Philadelphia College of Osteopathic Medicine

Annual Progress Report: 2007 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Philadelphia College of Osteopathic Medicine received $19,760 in formula funds for the grant award period January 1, 2008 through June 30, 2011. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Entry Mechanisms of Mouse Hepatitis Virus, a Model for Multiple Sclerosis - In order for viruses to infect cells, they must first bind to and enter potential host cells. There are several distinct pathways that viruses can use to enter cells, and understanding viral entry mechanisms provides important information on how viruses cause infections. This project investigates entry mechanisms of mouse hepatitis virus (MHV); the MHV strain used, MHV-A59, is studied as a model for multiple sclerosis (MS). Understanding more about MHV entry will provide information on how viruses infect the central nervous system, and may provide insight into how viral agents can contribute to changes observed in human demyelinating diseases such as MS.

Duration of the Project

1/1/2008 – 6/30/2011

Project Overview

Mouse hepatitis virus strain A59 (MHV-A59) can cause an acute meningoencephalitis, followed by a chronic demyelinating disease in animals surviving the acute infection. Understanding pathogenic properties of this neurovirulent virus might shed insight on how viruses could induce cytopathology and immunopathology associated with demyelinating disease. The ability of viruses to bind to and enter specific cell types is an important determinant of what cells get infected in vivo. Furthermore, the mechanism by which viruses enter cells will influence their ability to spread to uninfected cells, as well as influence virus-induced damage to the host cell and viral interactions with the host immune response.

Viruses can enter cells either at the level of the plasma membrane, or by receptor-mediated mechanisms. Receptor-mediated pathways may involve endocytosis utilizing structures called clathrin coated pits and endosomes, or membrane invaginations associated with protein complexes called caveolae. In addition, viral entry may occur at specific regions of the plasma membrane that are high in cholesterol and sphingolipid, called lipid rafts. This project will

examine the abilities of inhibitors of clathrin-dependent entry and caveolae-dependent entry, as well as cholesterol depletion, to interfere with viral replication. It will involve assays measuring viral titers, percent infected cells, and production of viral mRNA. In addition, both fluorescent and electron microscopy will be utilized to visualize aspects of viral entry and the affect of entry inhibitors. Of particular interest will be the investigation of entry mechanisms of MHV-A59 in primary neuronal cells, including mixed glial cells and cells enriched for astrocytes and microglial cells.

This project will also investigate the relationship between a virus's ability to induce cell-to-cell fusion, or syncytia formation, and use of entry pathways. Syncytia formation could allow viral entry through the plasma membrane, and could potentially allow the virus to infect adjacent cells that do not express virus-specific receptors, which could affect the in vivo pathogenesis of MHV.

Principal Investigator

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Other Participating Researchers and Employers

None

Expected Research Outcomes and Benefits

Multiple sclerosis (MS) is a human demyelinating disease where the cause is unknown. MS is considered an autoimmune disease in which patients develop an immune response to myelin, considered an insulator for the electrical impulses of nerve cells. It has been suggested that infectious agents might trigger the immune response observed in MS. The virus examined in this project, mouse hepatitis virus strain A59 (MHV-A59), is studied as a model for multiple sclerosis (MS). Thus, understanding how MHV-A59 infects cells might help explain how viruses can trigger events that lead to the development of MS.

The focus of this project is to better understand how MHV causes disease by examining the pathways that the virus uses to enter and infect different types of cells. We know that in some cell types, MHV-A59 infection produces fusion between membranes of adjacent cells, which, during an infection in the animal, would allow the virus to pass from cell to cell without exposure to the external environment and possible removal by the immune response. However, prior results have indicated that MHV-A59 does not produce cell-to-cell fusion in cells isolated directly from the brains of mice, suggesting that in the brain MHV-A59 might enter cells by a different pathway than that used to enter continuously cultured cell lines. This project proposes to compare entry mechanisms of MHV-A59 into cells where infection produces cell-to-cell fusion with entry into cells where cell fusion is not observed, which may more closely resemble infections in the animal.

