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TITLE: Engineered Herpes Simplex Viruses for the Treatment of Malignant Peripheral Nerve Sheath Tumors

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14. ABSTRACT The first year of our funding has been very productive and has led to new information regarding susceptibility of Malignant peripheral nerve sheath tumors (MPNST), which occur in patients with neurofibromatosis type-1, to oncolytic HSV (oHSVs). We have demonstrated that many of the MPNSTs that have tested are indeed sensitive to our oHSV, but that some are resistant (both murine and human). It is apparent from our data that ability to replicate in these tumor cells is related to additional factors besides simply status of HSV-1 receptors nectin-1 and nectin-2. We are examining further mechanisms that may be responsible for sensitivity and resistance in these lines which we hope to exploit in the future. We are currently testing 2 new hypotheses developed based upon the first year of data and are pursuing a surprising observation regarding the C134 and the M032 viruses and there ability to maintain late viral protein synthesis in the infected MPNST cells. This is an unanticipated finding that could have been discovered empirically these advances would not have been possible without these DOD funded studies.					
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March 11, 2013

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RE: DOD Award (11-1-0498) Annual Report

Dear Dr. Bekirov:

Thank you for your instructions and critique of our previously submitted annual report. We appreciated the guidance you have given us on the desired presentation of the annual reports. Since this is our first such report, we were not familiar with many of these and have made the adjustments as requested.

Specifically, to address the requests in our critique, we have cited the figures within the text to support key research findings. The results of the anti-tumor activity assays are now described in the body of the report, and legends have been provided to the figures. All placeholder figures for data that have not yet been collected have been removed. Figures now include descriptive legends.

A request for a description of statistical analyses was previously absent and now has been included with indicators of significance.

The western blots utilized in this study were for qualitative purposes only and not quantitative. Specifically, we felt it important for the purposes of this assay to include data utilizing the same number of cells for each blot. Glyco-protein D is produced at extraordinarily high levels by our herpes simplex virus, and thus, it is quite common in herpes simplex virology to have relative over-expression of this protein while other proteins are

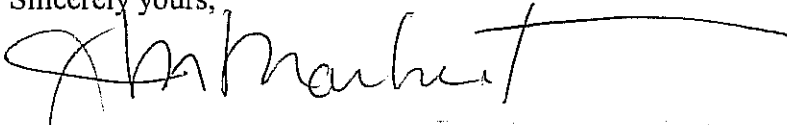
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expressed at much lower levels. This should explain the appearance of the blots as presented.

Finally, the research report has been rewritten against project tasks in the statement of work (SOW) and we have included additional analyses and descriptions of the data obtained for each task.

Thank you very much for your understanding in this, our first attempt at providing such a report for our DOD grant. Best wishes.

Sincerely yours,

A handwritten signature in black ink, appearing to read "J. Markert", with a long horizontal flourish extending to the right.

James M. Markert, M.D.
Division Director, Neurosurgery
James Garber Galbraith Professor
Neurosurgery, Physiology and Pediatrics, and Cell Biology

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The first year of our funding has been very productive and has led to new information regarding the susceptibility of MPNST to oHSVs. We are currently testing 2 new hypotheses developed based upon the first year of data and are pursuing a surprising observation regarding the C134 and the M032 viruses and their ability to maintain late viral protein synthesis in the infected MPNST cells. This is an unanticipated finding that could only have been discovered empirically. These advances would not have been possible without these DOD funded studies.

Our goals for this grant are summarized below and include:

- 1) To determine the molecular basis for the sporadic susceptibility or resistance to infection of MPNST cells to genetically engineered, oncolytic herpes simplex viruses (oHSVs) in our repository;
- 2) To examine inherent mechanisms expressed in MPNSTs that inhibit the replication of oHSVs and abrogate the ability of these viruses to kill infected cells and spread to neighboring tumor cells; and
- 3) To test the relative ability of our oHSVs to produce an anti-tumor effect alone and if this anti-tumor effect can be significantly enhanced by a low dose of radiation administered to the tumor.

Thus far our work during the initial year of funding has focused upon the first 2 aims listed. Our progress is summarized below and has been organized within the 4 major milestones listed in our Statement of work and addressed with regard to each of the Tasks and SubTasks.

Milestone 1: We will identify at least 2 oHSV-sensitive and 2 oHSV-resistant MPNST cell lines by completing the *in vitro* characterization of both human and mouse MPNST cell lines with respect to oHSV infection and killing. We have preliminary analysis of 2 human and two murine MPNST lines. They range from sensitive to resistant to oHSV infection and killing. This milestone will provide the prototypic MPNST cell lines that will be studied more extensively in all three aims.

Milestone 2: We will characterize each of the 9 human MPNST cell line and at least 18 of the 100+ mouse MPNST cell lines with regard to expression of HSV entry molecules expressed on the cell surface. This milestone will enable us to determine whether prevention of entry by down-regulation of appropriate receptors is the reason for oHSV resistance and, if so, whether we should define alternative receptors to which new oHSVs could be targeted.

Milestone 3: We will characterize the replication of oHSVs in each of the oHSV-sensitive and oHSV resistant MPNST cells identified in Milestone 1 by FACS and by titring virus at regular post-infection intervals. Within this context, we will establish the extent to which replication is enhanced in infected MPNST cells by oHSVs engineered to express proteins that directly promote virus replication. This milestone will allow us to select either the HCMV IRS-1 or the constitutively activated MEK gene as the most appropriate insert to overcome replication resistance.

Milestone 4: We will determine which of the oHSVs identified as “effective” in the first two aims of this proposal actually produce the expected anti-MPNST effect in oHSV-sensitive and oHSV-resistant tumors of human or mouse origin placed orthotopically in the appropriate strain of mouse (see below). Efficacy alone or in combination with enhancing adjunctive therapies will be defined. This milestone will serve to validate (or refute) the process for selection of effective oHSVs that could be advanced to clinical trials in patients with MPNSTs and identify which modality is most likely to have an impact on the natural history of this disease.

Task 1: Characterize the *in vitro* sensitivity of a panel of human and mouse MPNST cell lines to a panel of available oncolytic HSVs.

SubTask 1a. Using methods that we have described in Preliminary Findings in the Proposal, we will complete the screening of all 9 human MPNST cell lines and at least 18 of the 100+ mouse MPNST cell lines using FACS for detection of expression and modulation of HSV entry molecules (nectin-1, nectin-2) and alternative entry molecules (HVEM, IL13R α 2, uPAR, Her2/neu) recognized by fluorochrome-labeled antibodies.

Status: During the first year of funding, we examined whether the expression levels of the three principal HSV entry receptors (CD111, CD112, HVEM) correlated with viral recovery in the human MPNST tumor cell lines. Receptor expression levels were measured using antibodies against these major HSV entry molecules nectin-1 (CD111), nectin2 (CD112) and HVEM by immunofluorescence microscopy and by flow cytometry. The MPNST cell lines demonstrated greater nectin-2 surface expression than nectin-1 surface protein. While a peripheral blood leukocyte positive control sample stained with the antibody against HVEM, none of the human MPNST tested lines expressed HVEM based upon flow cytometry and immunofluorescence. The relative surface expression of Nectin 1 and Nectin 2 was then compared with viral recovery data as represented in **Figure 1**. The results show that surface expression of nectin 1 and 2

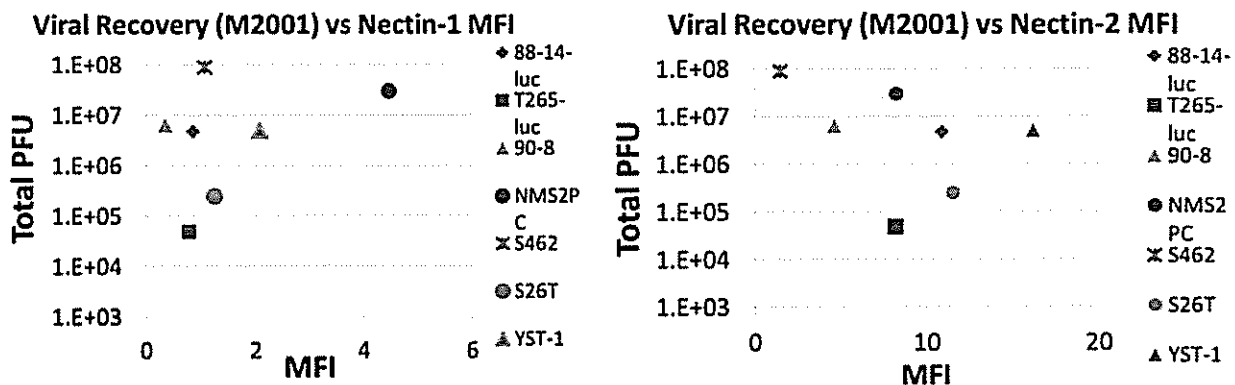


Figure 1 Comparison of Viral Recovery to Relative Receptor Expression. Nectin 1, Nectin 2 and HVEM receptor surface expression was measured for 7 of the MPNST cell lines and compared to CHO cells to determine the relative receptor expression. HVEM receptor expression was detectable in the lymphocyte positive control sample but was undetectable in the MPNST cell lines. The results show that viral recovery data does not directly correlate with relative Nectin 1 or Nectin 2 surface expression.

did not correlate directly with viral replication in these cell lines.

This suggests that entry molecule surface expression is not a rate-limiting step in viral infection in the MPNST cell lines. To further test this hypothesis, however, we created a lentivirus that expresses the human nectin-1 gene and have transduced both murine and human cell lines to test this hypothesis. The Lentivirus was created by PCR amplifying the human Nectin-1 coding domain (including the signal sequence) from a validated cDNA clone (Open Biosystems) and inserting it into a lentiviral targeting vector, pCK2015. This targeting vector contains the Nectin-1 coding domain followed by an internal ribosomal entry sequence and the puromycin resistance gene. We created the Nectin-1 Lentivirus by co-transforming pCK2015 with plasmids encoding the VSV envelope and accessory vector in 293-T cells and collecting the supernatants 48h post-transfection. The MPNST cells were incubated with these supernatants and as demonstrated in **Figure 2**, the lentiviral-transduced cell lines express abundant immunoreactive nectin-1 on their surface. Studies are currently ongoing to determine: 1) if increasing nectin-1 surface expression improves viral entry, 2) if increasing nectin-1 surface expression improves oHSV replication in MPNST cells, and 3) if oHSV entry and gene expression downmodulates lentiviral nectin-1 surface

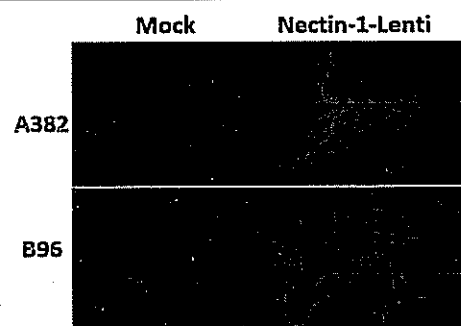


Figure 2 Immunofluorescence microscopy in 2 MPNST cell lines (A382 and B96) using antibody against Nectin 1 (Beckman Coulter, IM3451) in mock transduced (Left panels) and Nectin-1 expressing lentivirus transduced samples (Right panels). Greater Nectin-1 surface expression is detected in the lentivirus transduced cells. This has also been confirmed by flow cytometry studies. We have created 4 high Nectin-1 expressing MPNST cell lines thus far and will use them to test if increased nectin-1 expression improves viral entry and replication.

expression similar to that shown for native nectin-1 during infection.

SubTask 1b Using methods that we have described in Preliminary Findings in the Proposal, we will complete the screening of all 9 human MPNST cell lines and at least 18 of the 100+ mouse MPNST cell lines using oHSVs that express eGFP for detection of infection and cell death using FACS.

Status: We are starting these studies and have examined some of the cell lines as shown later in the progress report in Subtask 1

SubTask 1c. Screen each of the 9 human and 18 mouse MPNST cell lines for sensitivity to infection and killing by clinical candidate viruses G207, NV1020, M032 and C134 using classical virology techniques to measure cytopathic effect on monolayers, single step and multi-step replication assays .

Status: Over the past year, we focused on the in vitro characterization of the MPNST cell lines. We have tested 7 recombinant HSVs (4 of which are available as clinical grade virus), in 7 of the 9 human MPNST cell lines and 16 of the 18 murine MPNST cell lines proposed. The results of these studies are based upon increasing susceptibility / support for replication in the MPNST cell line (Figures 1 and 2). In order to finalize this milestone, we will test 2 additional human and 2 additional murine MPNST cell lines that Dr. Carroll's laboratory will supply to us.

Thus far we have detected a 100,000 fold (5 log) difference in viral replication between the least and most susceptible cell lines with our recombinant viruses (**Figure 3**). Of the murine MPNST cell lines, the A382 cell line is the least hospitable to viral replication. The oHSVs generate only $\sim 10^3$ (plaque forming units) pfu in single step replication assays in this cell line. Following the A382 cell line, the B91 cells are the second most restrictive cell line for three of the GLP quality oHSVs (G207, M032, and C134) whereas the A387 cell line is more restrictive to R7020 replication. With regard to murine MPNST cell lines that support viral replication, the 231 Trig and the A18 cell lines produced the highest overall viral recovery (10^8 PFU/ml).

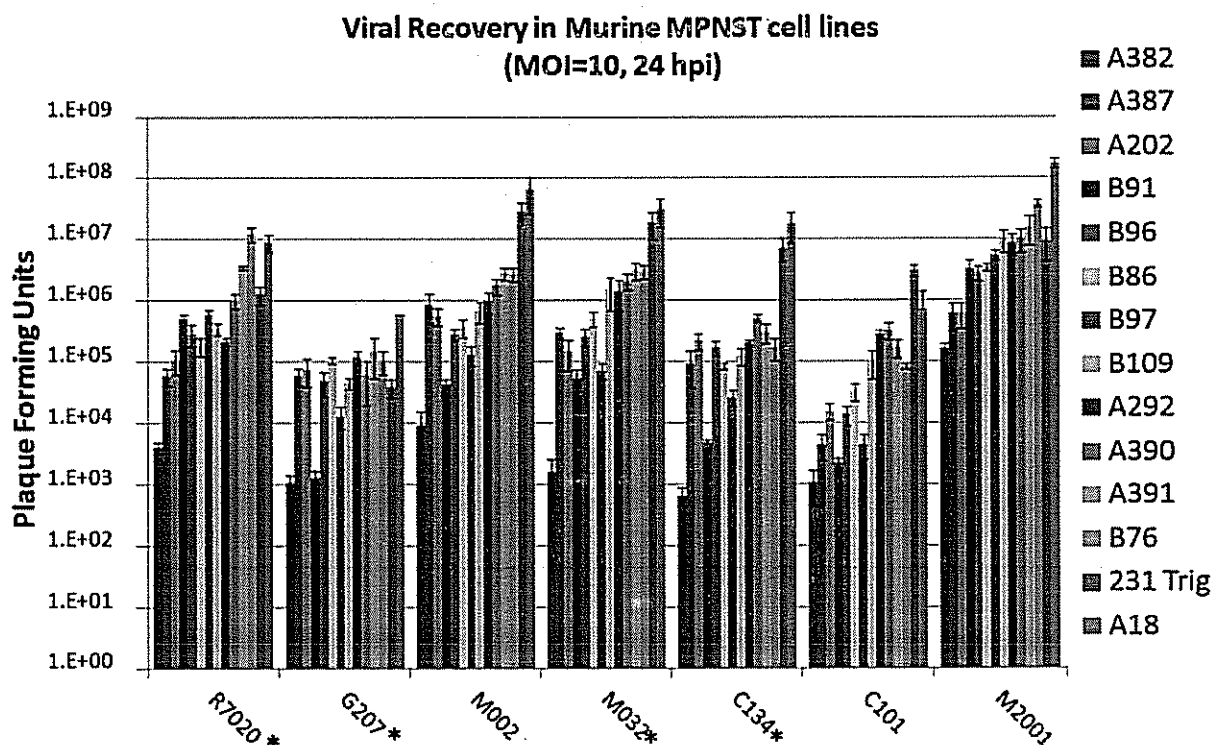


Figure 3: Summary of viral recovery studies in murine MPNST cell lines in order of least to greatest viral recovery. * denotes oHSVs with clinical grade virus available

For the Human MPNST cell lines, the choices for cell lines are more limited (7 cell lines tested). Most of the cell lines have been transduced to stably express luciferase (Luc). The most restrictive human MPNST cell lines to date are the T265T-LUC and S26T. These cell lines limited viral replication such that only 10^1 - 10^5 PFU of virus is produced following single step replication assays. The most susceptible human cell line was the S462 cell line which generated $\sim 10^7$ - 10^8 pfu for all of the viruses tested. Identification of the second most susceptible cell line was more complex. Depending upon the virus used, certain cell lines were more permissive than others in these assays (**Figure 4**). For two of the clinical grade oHSVs (R7020 and C134), the YST-1 cell line produced the greatest amount of virus (7.7×10^5 and 9.3×10^6 pfu). For the G207 and M032 oHSVs, NMS2PC was the next most susceptible cell line producing 4.33×10^5 pfu and 2.07×10^6 pfu, respectively.

