Wound Healing and Cancer: Role of Botanicals and Phytochemicals. *Journal of Horticulture* 720,129-136 .

12. Thangapazham R. L, Sharma A, **Maheshwari R. K.** (2006). Multiple Molecular Targets in Cancer Chemoprevention by Curcumin. *Journal of the American Association of Pharmaceutical Sciences* 8(3):E 443-449.

13. Sharma A., Singh, A.K., Warren J., Thangapazham, R., and **Maheshwari, R.K.** (2006) Differential Expression of Angiogeneic Genes in diabetic Wound Healing. *Journal of Investigative Dermatology* 126:2321-2331.

14. Sundar, Shirin V., Li, Ying-Yue, Rollwagen, Florence M., and **Maheshwari, Radha K.** (2005). Hemorrhagic shock induces differential gene expression in mouse liver. *Biochem. Biophy. Res. Comm.* 332,688-696.