Summary of Research Completed

Viruses can enter cells through several mechanisms, two common ones being clathrin-mediated and caveolin-mediated endocytosis. The focus of this project has been to examine the entry mechanism(s) utilized by two strains of mouse hepatitis virus, MHV-A59 and MHV-2. These two viruses differ in their abilities to induce cell-to-cell fusion when they infect different cell lines, thus might be expected to differ in the mechanism(s) used to enter cells. In previous years a reverse transcriptase, real-time polymerase chain reaction (RT^2-PCR) assay was developed to quantify levels of viral RNA in cells at early times post infection as a measure of entry of two recombinant viruses, $RA59_{SA59}$ and $RA59_{S2}$, that express the MHV-A59 or MHV-2 S proteins in an MHV-A59 background. Two cell lines, L2 cells (murine fibroblast cells) and bEnd cells (murine brain endothelial cells) have been used for these studies. Prior studies have indicated that infection by $RA59_{SA59}$ and $RA59_{S2}$ is reduced in the presence of two inhibitors of clathrin-dependent endocytosis, bafilomycin and chlorpromazine, suggesting a role for clathrin-mediated endocytosis in viral entry. Previous studies have also implicated lipid rafts as being important in viral entry, which suggests the possibility that caveolin may be involved since this protein is a component of lipid rafts.

The data obtained using these inhibitors suggest that in L2 cells both strains of virus travel to endosomes through the clathrin-mediated pathway, but have different requirements for exiting endosomes. Interestingly, the inhibitory affects of chlorpromazine on the two viruses differs between cell lines, indicating that viral entry mechanisms may be cell line dependent (2009-2010 progress report). However, it is possible that the inhibitors used in these initial studies have multiple effects on host cells that influence several steps of the viral replication cycle, not just viral entry. To better evaluate the possible importance of both clathrin- and caveolin-mediated entry mechanisms, experiments during the 2010-2011 fiscal year have focused on using siRNAs specific for clathrin and caveolin sequences to down-regulate expression of these proteins on the cell surface, then examining the abilities of $RA59_{SA59}$ and $RA59_{S2}$ to infect cells expressing decreased levels of clathrin or caveolin. This approach was pursued to provide for a more direct evaluation of the relative importance of clathrin- or caveolin-mediated endocytosis in viral entry.

Initial experiments dealt with optimizing an RNA interference (RNAi) protocol to inhibit the expression of either clathrin or caveolin in L2 cells. Both small interfering RNA (siRNA) and small hairpin RNA (shRNA) technologies were tested for efficiency of mRNA down-regulation. RT^2-PCR and Western blot analysis were used to examine clathrin or caveolin mRNA and protein levels, respectively, following inhibition of gene expression by RNAi. Immunofluorescence microscopy was used to assess efficiency of siRNA and shRNA transfections, as well as visualize caveolin expression after transfection with caveolin-specific siRNA. Once the siRNA protocol was optimized, the ability of $RA59_{SA59}$ and $RA59_{S2}$ to infect transfected cells could be examined. Most of the experiments performed to date have been using caveolin-specific siRNA to down-regulate expression of caveolin in L2 cells; currently, experiments are being expanded to include clathrin-specific siRNA and bEnd cells.

Transfection of L2 cell monolayers were performed using caveolin or clathrin specific siRNA and shRNA constructs (purchased from Santa Cruz or Invitrogen) and Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen) diluted into OPTI-MEM® Reduced Serum

Medium following the manufacturers recommended protocol. Controls included nonsense RNAi (negative controls), transfection reagent alone (sham transfected) or fluorescent siRNA or shRNA constructs (positive controls). Cells transfected with fluorescent positive controls were examined by fluorescence microscopy and provided visualization of transfection efficiency. As seen in figure 1, fluorescent siRNA entered nearly 100% of the cells whereas fluorescent shRNA transfection resulted in labeling in only ~10% of cells in the monolayer. These results were consistent with Western blot analysis of cell lysates derived from caveolin-specific siRNA and shRNA transfection of L2 cells (figure 2). Densitometry quantitation of Western blots consistently showed that the siRNA protocol down-regulated caveolin approximately 80-85% while shRNA transfection resulted in only 30-50% down regulation of the protein. Given these results, subsequent experiments used the siRNA constructs.