We have also provided a summary of the MPNST susceptibility to oHSV anti-tumor activity based upon two independent assays (alamar blue and virus induced cytopathic effect [CPE]) These studies are ongoing and show that late viral protein synthesis and regulation of protein synthesis initiation limits some of the oHSVs to replicate in the tumor cells. Viruses that are capable of enhanced late viral protein synthesis, as demonstrated by gD protein production in infected cells on the whole replicate better than those that undergo translational arrest, which is a common antiviral response in infected cells. It has been well described (Mohr, 1995, Cassady 1998, Cassady 2005, Shah 2006) that viruses capable of evading this host translation shutoff response synthesize greater amounts of late viral proteins and replicate better than $\Delta\gamma_{134.5}$ recombinant viruses incapable of blocking this anti-viral response. It is therefore not surprising that the R7020 and C134 recombinant, both of which contain PKR evasion genes (R7020 a single copy of the $\gamma_{134.5}$ gene, and C134 the HCMV IRS1 gene), synthesized late viral proteins and replicated better than two of the $\Delta\gamma_{134.5}$ viruses tested (C101 and G207) in MPNST cell lines. Immunostaining studies show that the R7020 and C134 recombinants evaded this host antiviral response whereas the two $\Delta\gamma_{134.5}$ recombinants (C101 and G207) without PKR-evasion genes were incapable of blocking translational arrest as indicated by phosphorylation of eIF2 α .

Composites of the viral recovery results for all of the cell lines are provided in Figures 3 and 4. These allow comparison between the cell lines. In addition to this broad overview of all of the results, we

have also included the results from viral replication (multistep replication studies with CPE images, single step replication results with CPE images, cytotoxicity studies, and western blot data for the cell lines) for each of the cell lines in Figures 5-41.

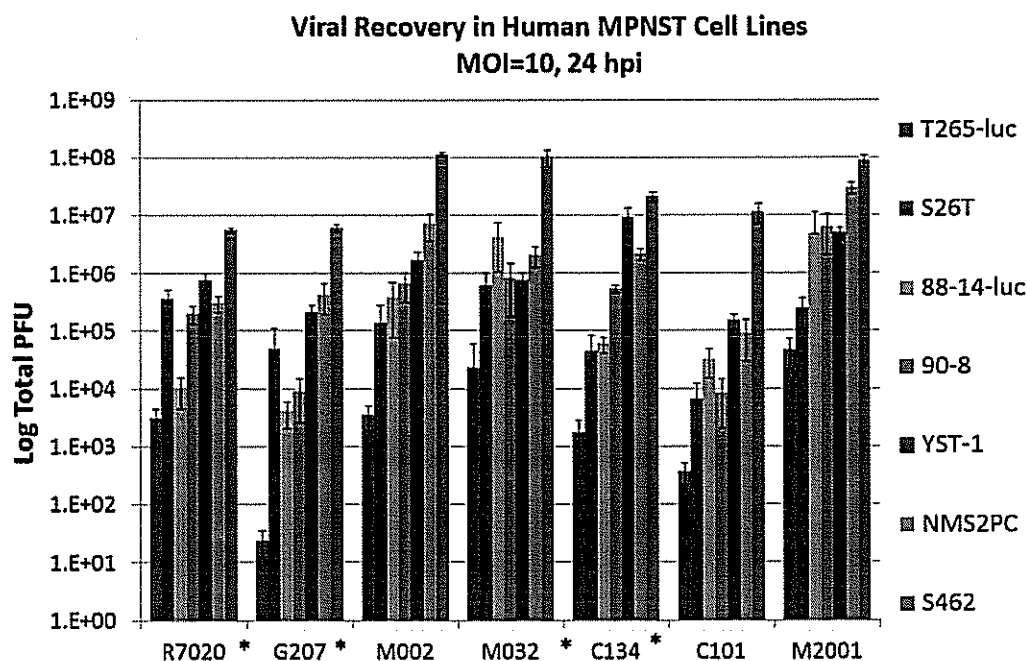


Figure 4: Summary of viral recovery studies in human MPNST cell lines in order of least to greatest viral recovery. * denotes oHSVs with clinical grade virus available

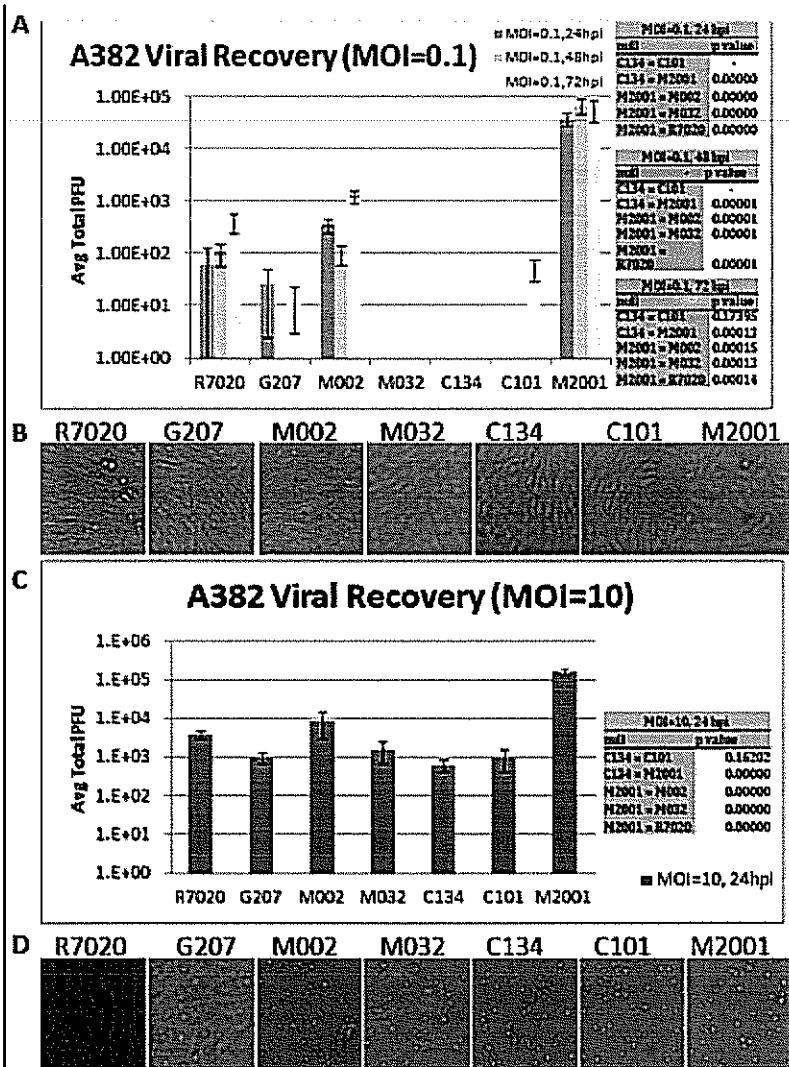


Figure 5: Composite of A382 murine MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on A382 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A382 cells (density of 1.5e5 cells/well; 100X magnification).

Summary and Analysis (A382)

The A382 line is one of the more restrictive cell lines to HSV replication. Even wild-type HSV encoding the GFP gene replicates poorly in this MPNST generating only 10^4 PFU on D1-3 post infection. The clinical grade viruses (C134, M032, G207, R7020) produce 10-100x less virus than wild-type in single step replication assays. (Figure 5A) At high MOI the cells exhibit early CPE (Figure 5B, D). C134 exhibits no replication advantage over its $\Delta\gamma_134.5$ parent virus (C101) in this cell line. The multistep replication studies are incomplete but based upon the single step replication studies, the cell line is highly restrictive to the clinical grade viruses (Figure 5D). As discussed below, this cell line is restrictive to HSV replication and we intend to further investigate this cell line as one of the two restrictive murine cell lines in future studies (as discussed in sub-task 1d)

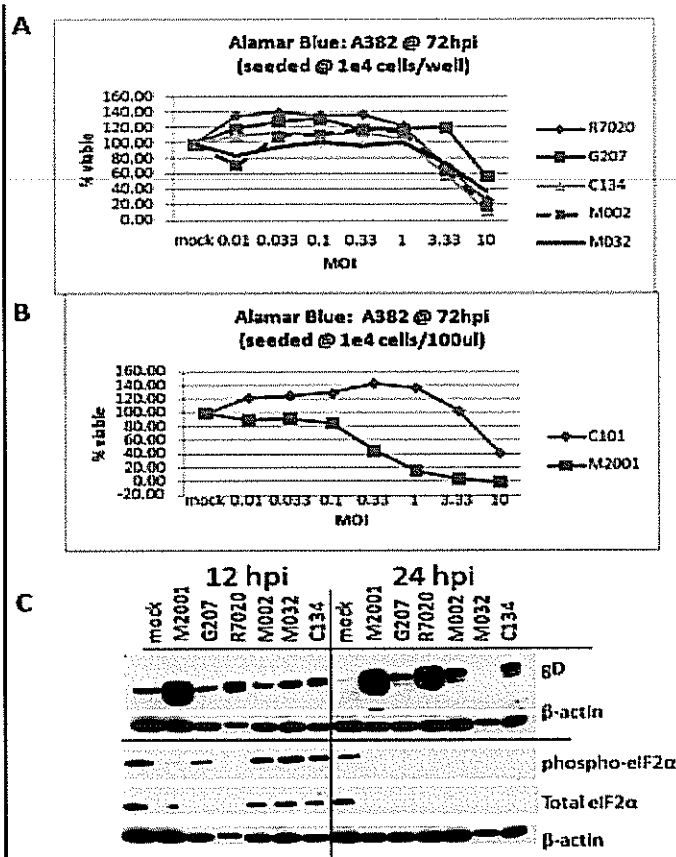


Figure 6: Composite of A382 murine MPNST cell line *in vitro* studies. A. Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. B. Alamar Blue cell viability assay comparing nondinical grade oHSVs C101 ($\Delta\gamma 1$ 34.5) vs. M2001 (wt) at 72hpi. C. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (A382 cont'd.)

Consistent with the CPE images shown in the prior figure, there is evidence of cell killing by Alamar blue assay. There is no difference in A382 cell killing between R7020, C134, M002 or M032). Alamar blue assay, however, shows that M2001 (wild-type HSV expressing EGFP) is more effective than $\Delta\gamma 1$ 34.5 viruses at killing the A382 cell line (Figure 6A,B).

Western blot images (Figure 6C) show that viruses that in other cell lines are capable of PKR evasion (R7020, M2001, C134) at 12hpi synthesize greater gD at 24hpi. In addition the M002 virus also accumulates gD. M032 is incomplete based upon less protein loading as shown by actin staining. While eIF2 α staining is interpretable in the 12h samples, by 24hpi there is no staining detected in any of the virus infected samples (total or phosphorylated). At this time, we do not know if this is a technical limitation with sample preparation or if this is a due to loss of eIF2 α in infected cells. The presence of eIF2 α in the mock sample suggests that this is due to loss of the protein in infected cells.

Conclusion: This is one of the more resistant mouse cell lines tested and we intend to use this as one of the two resistant

mouse cell lines for future studies. Of particular interest is the change in the protein synthesis phenotype and the eIF2 α changes in infected cells.

Summary and Analysis (A387)

A387 does not support efficient HSV replication. In contrast to the previous cell line however, M002 replicates as well as M2001 and exhibits a similar replication kinetic as M2001 (Figure 7A,C). The cells round lose contractility and form clumped spheroid forms during HSV infection as shown in the early CPE images (Figure 7B). Of note the cells also form these rounded structures during growth in cell culture making it difficult to establish a uniform and reproducible infection results in vitro and test-retest reliability. This may limit the interpretation of subsequent studies such as western blotting and alamar blue

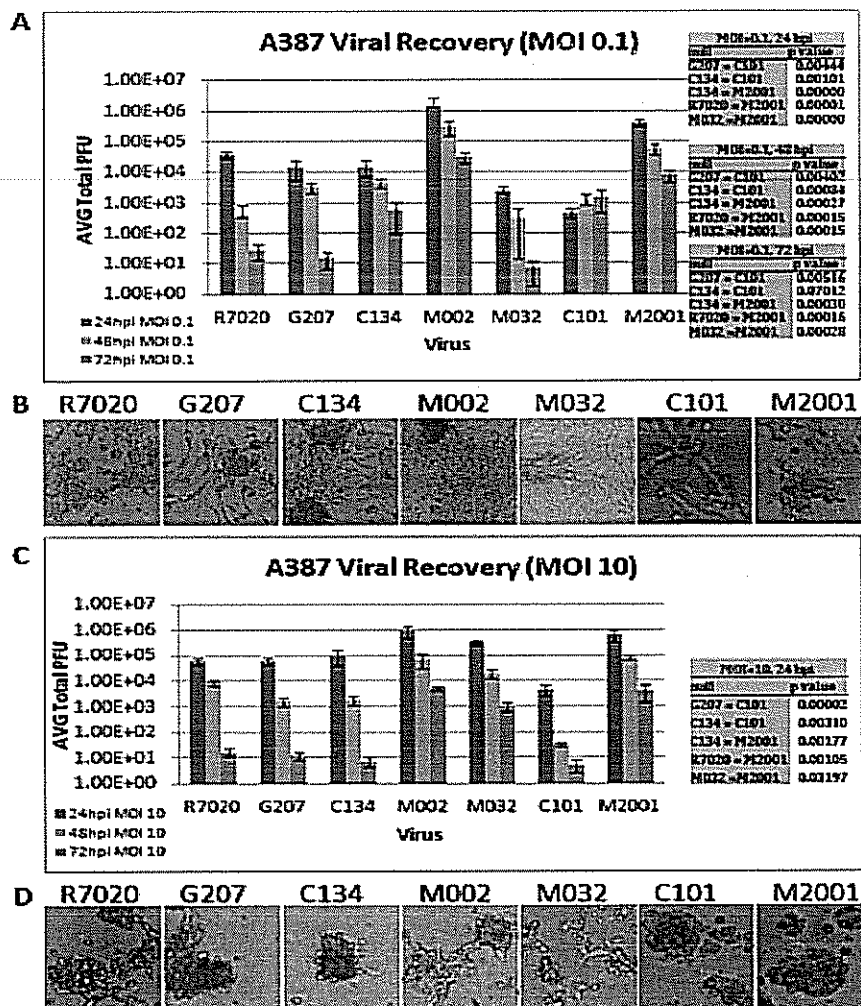


Figure 7: Composite of A387 murine MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on A387 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A387 cells (density of 1.5e5 cells/well:100X magnification).

Summary and Analysis (A387 cont'd.)

In alamar blue studies the M002 and M032 (murine and human IL-12 expressing viruses) produced paradoxical results (Figure 8A,B). Whereas M002 (along with R7020) produced the greatest cell killing in alamar blue cell killing assays, M032 performed the worst.

Immunostaining studies show that the $\gamma_{134.5}$ containing viruses (R7020 and M2001) are capable of producing appreciable gD after high MOI infection (Figure 8C). It is also interesting that in the M2001 and R7020 infected samples there is loss of actin staining over the initial 24hpi. C134 infected cells also exhibit loss of actin staining.

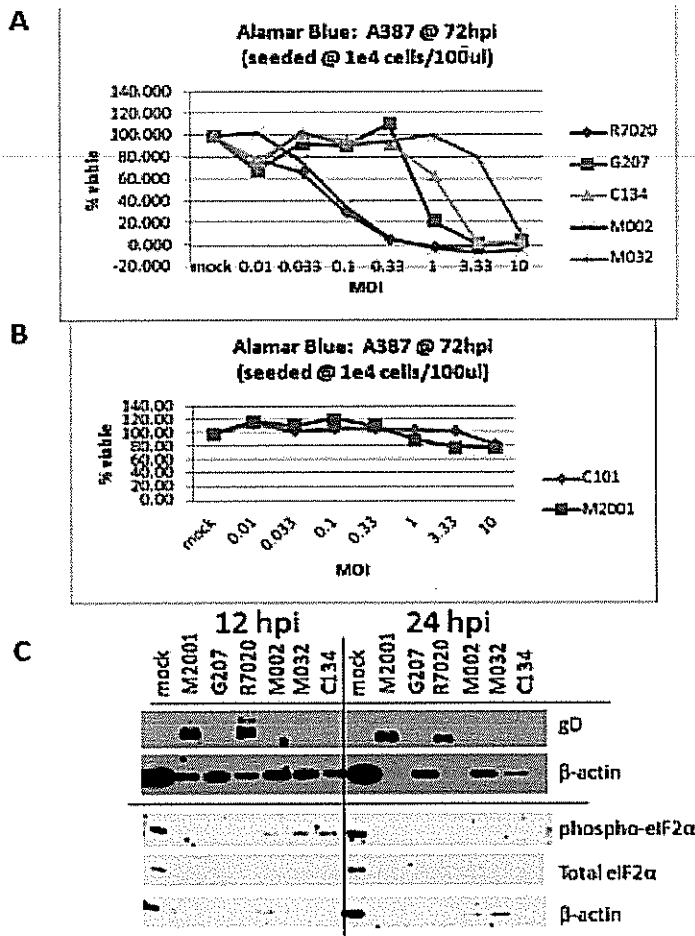


Figure 8: Composite of A387 murine MPNST cell line *in vitro* studies. **A.** Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. **B.** Alamar Blue cell viability assay comparing nondinical grade oHSVs C101 ($\Delta\gamma_{134.5}$) vs. M2001 (wt) at 72hpi. **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

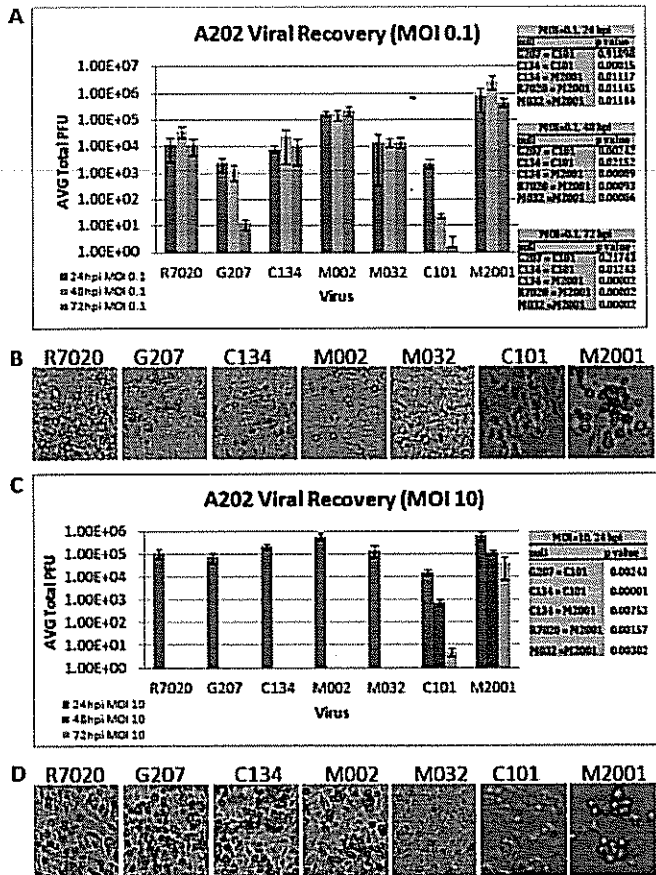


Figure 9: Composite of A202 murine MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/48/72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on A202 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A202 cells (density of 1.5e5 cells/well; 100X magnification).

Summary and Analysis (A202)

Most of the viruses tested were capable of sustained replication over the initial 72h of infection. The two viruses incapable of sustained replication (C101 and G207) were the prototypical $\Delta\gamma_134.5$ viruses. M2001 replicated the best followed by M002. The M032, C134 and R7020 viruses exhibited similar replication patterns (Figure 9A,C)

CPE images reveal that M2001 produced the greatest early CPE. While C134, M002, G207, and R7020 also induced cytopathic changes. In contrast, M032 and C101 infection did not elicit significant cytopathic effect (Figure 9B,D).

Conclusion: the A202 cell line is considered resistant to HSV replication and cytopathic effect. We intend to further study this line as one of the 2 resistant murine cell lines. We have chosen it because there is differential replication of the $\Delta\gamma_134.5$ viruses in this cell line.

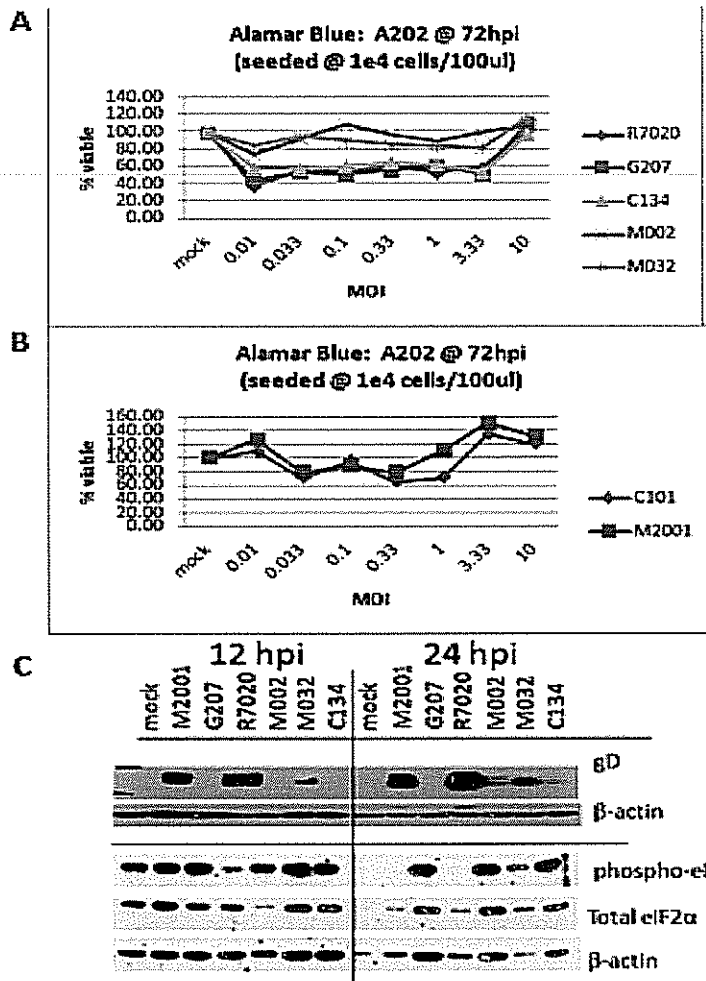


Figure 10: Composite of A202 murine MPNST cell line *in vitro* studies. **A.** Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. **B.** Alamar Blue cell viability assay comparing nondclinical grade oHSVs C101 ($\Delta\gamma 1$ 34.5) vs. M2001 (wt) at 72hpi. **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (A202 cont'd.)

Alamar Blue studies are difficult to interpret. No killing is seen by M002 and M032 (Figure 10A,B). The remaining viruses shows some killing at low to moderate MOI, but none at high MOI. Our working hypothesis is that the this MPNST cell line has aberrant redox properties that limit the alamar blue assay and render it uninterpretable.

Immunostaining studies (Figure 10C) show that the $\gamma 1$ 34.5 containing viruses (R7020 and M2001) accumulate the greatest gD. In the revised image, the actin staining shows similar loading between samples, The p-eIF2 α studies show that C134 is incapable of PKR evasion in this cell line based upon limited gD production and the presence of p-eIF2 α .

As discussed previously (Figure 9), we intend to further study the A202 cell line as one of the two resistant murine MPNST cell lines. Of particular interest is its metabolic profile and whether this may alter interpretation of the alamar blue cytotoxicity assay. The cell line is also interests us because of the C134 virus phenotype in this cell line. Preliminary results show that C134 acts similar to its parent virus (C101) in certain assays (p-eIF2 α and gD immunostaining studies) but is capable

of sustained replication in the multistep replication studies unlike C101.

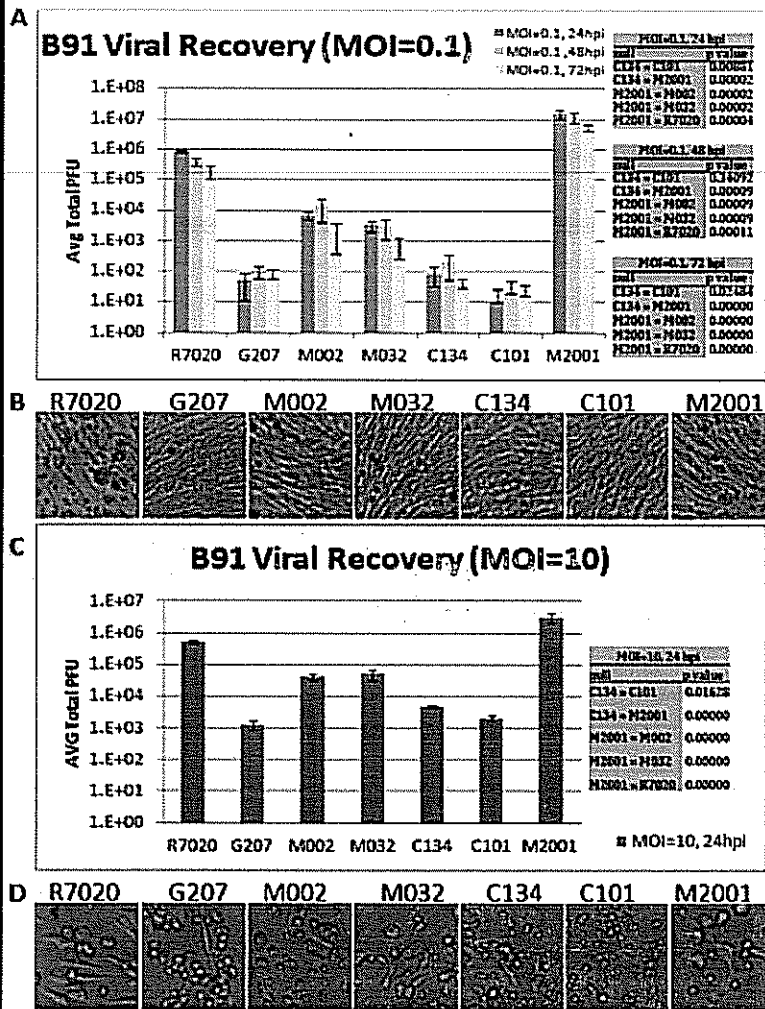


Figure 11: Composite of B91 murine MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on B91 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on B91 cells (density of 1.5e5 cells/well; 100X magnification).

Summary and Analysis (B91)

The $\gamma_{134.5}$ containing viruses (R7020 and M2001) replicate best in B91 cells (Figure 11A,B). Of the $\Delta\gamma_{134.5}$ viruses (M032 and M002) replicate better than G207, C101 or C134. The chimeric HSV replicates only marginally better than its $\Delta\gamma_{134.5}$ parent virus C101. This cell line is considered resistant to HSV replication and to early viral cytopathic effect (Figure 11C) based upon the multistep replication studies. In high MOI infection there is evidence of early CPE indicating that it is possible to overcome this resistance.

Conclusion: This is another resistant murine cell line (similar to A202) where the clinical grade viruses exhibit differential replication profiles and may be of interest in future studies.

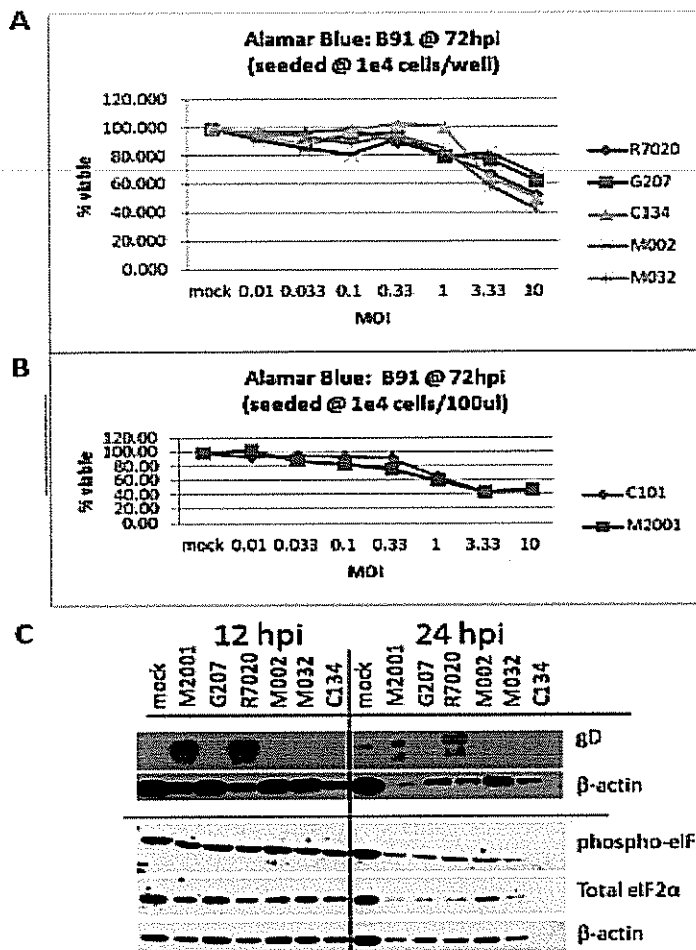


Figure 12: Composite of B91 murine MPNST cell line *in vitro* studies. **A.** Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. **B.** Alamar Blue cell viability assay comparing nonclinical grade oHSVs C101 ($\Delta\gamma_1$ 34.5) vs. M2001 (wt) at 72hpi. **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (B91 cont'd.)

Despite significant differences in viral replication and CPE, Alamar blue measurement of cell viability showed no difference between the recombinants tested (Figure 12A). Even more surprising there was no difference detected between wild-type and $\Delta\gamma_1$ 34.5 virus (Figure 12B).

Western blots (Figure 12C) show gD accumulation only in the γ_1 34.5 containing viruses (M2001 and R7020). Also surprising was presence of p-eIF2 α in infected and uninfected B91 cells (12hpi). This suggests that the γ_1 34.5 containing viruses were still capable of late viral protein synthesis. The 24hpi images are difficult to interpret and show differential loss of actin staining suggestive of loss of viable cells in some samples. This is especially evident in the M2001 samples where there is loss of gD staining and actin suggestive of overall cell loss secondary to cytopathic effect. The C134 virus acts similar to the C101 parent virus in this cell line with limited gD accumulation and lower viral replication.

Summary and Analysis (B96)

The B96 murine MPNST line, provides an intermediate replication environment for HSV (in single step replication studies) (Figure 13A). The $\gamma_134.5$ containing viruses (M2001, R7020) replicate the best in these cells with M2001 replicating the best. The M002, M032, and C134 recombinants replicate similarly in this cell line with G207 and C101 replicating the poorest.

Early cytopathic effects are seen in all cell lines at high MOI (Figure 13B).

Immunostaining studies (Figure 13C) show inconsistent gD, actin staining at 12-24h likely related to cell loss and CPE (as shown above in figure 13b).