With siRNAs, transfections can be performed on a monolayer of cells (forward transfection) or as cells are seeded onto the appropriate tissue culture plate or flask (reverse transfection). Both types of protocols were tested and the success of caveolin down-regulation at various times post transfection was assessed by Western blot analysis. Denistometry analysis on Western blots revealed that the reverse protocol resulted in the greatest and most consistent down-regulation of caveolin; a representative Western blot is shown in figure 3. Using the reverse protocol, the levels of down regulation were similar for all time points tested (24-72 hours post transfection; data not shown). Immunofluorescence labeling for caveolin was used to visualize down regulation of this protein following reverse transfection (figure 4). The reverse transfection protocol was used in subsequent experiments to evaluate viral entry; viral infections were performed at 24 hours post transfection.

Once the parameters for RNAi to down-regulate caveolin were established, transfected cells were infected with $RA59_{SA59}$ or $RA59_{S2}$ and viral RNA was quantified by RT^2-PCR at 4 hours post infection. The same RNA samples tested for viral sequences could be assayed for caveolin mRNA by RT^2-PCR using a TaqMan gene expression assay (Applied Biosciences). As shown in figure 5, caveolin protein expression and mRNA were inhibited to a similar extent by caveolin-specific siRNA transfections. RT^2-PCR assays for viral sequences indicated that replication of both $RA59_{SA59}$ and $RA59_{S2}$ was inhibited approximately 50% in caveolin-specific siRNA transfected cells relative to cells transfected with negative control siRNA (figure 6). The data from the siRNA experiments suggests that caveolin may play a role in MHV-A59 and MHV-2 entry into host cells, but is not the only pathway used to enter host cells.

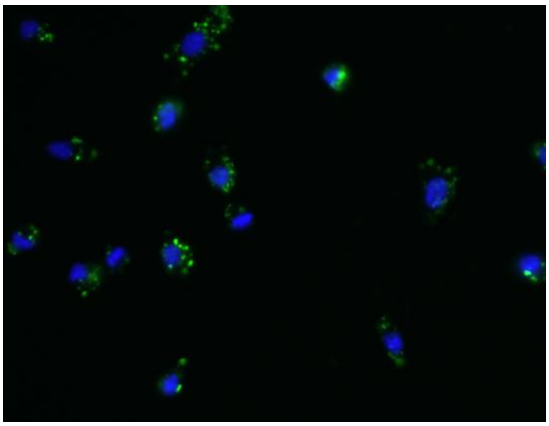
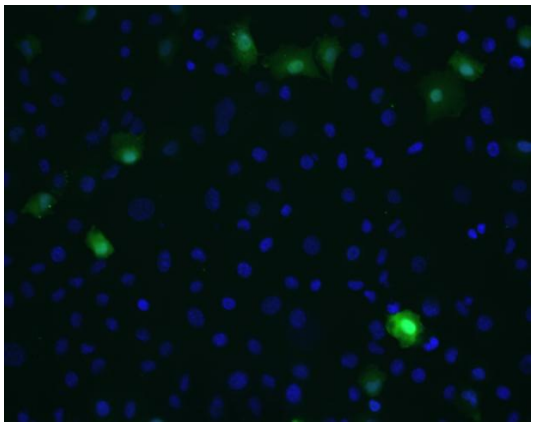
A.  B. 

Figure 1. Transfection of L2 cell monolayers with fluorescent siRNA (A) or shRNA (B). Green fluorescence indicates cells that were successfully transfected; blue labeling indicates cell nuclei.