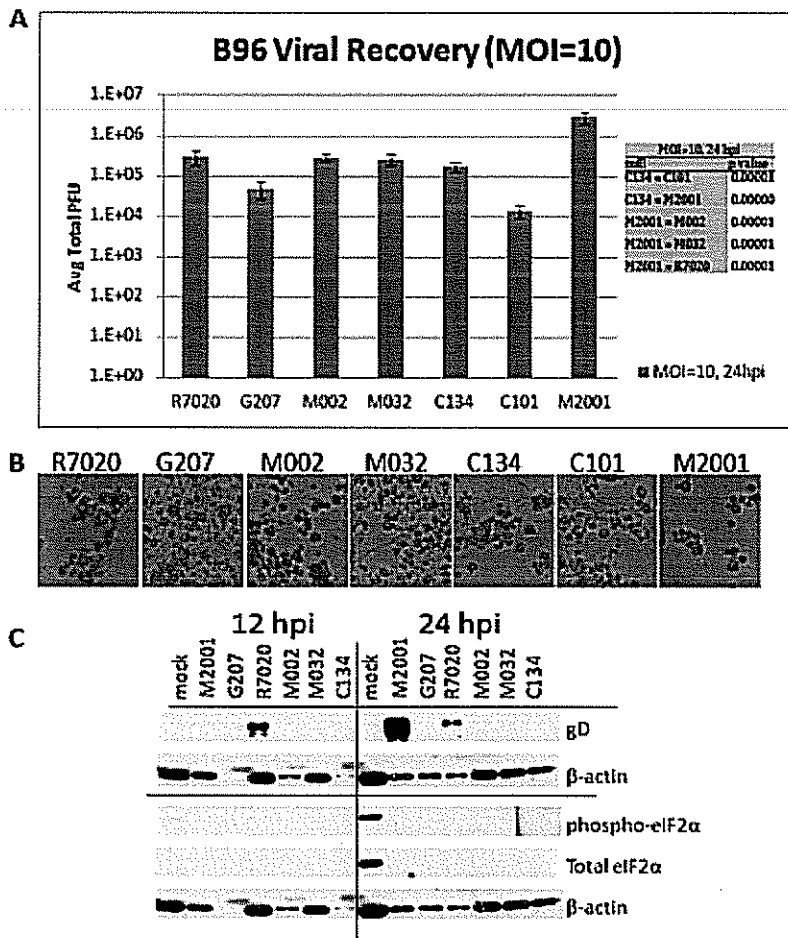


Figure 13: Composite of B96 murine MPNST cell line *in vitro* studies. A. Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **B.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on B96 cells (density of 1.5×10^5 cells/well; 100X magnification). **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2α along with β-actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

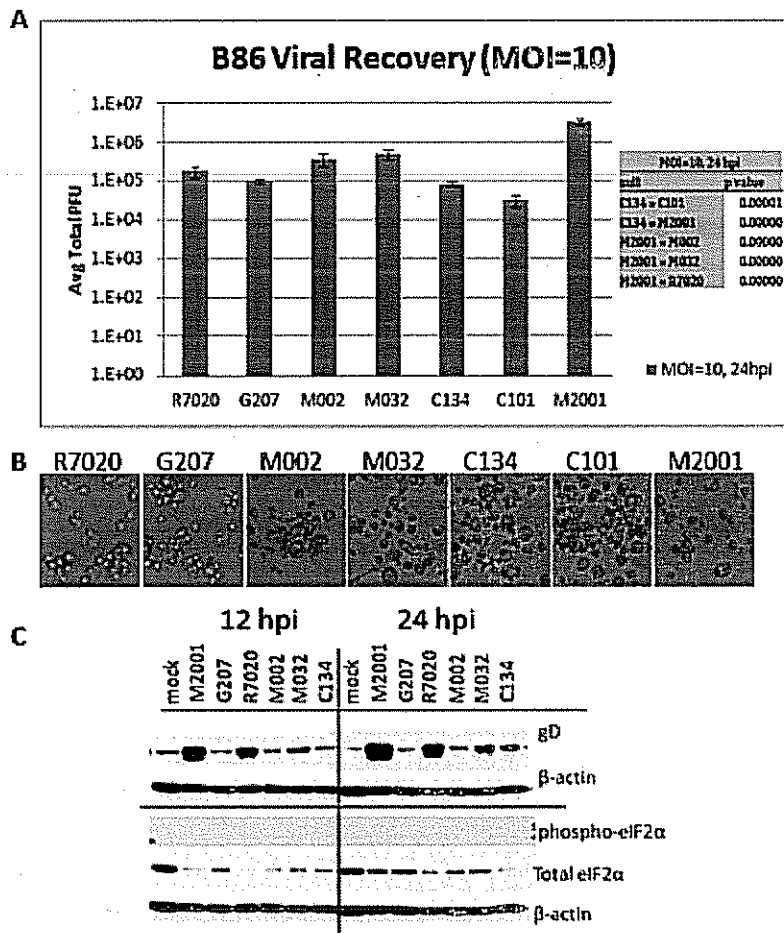


Figure 14: Composite of B86 murine MPNST cell line *in vitro* studies. A. Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **B.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on B86 cells (density of 1.5e5 cells/well; 100X magnification). **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2α along with β-actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (B86)

The B86 MPNST cell line similar to B96 provides an intermediate replication environment for HSV (Figure 14A). Again, M2001 replicates the best in this tumor line. In contrast with the B96 cell line discussed above (Figure 13), M002 and M032 replicate better than the R7020 ($\gamma_{134.5}$ single copy) recombinant. C134 and C101 exhibit the lowest replication of the viruses tested in spite of its ability to synthesize similar levels of gD as M032 at 24hpi (Figure 14C). Again, early cytopathic effect is seen with all viruses at high MOI (Figure 14B).

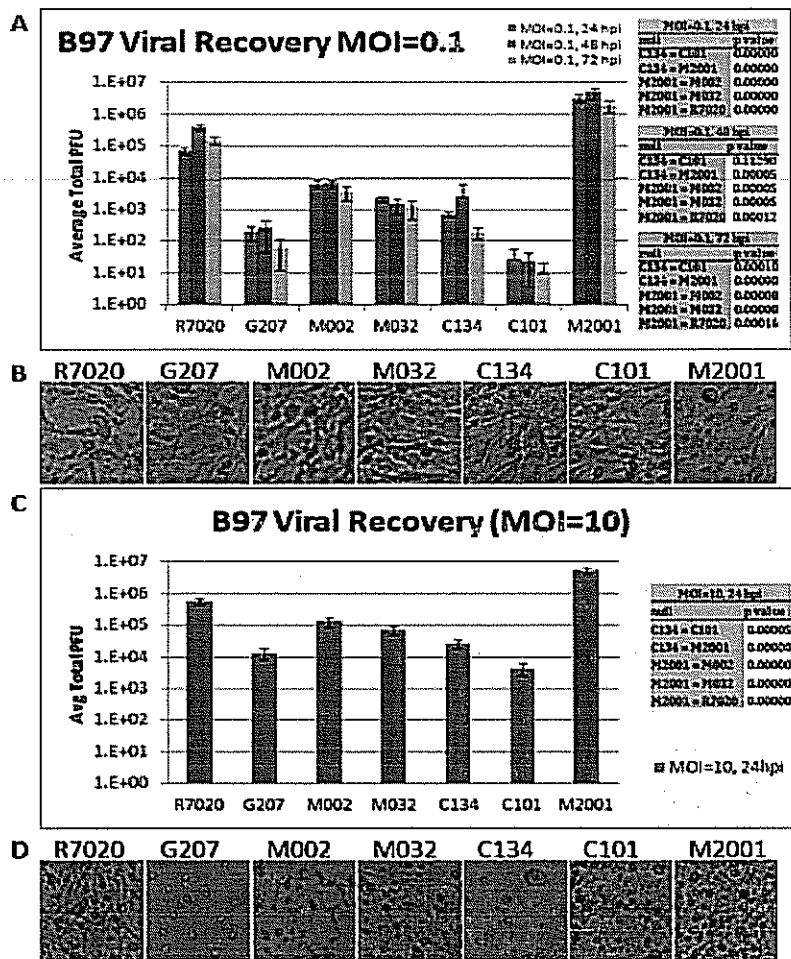


Figure 15: Composite of B97 murine MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on B97 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on B97 cells (density of 1.5e5 cells/well;100X magnification).

Summary and Analysis (B97.) The B97 MPNST cell line produces the highest differential replication rates between the γ_1 34.5 and $\Delta\gamma_1$ 34.5 viruses tested (100,000x fold difference between C101 and M2001 in multistep replication assays) (Figure 15A,C). The γ_1 34.5 containing viruses (R7020 and M2001) produce early CPE at low and high MOI (Figure 15B,D). The $\Delta\gamma_1$ 34.5 recombinants only produced significant CPE at high MOI in this cell line. C134 replicates better than its parent virus C101 in this cell line but the M032 and M002 virus replicate better than C134.

A

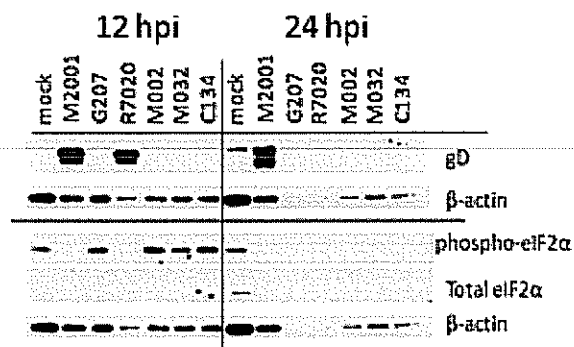


Figure 16: Composite of B97 murine MPNST cell line *in vitro* studies. A. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2α along with β-actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (B97 cont'd.)

Immunostaining studies (Figure 16A) are interpretable for the 12h timepoint and show gD accumulation only in the M2001 and R7020 samples. Despite their improved replication over C101, the M002, M032, and C134 samples show no significant accumulation of gD suggestive of protein shutoff. The p-eIF2α staining also suggests this. The total eIF-2α staining is uninterpretable. There is differential actin staining which when combined with the replication data in Figure 15 suggests that there is greater cell loss in the R7020 and M2001 samples than in the mock and Δγ_{134.5} infected cells.

Summary and Analysis (B109)

The murine B109 MPNST cell line supports HSV replication. As is true for all the cell lines, M2001 replicated best. The IL-12 expressing viruses (M002, M032) and the single $\gamma_134.5$ copy virus (R7020) were the next best replicating recombinants (Figure 17A). C134 replicates similar to its parent virus C101 in this cell line suggesting that it is restricts the IRS1 PKR evasion (as shown by p-eIF2 α staining and the lack of appreciable gD accumulation in panel C, (Figure 17C)). All viruses exhibited early CPE at high MOI (Figure 17B).

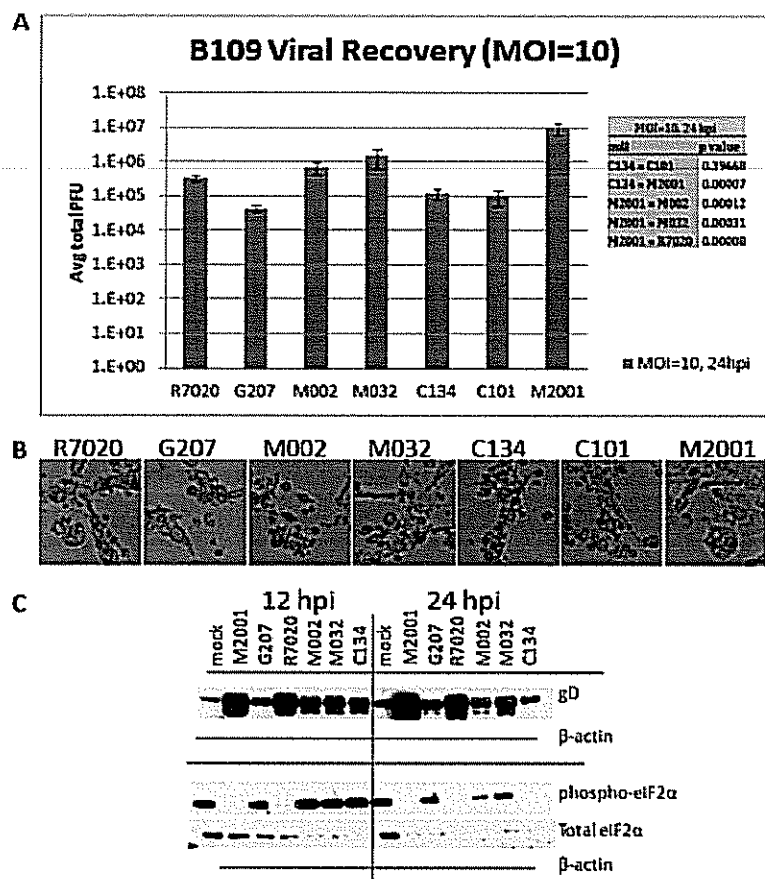


Figure 17: Composite of B109 murine MPNST cell line *in vitro* studies.
A. Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **B.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on B109 cells (density of 1.5e5 cells/well; 100X magnification). **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (A292.)

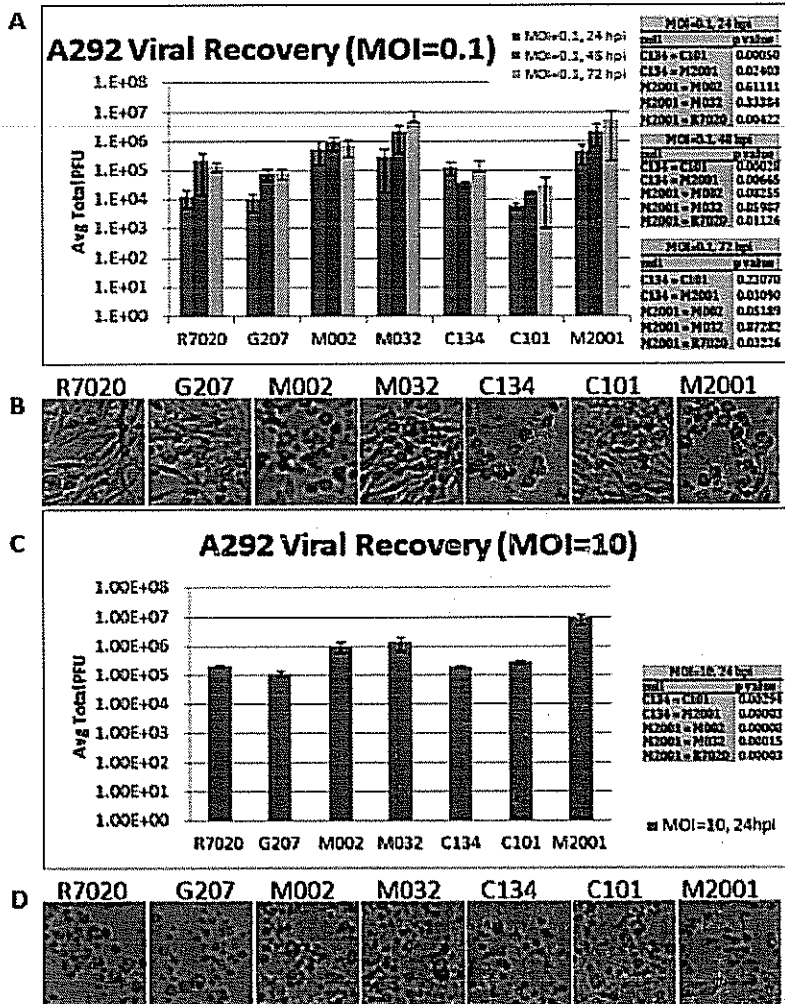


Figure 18: Composite of A292 murine MPNST cell line *in vitro* studies.

A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/48/72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on A292 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A292 cells (density of 1.5e5 cells/well; 100X magnification).

The A292 murine MPNST cell line supports HSV replication. Recombinant viral replication approaches that of the wild-type-GFP virus M2001. The M032 virus replicates better than the single copy γ_1 34.5 virus R7020 and C134 (Figure 18A,B). In the early CPE images, the C134 infected cells show the greatest CPE of the non-M2001 samples. It is interesting that in multistep replication studies, in contrast to the other viruses tested, it is the only recombinant incapable of increasing virus recovery over time (Figure 18A)

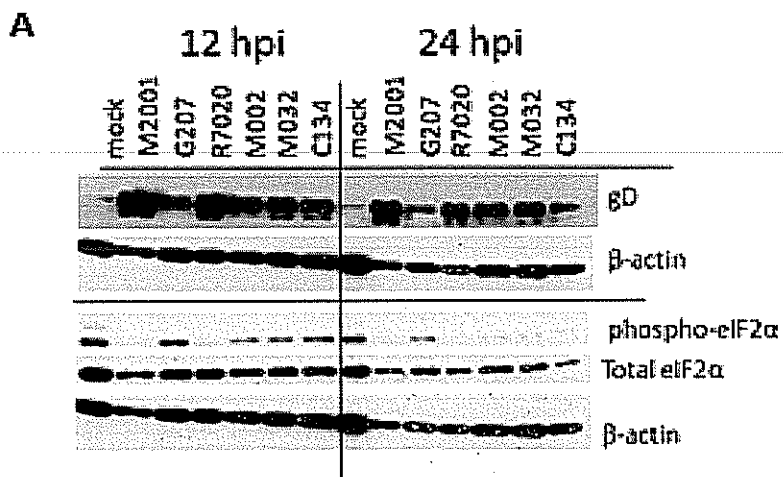
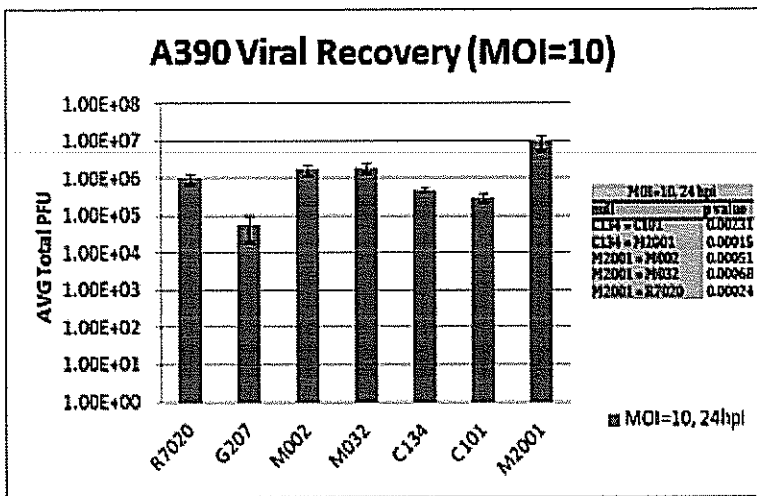


Figure 19: Composite of A292 murine MPNST cell line *in vitro* studies. A. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (A292 cont'd.)

In immunostaining studies (Figure 19A) the viruses initially synthesize gD (of note, G207 produces the least of the viruses in the 12h samples). By 24h however, the C134 virus produces less gD, similar to G207. Immunostaining for p-eIF2 α at 12hpi shows that there is baseline phosphorylation in uninfected A292 cells but that the $\gamma_{134.5}$ containing virus infected cells (R7020 and M2001) there is less p-eIF-2 α detected.

A



B

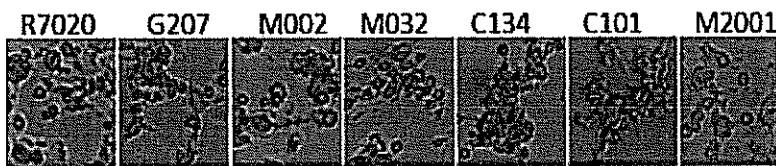


Figure 20: Composite of A390 murine MPNST cell line *in vitro* studies.
A. Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A390 cells (density of 1.5e5 cells/well; 100X magnification).

Summary and Analysis (A390)

In preliminary single step replication studies, the viruses tested replicated within 100x of one another (Figure 20A). The M2001 produced a significantly greater amount of virus followed by M032, M002 and R7020. G207 produced the least amount of virus. C134 and C101 produced virus at an intermediate level between G207 and R7020). All of the viruses produced early cytopathic effect at high MOI.

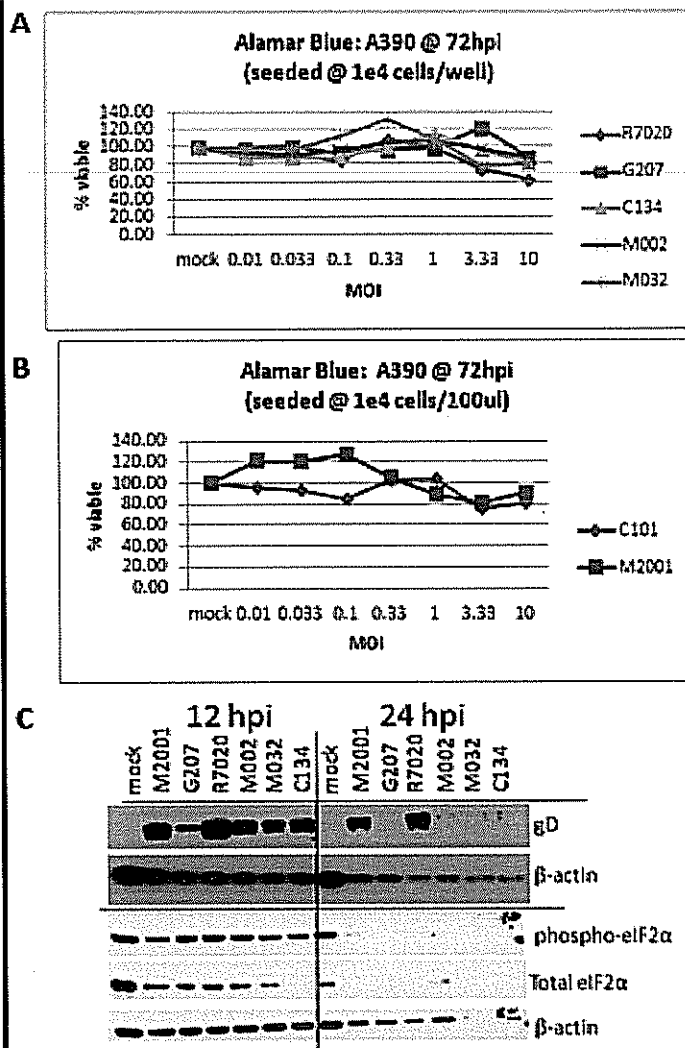


Figure 21: Composite of A390 murine MPNST cell line *in vitro* studies. **A.** Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. **B.** Alamar Blue cell viability assay comparing nondinical grade oHSVs C101 ($\Delta\gamma1$ 34.5) vs. M2001 (wt) at 72hpi. **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (A390 cont'd.)

Alamar blue cytotoxicity studies were inconsistent with the CPE analysis and replication based studies, showing viability in the 60-90% range at 72 hpi. C101 showed early CPE exceeding M2001, which is an uninterpretable result. (Figure 21A,B).

The immunostaining studies (Figure 21C) showed the greatest gD accumulation in the M2001 and R7020 infected cells. There was baseline eIF2 α phosphorylation detected in A390 MPNST cells. HSV infection did not appear to alter the accumulation of eIF2 α accumulation.

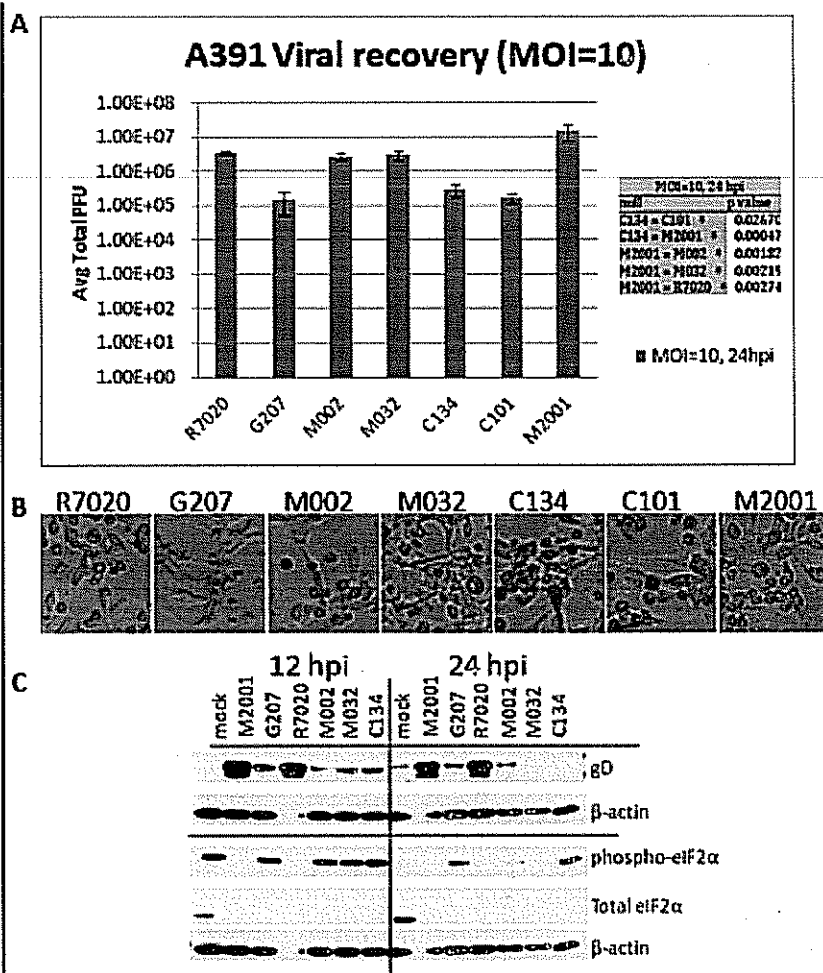


Figure 22: Composite of A391 murine MPNST cell line *in vitro* studies.
A. Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A391 cells (density of 1.5e5 cells/well; 100X magnification). **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2α along with β-actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (A391)

The A391 MPNST cell line is sensitive and supports HSV replication. All of the viruses tested were capable of generating $>10^5$ PFU in single step replication assays (Figure 22A) with CPE at 24 hpi (Figure 22B) at high MOI.

Immunostaining studies (Figure 22C) showed greater gD accumulation in the M2001 and R7020 infected cells (consistent with the replication data), and p-eIF-2α in the Δγ134.5 viruses, particularly at 12h.

Summary and Analysis (B76)

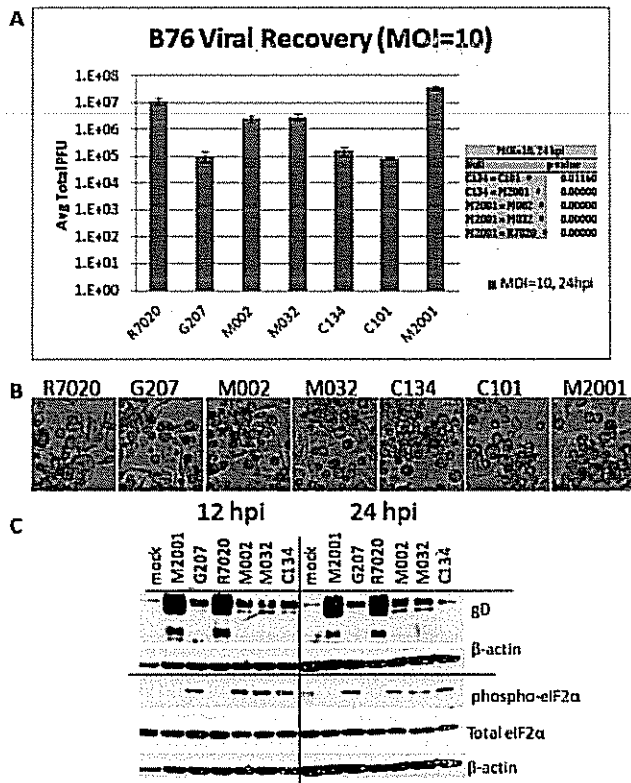


Figure 23: Composite of B76 murine MPNST cell line *in vitro* studies. A. Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **B.** Photomicrographs of cytopathic effect at high MOI for all α HSVs tested on B76 cells (density of 1.5×10^5 cells/well; 100X magnification). **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

The B76 MPNST cell line supports HSV replication and shows differential replication of $\Delta\gamma_{134.5}$ viruses (Figure 23A). Of the $\Delta\gamma_{134.5}$ viruses tested, M002 and M032 replicate best. C134 only generates 10^5 pfu and behaves similar to its parent virus C101 and G207 in the B76 cell line. The replication of M002 and M032 does not appear to be an eIF2 α dependent phenotype. Immunostaining (Figure 23C) shows the greatest gD accumulation in the M2001 and R7020 infected samples. The M002, M032 and C134 infected samples show evidence of eIF2 α phosphorylation at 12hpi and less gD accumulation than the M2001 and R7020 infected samples, yet viral replication differs by 10x between these $\Delta\gamma_{134.5}$ viruses.

Summary and Analysis (231-Trig)

The 231-Trig cell line is an HSV sensitive murine MPNST that supports clinical grade virus replication. M032 replicates similar to M2001 in multistep and single step replication assays generating 10^7 PFU in both assays. The 231-Trig line supports efficient viral replication, including C101. It is most restrictive to G207 growth (Figure 24A,C).

In terms of early cytopathic effects (Figure 24B,D) minimal cytopathic effects were seen when low MOI was used, and all viruses produced cytopathic effects at high MOI.

Conclusion: The 231-Trig MPNST cells are a sensitive cell line. We intend to include this as one of the 2 oHSV sensitive MPNST cell lines in future studies as described in Subaim 1d.

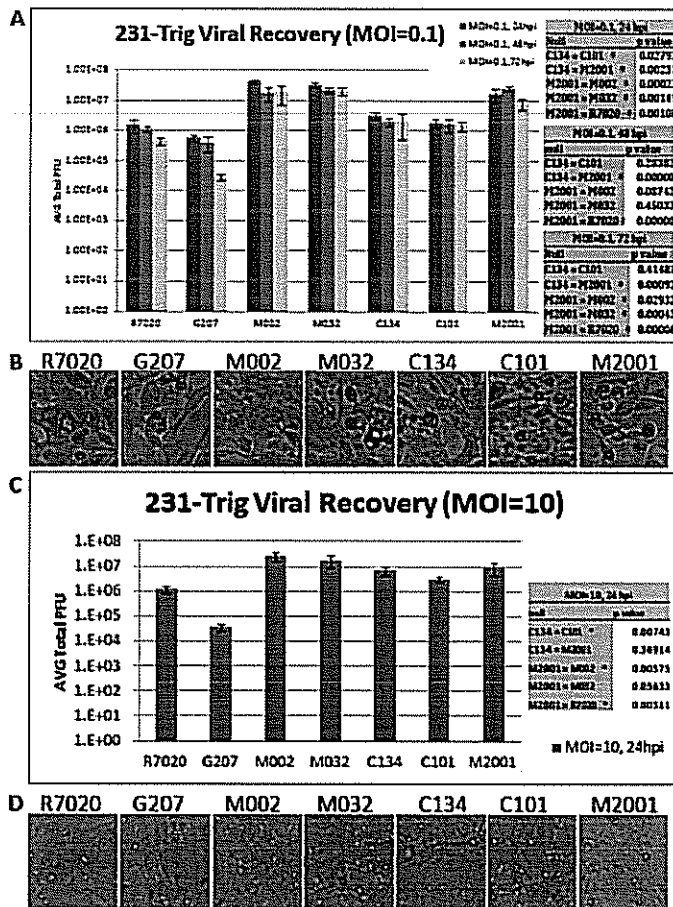


Figure 24: Composite of 231-Trigeminal murine MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). B. Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on 231-Trig cells (100X mag). C. Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. D. Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on 231-Trig cells (density of 1.5×10^5 cells/well; 100X magnification).

Summary and Analysis (231-Trig cont'd.)

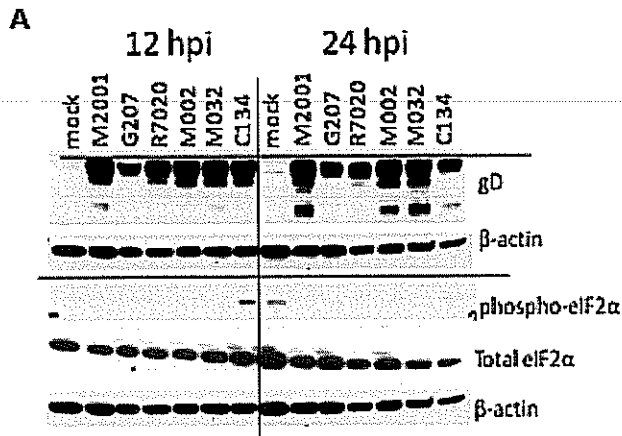


Figure 25: Composite of 231-Trigeminal murine MPNST cell line *in vitro* studies. A. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2α along with β-actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

All of the viruses tested produce abundant gD in the 231-Trig cell line. In terms of early cytopathic effects (Figure 25A) minimal cytopathic effects were seen when low MOI was used, and all viruses produced cytopathic effects at high MOI. M2001, M002, and M032 produce the greatest gD at 24hpi followed by C134. There is no evidence of p-eIF2α in the samples at 24hpi consistent with the permissive late protein phenotype of this cell line.

Conclusion: We intend to include this as one of the 2 oHSV sensitive MPNST cell lines in future studies as described in Subaim 1d.

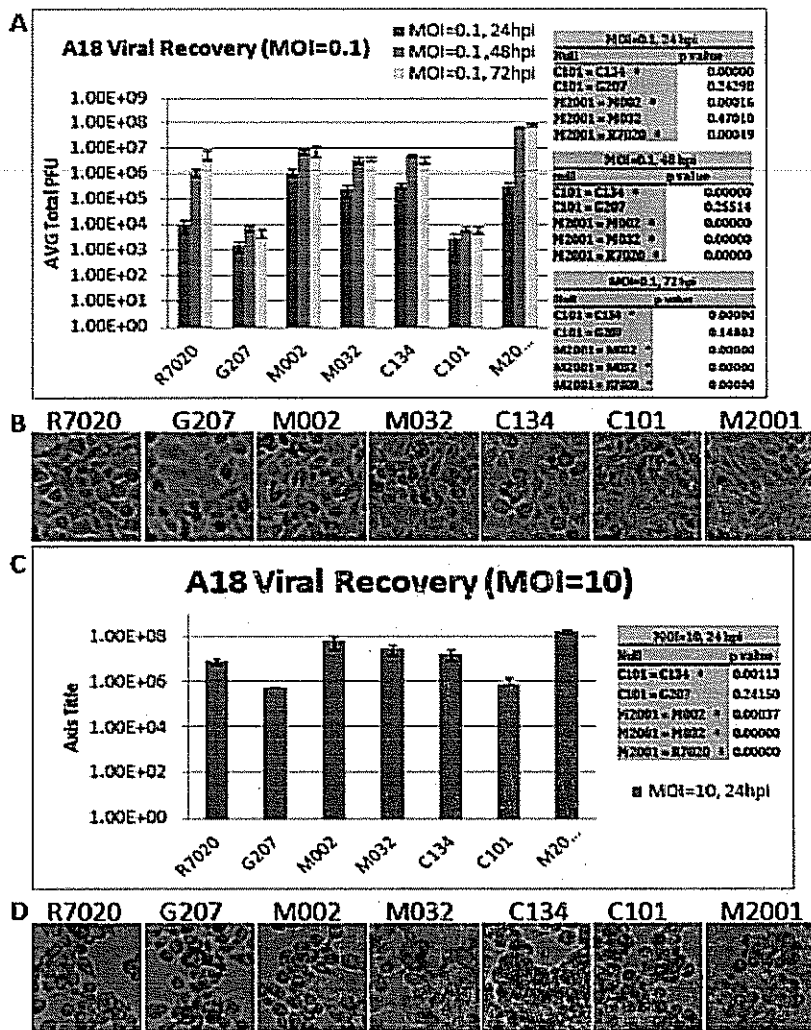


Figure 26: Composite of A18 murine MPNST cell line *in vitro* studies. **A.** Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on A18 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A18 cells (density of 1.5e5 cells/well; 100X magnification).