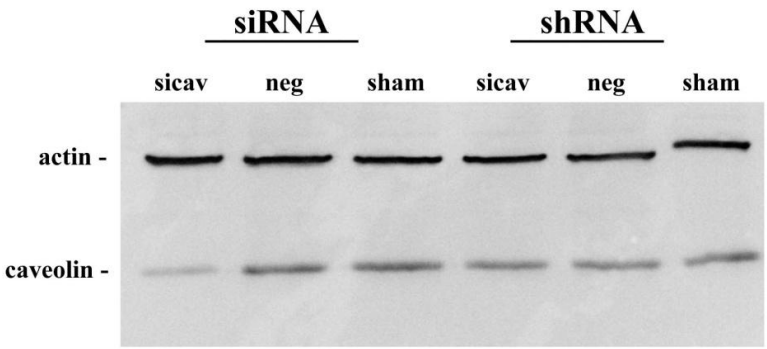


Figure 2. Representative Western blot of L2 cell lysates derived from transfections. L2 cells were either sham transfected, or transfected with negative control siRNA/shRNA or caveolin siRNA/shRNA. Caveolin migrates at 23 kDa, while actin, included as a loading control, migrates at 42 kDa.

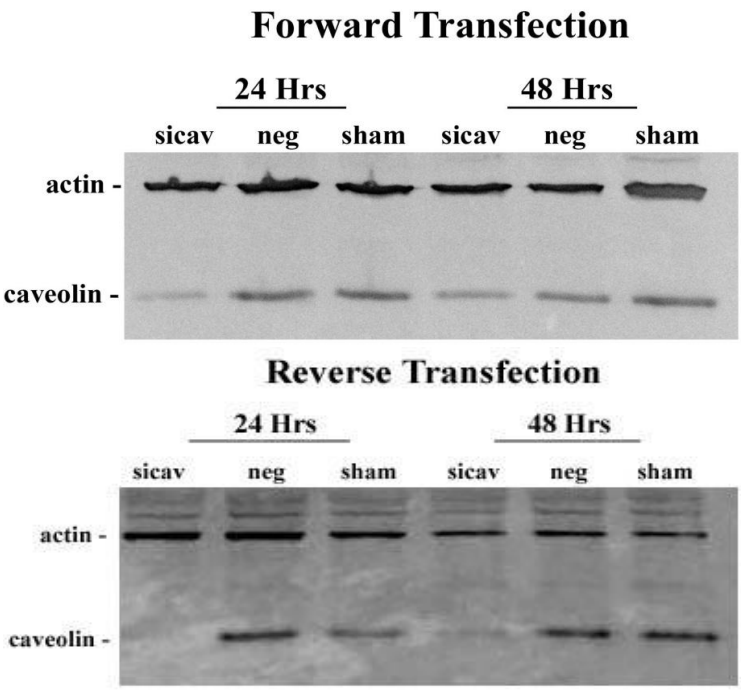


Figure 3. Representative Western blots from the forward (A) and reverse (B) transfection protocols. Protein lysates were obtained at 24 and 48 hours post transfection; actin is included as a loading control.

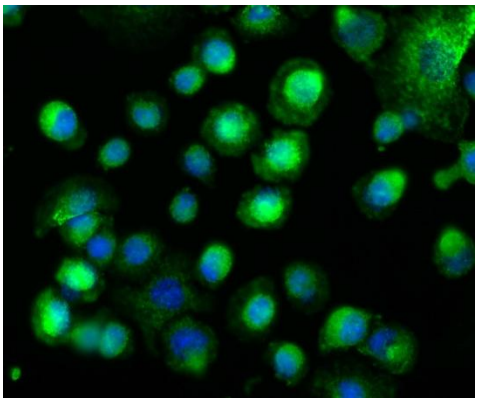
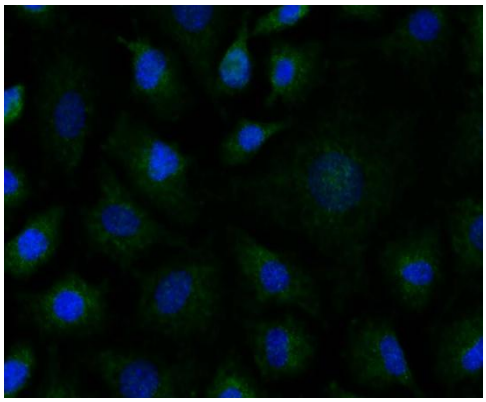
A.  B. 