*Summary and Analysis (A18)

The A18 cell murine MPNST cell line is also sensitive to HSV infection and replication. Unlike the 231-Trig line, however, there is greater replication variation between $\Delta\gamma_{134.5}$ viruses in this cell line. Of the $\Delta\gamma_{134.5}$ viruses tested, the M002, M032, and C134 viruses replicate the best (Figure 26A,C). The R7020 replicates well but initially produces less virus than the chimeric and IL12 containing viruses. The prototypical $\Delta\gamma_{134.5}$ viruses (G207 and C101) that induce protein shutoff in non-MPNST cell lines generate the least amount of virus in this cell line in multistep replication assays.

In terms of early cytopathic effects (Figure 26B,D) minimal cytopathic effects were seen when low MOI was used, and all viruses produced cytopathic effects at high MOI.

Conclusion The A18 cell line will be one of the 2 sensitive murine MPNST cell lines that we will use in future studies because it shows a greater differential effect on $\Delta\gamma_{134.5}$ replication.

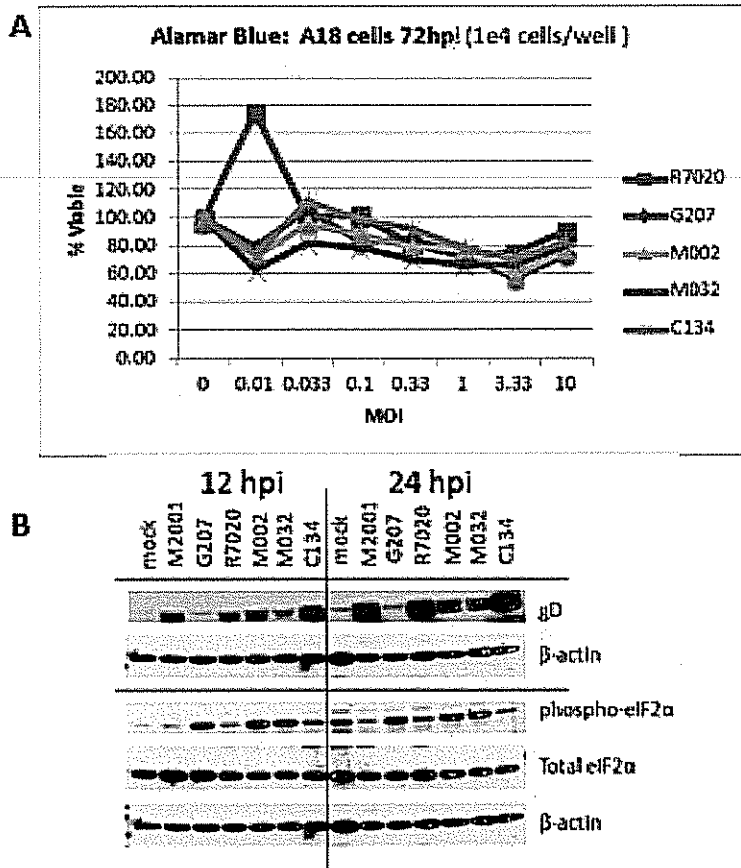


Figure 27: Composite of A18 murine MPNST cell line *in vitro* studies. **A.** Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. **B.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

human tumor cell lines tested (Shah, 2007)

Summary and Analysis (A18 cont'd.)

In the Alamar Blue assay of cell viability, all viruses were somewhat effective against A18 (Figure 27 A).

Western blot analysis of the mutant viruses on A18 (Figure 27B) demonstrate decreased gD production in G207 and to some degree M002 and M032 infected cell samples. As expected, the greatest phosphorylation of eIF 2 α , was seen in G207, followed by M032 and M002, although these were not drastically different from R7020 and C134. By 24 hpi the greatest gD accumulation occurred in the M2001, R7020, and C134 infected cells. Consistent with the gD findings, less p-eIF2 α is detected in the M2001, R7020 and C134 infected samples.

Conclusion The A18 cell line will be one of the 2 sensitive murine MPNST cell lines that we will use in future studies because it shows a greater differential effect on $\Delta\gamma_{134.5}$ replication. The C134 recombinant differs from its parent virus (C101) in this cell line and is capable of maintaining late viral protein synthesis and precludes PKR mediated shutoff similar to that shown in other

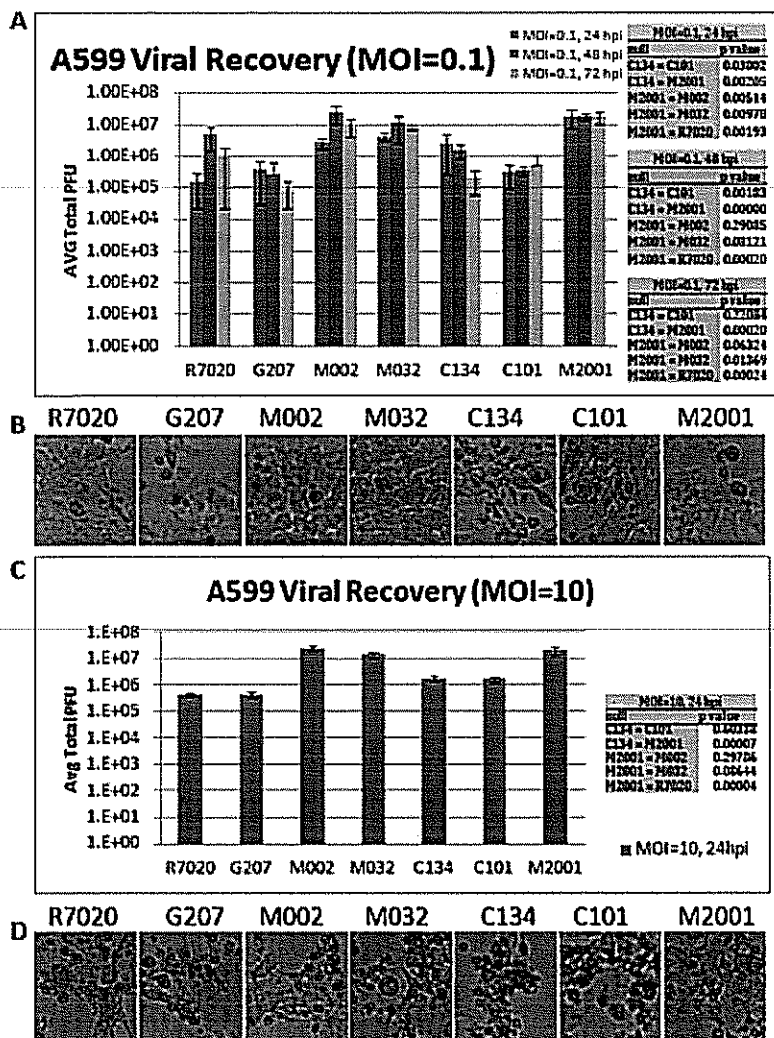


Figure 28: Composite of A599 murine MPNST cell line *in vitro* studies.
A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on A599 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on A599 cells (density of 1.5e5 cells/ well; 100X magnification).

Summary and Analysis (A599.)

The A599 MPNST cell line is sensitive to oHSV replication. M2001 produces 10^7 pfu in multistep replication assays (Figure 28A). M032 and M002 generate a similar amount of virus at 48 and 72h post infection as M2001. C134 initially replicates well in this cell line but over the next 48h of infection replication declines and the chimeric HSV replicates similar to its $\Delta\gamma_134.5$ parent virus C101. It is interesting that C134 infected cells appear pyknotic in CPE studies at low and high MOI and have a different appearance than the other $\Delta\gamma_134.5$ and the M2001 infected cells (Figure 28B and 28D). In single step replication assay, M032 and M002 performed similarly to M2001, while R7020 and G207 performed less well (Figure 28C).

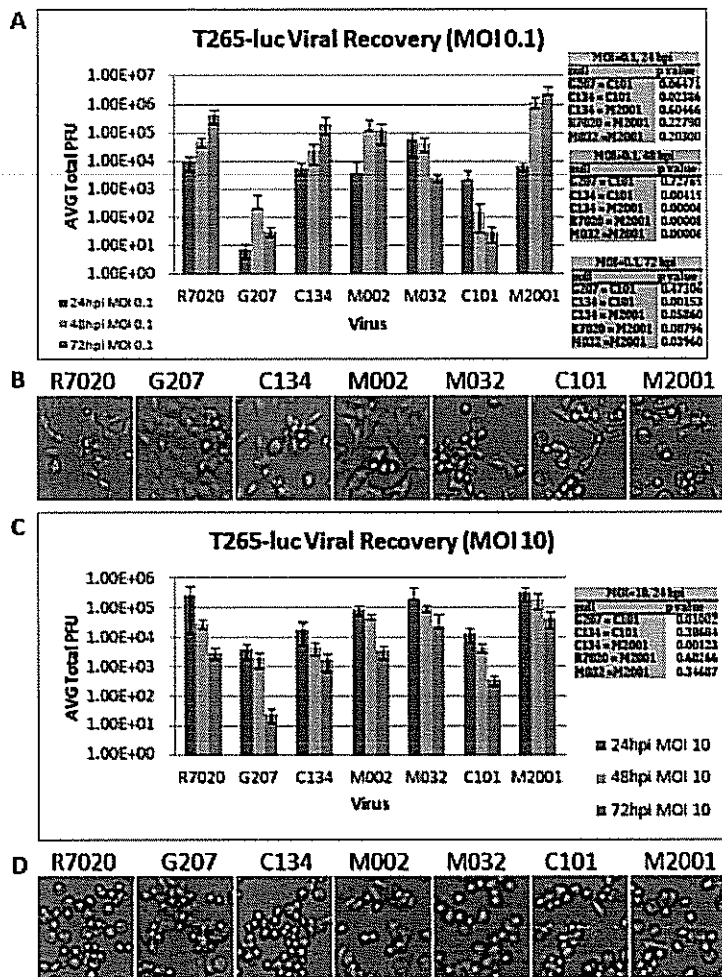


Figure 29: Composite of T265-luciferase human MPNST cell line *in vitro* studies. **A.** Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on T265-luc cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on T265-luc cells (density of 1.5e5 cells/well; 100X magnification).

Summary and Analysis (T265-luc)

The T265-luc human MPNST cell line is resistant to HSV infection. In multistep replication studies M2001 replicates the best followed by C134 and R7020. The replication pattern is interesting: in the R7020, C134, and to some extent M002 infected cells there is progressive increase in virus replication at 24, 48 and 72 hpi. In contrast M032 and C101 show a decline in viral recovery over the 24, 48 and 72 h timepoints. While C134 and M032 generate similar peak viral levels they appear to do this with a different kinetic pattern (M032 early and C134 late) (Figures 29A,C)

Early cytopathic effect (Figure 29B,D) was particularly evident in the high MOI assay. As was seen in the Alamar blue assay below, this was likely due to non-replicative killing.

Conclusion: This is an interesting cell line that we intend to include in future studies as a representative resistant human cell line for future studies as described in Subaim 1d.

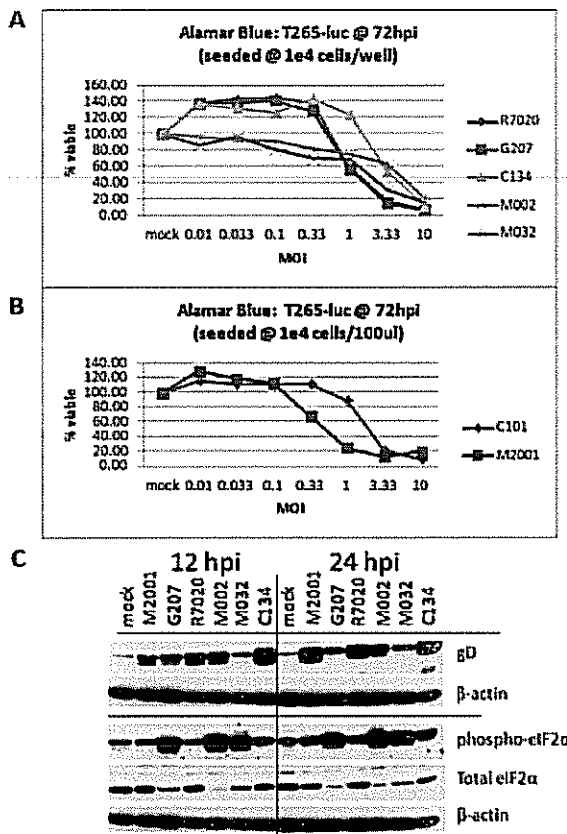


Figure 30: Composite of T265-luciferase human MPNST cell line *in vitro* studies. A. Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. B. Alamar Blue cell viability assay comparing nonclinical grade oHSVs C101 ($\Delta\gamma 1$ 34.5) vs. M2001 (wt) at 72hpi. C. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (T265-luc cont'd.)

In the Alamar Blue assay of cell viability, all viruses were particularly effective against T265-luc (Figure 30 A,B). At the highest MOI, the viruses were extremely effective producing about 80-100% CPE.

Western blot analysis of the mutant viruses on T265-luc (Figure 30C) demonstrated decreased production of gD, a late gene product, in M032 and to a lesser degree, G207. As expected, the greatest phosphorylation of eIF 2 α , was seen in G207, followed by M032 and M002. R7020 and M2001 showed similar p-eIF2 α staining as mock infected cells. C134 produced an intermediate level of eIF2 α staining somewhere between that of the $\gamma 134.5$ and M032 infected cell samples. This interesting pattern of eIF2 α phosphorylation combined with the replication findings shown in (Figure 29), warranted further study and thus this line was selected as one of the lines we will study in depth.

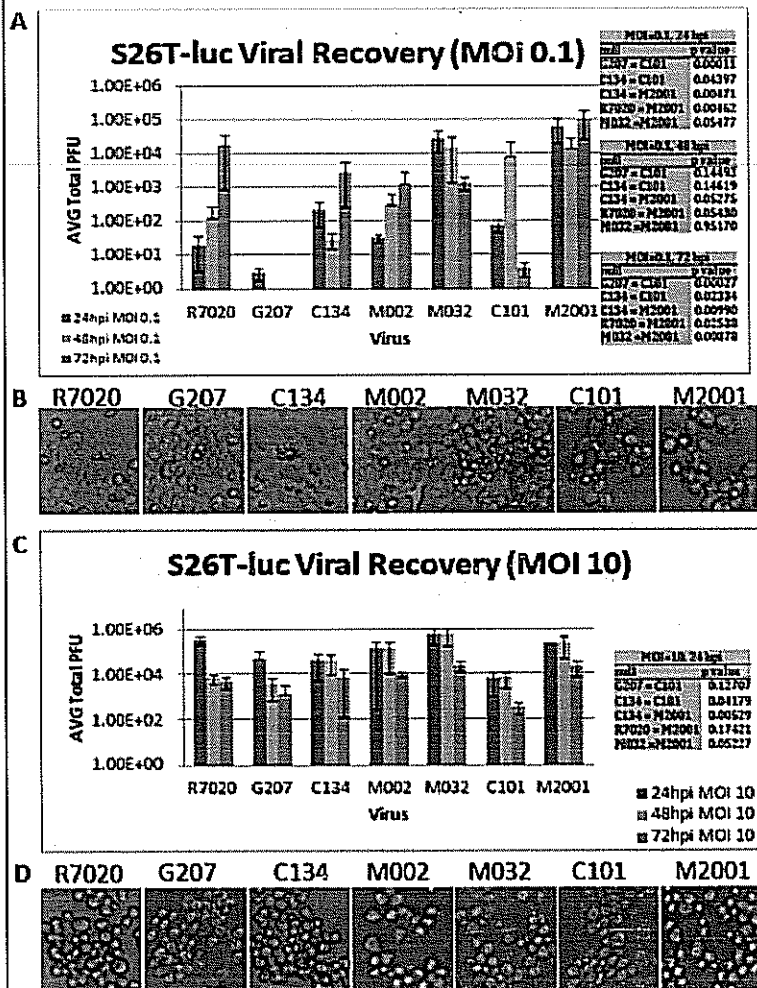


Figure 31: Composite of S26T-luciferase human MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on S26T-luc cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on S26T-luc cells (density of 1.5e5 cells/well:100X magnification).

Summary and Analysis (S26T-luc)

The S26T-luc Human MPNST cell line is highly resistant to most of the $\Delta\gamma_{134.5}$ viruses tested. Wild-type HSV expressing GFP (M2001) was capable of sustained replication over a 72h period and generated between 10^4 and 10^5 PFU. R7020 by 72hpi generated virus in at 10^4 pfu. M032 replicates well initially (10^4 pfu 24hpi) but then declines over time (10^3 pfu 72hpi). C134 replicates poorly in this human MPNST cell lines behaving similar to the $\Delta\gamma_{134.5}$ parent virus C101 (figure 31 A).

Differences in replication kinetics between the viruses are less apparent in single step replication assays (Figure 31C)

Early cytopathic effect was particularly evident in the high MOI assay. (Figure 31B,D).

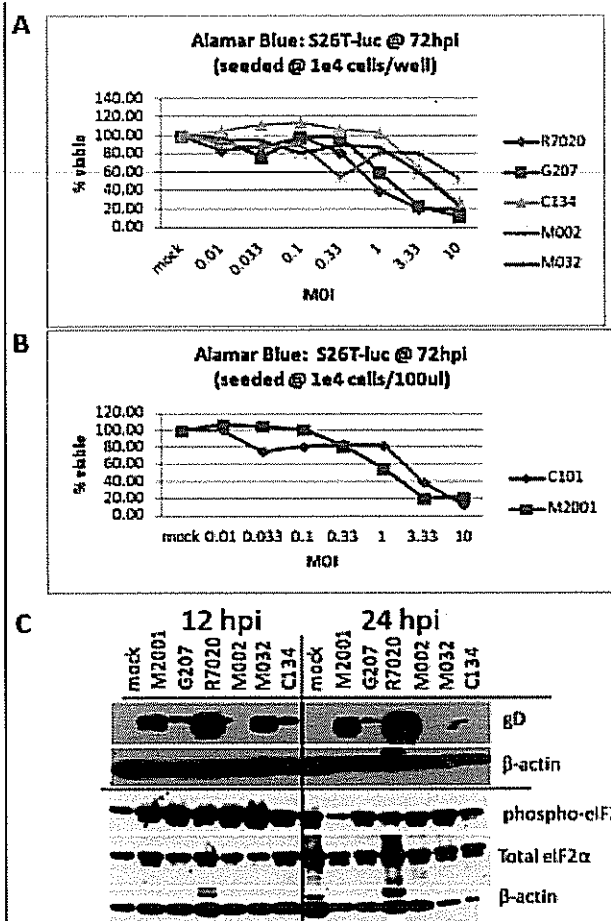


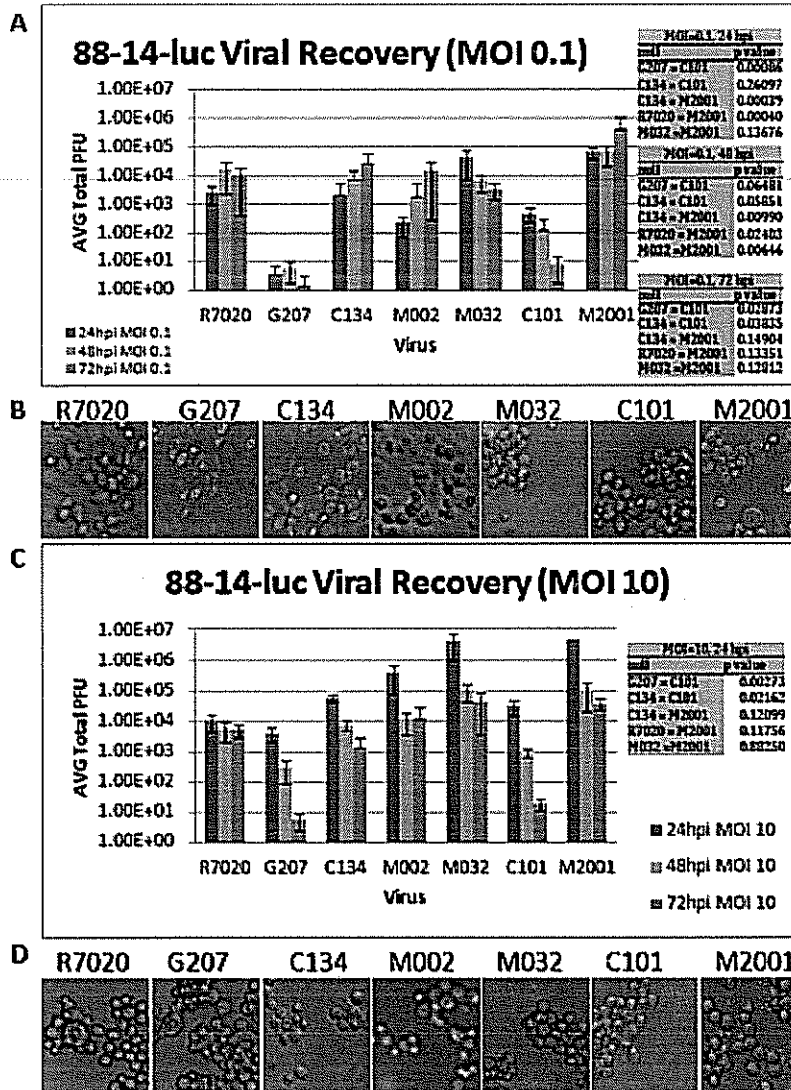
Figure 32: Composite of S26T-luciferase human MPNST cell line *in vitro* studies. A. Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. B. Alamar Blue cell viability assay comparing nonclinical grade oHSVs C101 ($\Delta\gamma 1$ 34.5) vs. M2001 (wt) at 72hpi. C. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (S26T-luc cont'd.)

In the Alamar Blue assay of cell viability, all viruses were particularly effective against S26T-luc (Figure 32 A,B). At the highest MOI, the viruses were extremely effective producing about 65-80% CPE.

Western blot analysis of the mutant viruses on S26T-luc (Figure 39C) demonstrated significant production of gD, a late gene product, by R7020, M2001, and to a lesser extent, G207. M002, M032, and C134 had little to no expression of gD. There was an interesting pattern of phosphorylation of eIF 2 α , in all viruses, even M2001.

Conclusion: The 26T-luc is an interesting cell line that we intend to include in future studies as a representative resistant human cell line in Subaim 1d.



Summary and Analysis (88-14-luc)

The 88-14-luc human MPNST cell line is resistant to HSV replication, similar to that seen in the S26T cell line shown in Figure 31. The viruses tested exhibit different replication patterns in multistep replication assays (Figure 33A). M2001, C134, and M002 increase viral replication over time whereas G207, C101, and M032 replication declines over the 72h culture period. At high MOI M032 produces as much virus as the wild-type HSV M2001.

Figure 33: Composite of 88-14-luciferase human MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). B. Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on 88-14-luc cells (100X mag). C. Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. D. Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on 88-14-luc cells (density of 1.5e5 cells/well; 100X magnification).

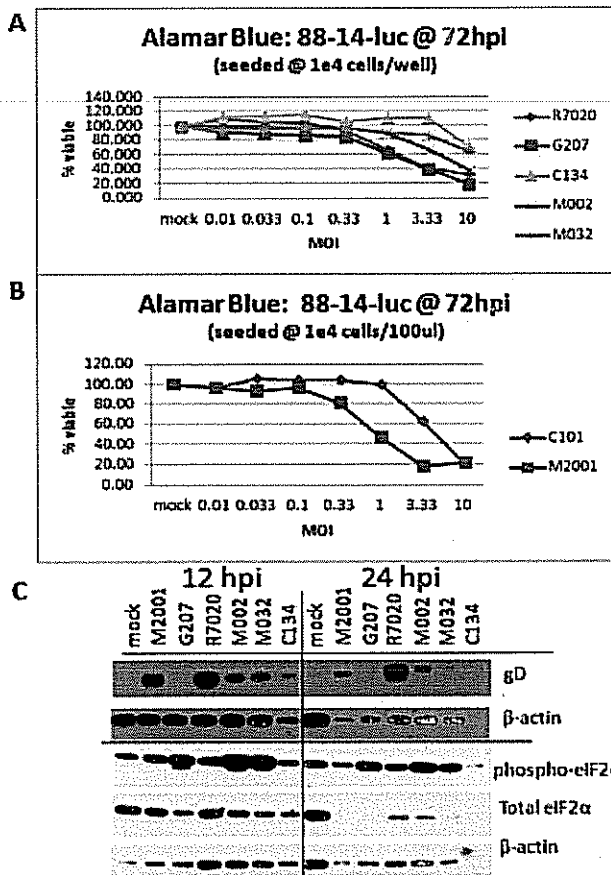


Figure 34: Composite of 88-14-luciferase human MPNST cell line *in vitro* studies. A. Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. B. Alamar Blue cell viability assay comparing nonclinical grade oHSVs C101 ($\Delta\gamma$ 34.5) vs. M2001 (wt) at 72hpi. C. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

In the Alamar Blue assay of cell viability, all viruses were particularly effective against S26T-luc (Figure 34 A,B). At the highest MOI, the viruses were extremely effective producing about 65-80% CPE.

Western blot analysis of the mutant viruses on 88-14 (Figure 34C) demonstrate significant production of gD, a late gene product, in M2001 and R7020. Lesser amounts were made in M002 and M032. As expected, the greatest phosphorylation of eIF 2 α , was seen in G207, followed by M032 and M002, although these were not drastically different from R7020 and C134. C134 samples are incapable of being interpreted in the western blot studies due to insufficient protein loading based upon the actin staining.

Summary and Analysis (90-8)

The 90-8 cell line exhibits intermediate sensitivity to HSV infection. M2001 replicates the best. The M002, M032, R7020, and C134 viruses replicate similarly. The G207 and C101 virus replicate poorly in this cell line (Figure 35 A,C). All of the viruses tested elicit early CPE, with some present even in the low MOI group (Figure 35 B, D)

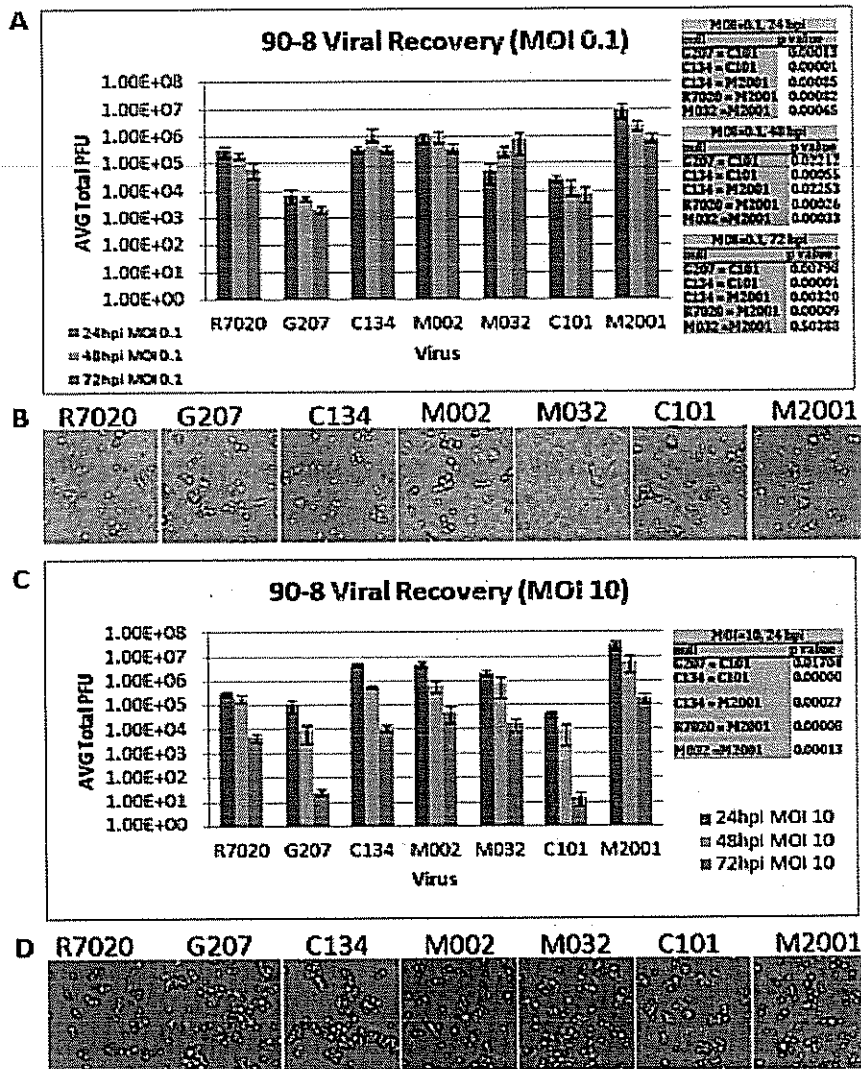


Figure 35: Composite of 90-8 human MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on 90-8 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell), viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on 90-8 cells (density of 1.5e5 cells/well; 100X magnification).

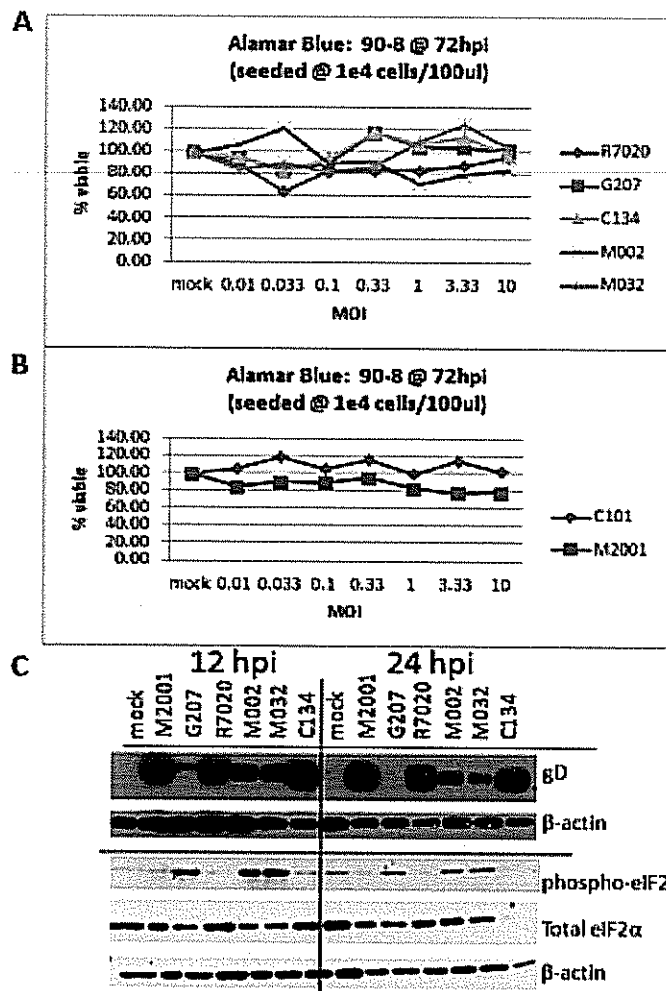


Figure 36: Composite of 90-8 human MPNST cell line *in vitro* studies. **A.** Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. **B.** Alamar Blue cell viability assay comparing nondinical grade oHSVs C101 ($\Delta\gamma 1$ 34.5) vs. M2001 (wt) at 72hpi. **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (90-8 cont'd.)

In the Alamar Blue assay of cell viability, all viruses were particularly ineffective against 90-8. These results are inconsistent with the CPE studies (shown in Figure 36 B and D) in which all of the viruses tested elicited morphologic evidence of cell damage and death.

Western blot analysis of the mutant viruses on 90-8 (Figure 36C) demonstrate significant production of gD, a late gene product, with the exception of G207, which had minimal expression of gD. M002 and M032 were intermediate. As expected, the greatest phosphorylation of eIF 2 α , was seen in G207, followed by M032 and M002.