Figure 4. Expression of caveolin in L2 cells transfected with negative control siRNA (A) or caveolin-specific siRNA (B). Caveolin labeling is in green, while cell nuclei are labeled blue.

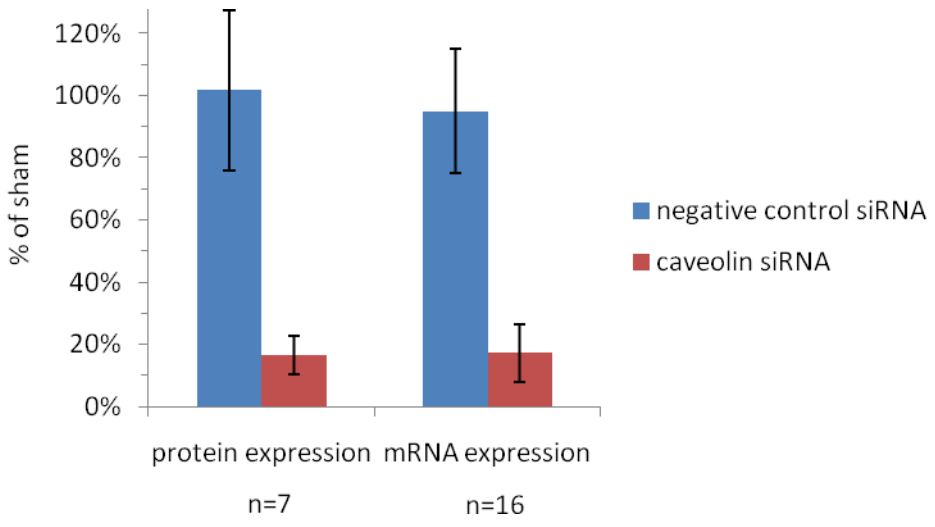


Figure 5. Down-regulation of caveolin protein expression and mRNA as determined by Western blot analysis and RT^2-PCR, respectively. Results are expressed as a percentage of sham-transfected cells.

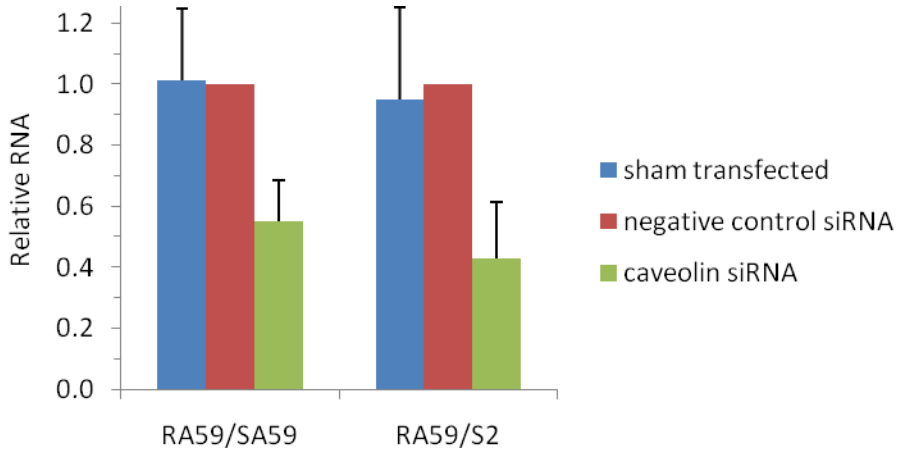


Figure 6. Relative levels of viral RNA, as measured by RT^2-PCR, in sham transfected cells or cell transfected with negative control siRNA or caveolin-specific siRNA. Viral RNA levels were calculated relative to viral RNA in negative control siRNA samples and represent the mean + standard deviation from 8 separate samples.