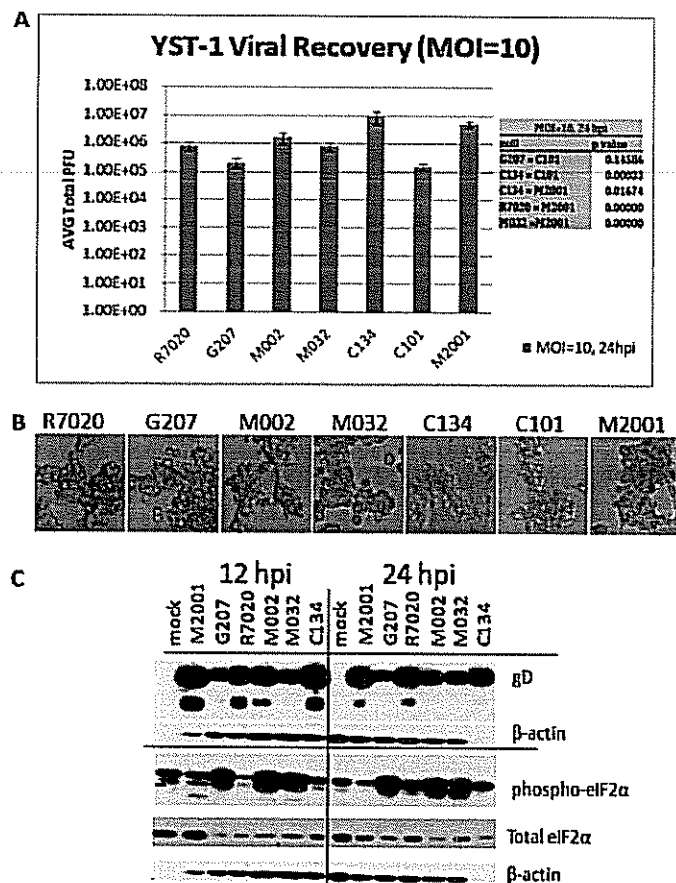


Figure 37: Composite of YST-1 human MPNST cell line *in vitro* studies.
A. Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **B.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on YST-1 cells (density of 1.5e5 cells/well; 100X magnification). **C.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

MPNST cell line allows the replication of all of our viral mutants under study and is sensitive to the virus as a cytotoxic agent.

Summary and Analysis (YST-1)

Human MPNST cell line YST-1 was sensitive to viral replication and cytopathic effects as shown in the accompanying figures. In the single step replication assay, all viruses were able to replicate, producing at least 1×10^5 pfu, with C134 resembling wild-type virus at 1×10^7 . This virus statistically replicated superiorly to C101 (Figure 37A).

In terms of cytopathic effects (37B) all viruses cytopathic effects at high MOI. This cell line this was categorized as *sensitive*.

Western blot analysis of the mutant viruses on YST-1 (Figure 37C) demonstrate significant production of gD, a late gene product, with the exception of G207, which had moderate expression of gD, along with M032. As expected, the greatest phosphorylation of eIF 2 α , was seen in G207, and M032 and M002, followed by R7020 and C134.

Conclusion: YST-1 represents a human

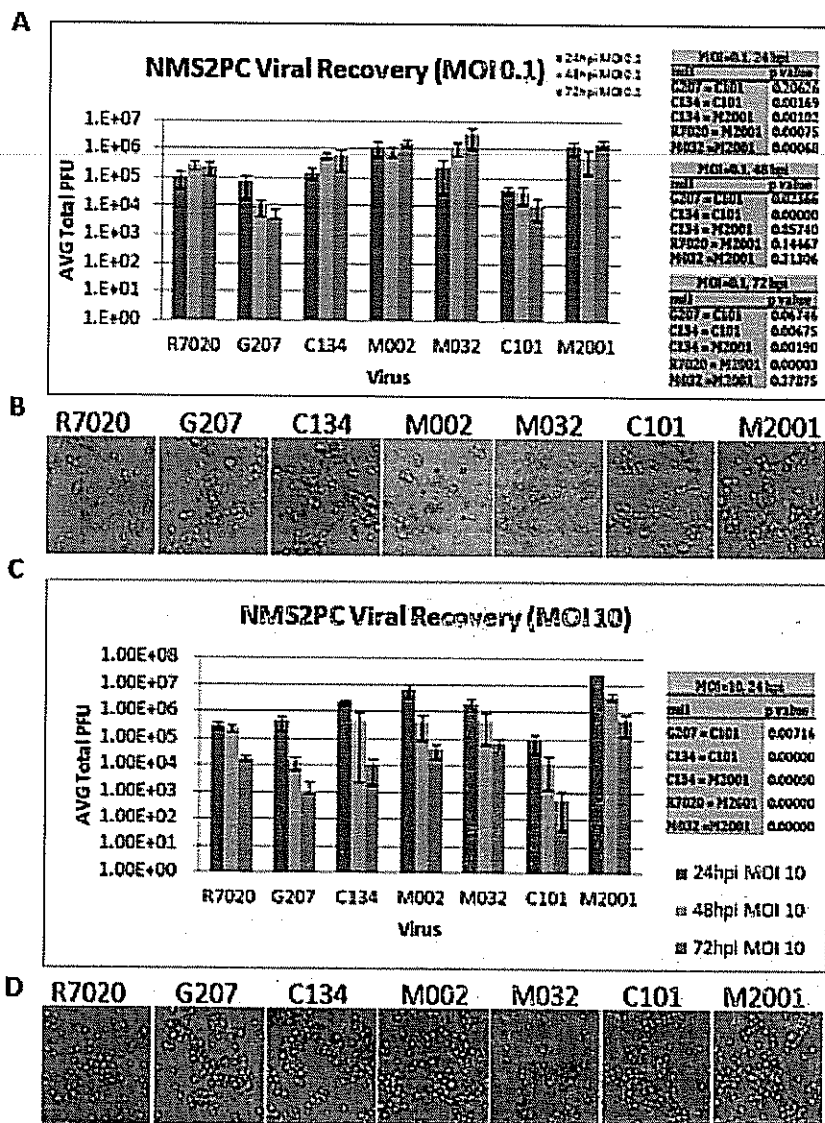


Figure 38: Composite of NMS2PC human MPNST cell line *in vitro* studies. A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). B. Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on NMS2PC cells (100X mag). C. Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. D. Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on NMS2PC cells (density of 1.5e5 cells/well; 100X magnification).

Summary and Analysis (NMS2PC)

Human MPNST cell line NMS2PC was in general sensitive to viral replication and cytopathic effects as shown in the accompanying figures. In the multistep replication assay, no viruses reached to 1×10^7 pfu, with M002, M032, and C134 reaching or exceeding 1×10^6 pfu (Figure 38A). In the single step replication assay, all viruses were likewise intact for replication, replicating to at least 5×10^6 pfu, with M002 and M032 resembling wild-type virus at 1×10^8 pfu. These same 3 viruses statistically replicated superiorly to G207, C101, and R7020 (Figure 38C).

In terms of cytopathic effects (Figure 38B,D) all viruses produced some early cytopathic effect at both low and high MOI, although not to the degree of the line S462.

Conclusion: NMS2PC represents a human MPNST cell line that both allows the replication of all of our viral mutants under study and is more sensitive to the virus as a cytotoxic agent than most other human lines.

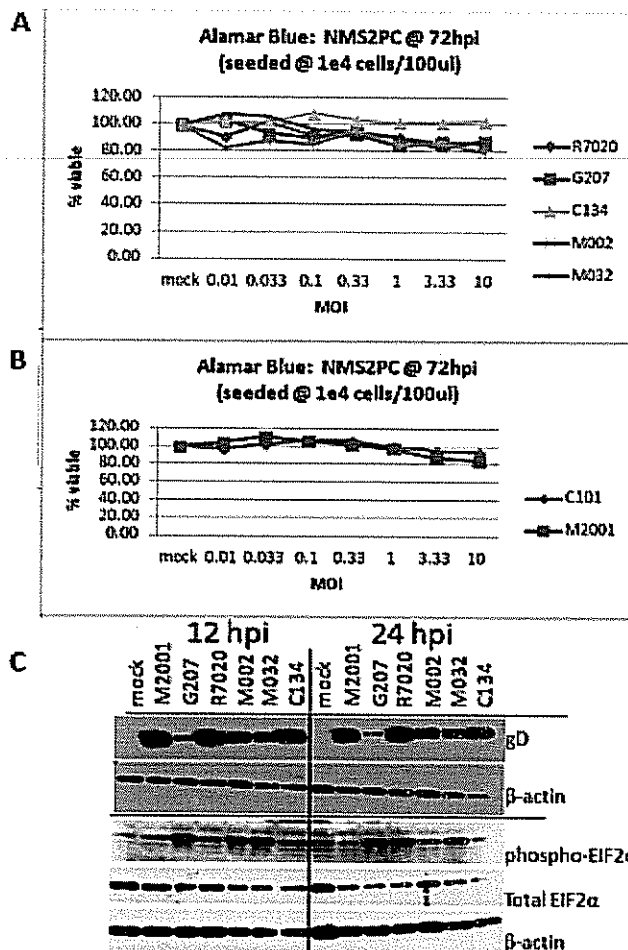


Figure 39: Composite of NMS2PC human MPNST cell line *in vitro* studies. A. Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. B. Alamar Blue cell viability assay comparing nonclinical grade oHSVs C101 ($\Delta\gamma1$ 34.5) vs. M2001 (wt) at 72hpi. C. Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2 α along with β -actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (NMS2PC cont'd.)

In the Alamar Blue assay of cell viability, all viruses were particularly ineffective against NMS2PC, even M2001 (Figure 39 A,B). At the highest MOI, the viruses were minimally effective producing about 20% CPE, except for C134.

Western blot analysis of the mutant viruses on NMS2PC (Figure 39C) demonstrate significant production of gD, a late gene product, with the exception of G207, which had minimal expression of gD. As expected, the greatest phosphorylation of eIF 2 α , was seen in G207, followed by M032 and M002, although these were not drastically different from R7020 and C134.

This interesting pattern of phosphorylation of eIF 2 α , combined with the replication findings above, warranted further study and thus this line was selected as one of the lines we will study in depth.

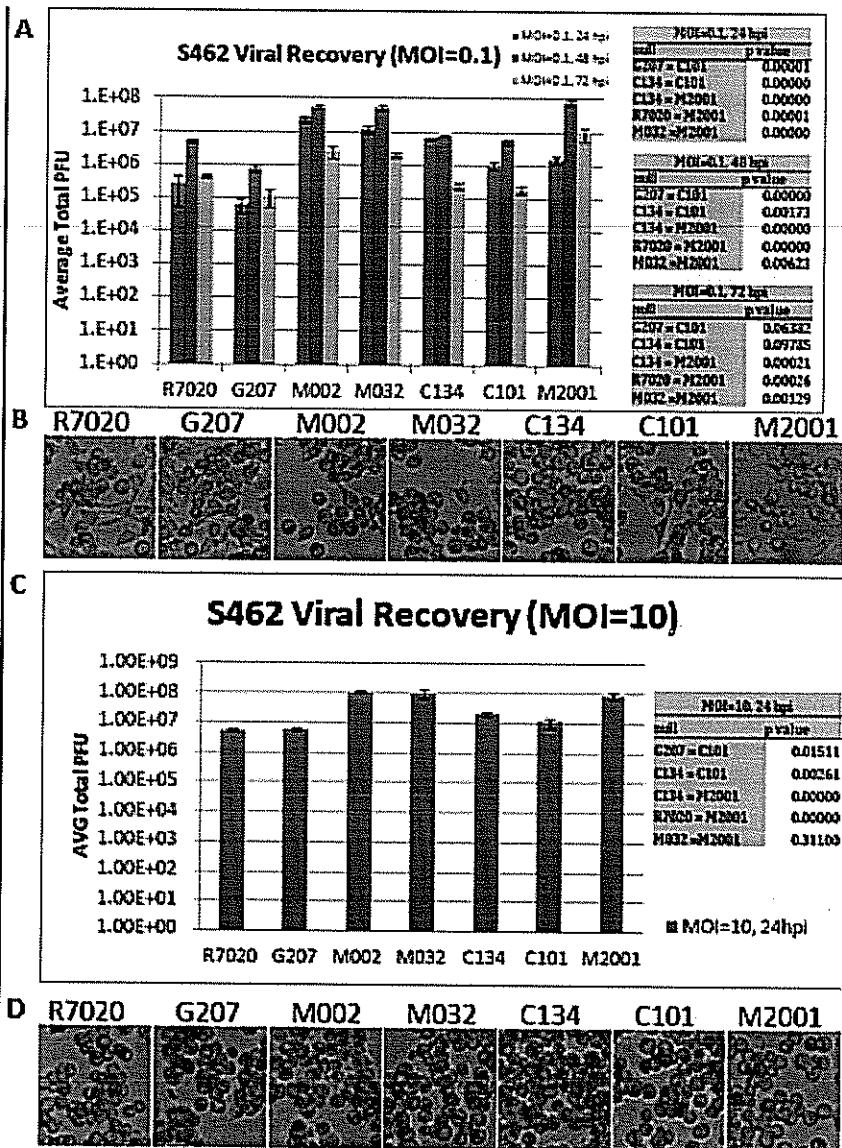


Figure 40: Composite of S462 human MPNST cell line *in vitro* studies.
A. Multistep Replication Study using a low multiplicity of infection (MOI: 0.1 plaque forming unit/cell) (PFU/cell). Viral recovery data shown at 24/ 48/ 72hpi with standard deviation (SD). **B.** Photomicrographs demonstrate cytopathic effect (CPE) in multistep replication study (24hpi) for all oHSVs tested on S462 cells (100X mag). **C.** Single-step replication study using a high MOI (10 PFU/cell). viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on S462 cells (density of 1.5e5 cells/well:100X magnification).

particularly well in this line.

Summary and Analysis (S462)

Human MPNST cell line S462 was in general favorable for viral replication and cytopathic effects as shown in the accompanying figures. In the multistep replication assay, all viruses reached to at least 1×10^6 pfu, with M002, M032, and C134 reaching or exceeding 1×10^7 pfu (Figure 40A). In the single step replication assay, all viruses replicated to at least 5×10^6 pfu, with M002 and M032 resembling wild-typ virus at 1×10^8 . These same 3 viruses statistically replicated superiorly to G207, C101, and R7020 (Figure 40C).

In terms of cytopathic effects (40B,D) all viruses except R7020 and G207 produced significant cytopathic effects when low MOI was used, and all viruses produced cytopathic effects at high MOI. This cell line this was categorized as *sensitive*

Conclusion: S462 represents a human MPNST cell line that both highly supports the replication of all of our viral mutants under study and is uniformly sensitive to the virus as a cytotoxic agent. Viral mutants M002 and M032, and to a lesser degree, C134, replicated

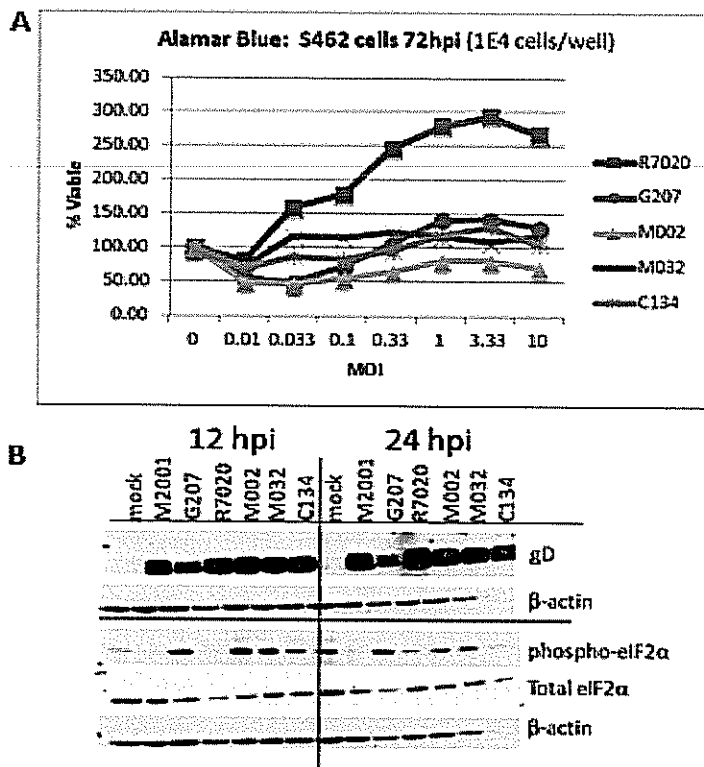


Figure 41: Composite of S462 human MPNST cell line *in vitro* studies. A. Alamar Blue cell viability assay comparing clinical grade oHSVs (and the non-clinical grade oHSV M002) antitumor effect at 72hpi. **B.** Immunostaining studies demonstrating synthesis of late gene product (glycoprotein D), phosphorylated and total eIF-2α along with β-actin protein loading controls at 12hpi (left panel) and 24hpi (right panel).

Summary and Analysis (S462 cont'd.)

In the Alamar Blue assay of cell viability (Figure 41A), R7020 was particularly ineffective against S462 when compared to M2001. M002 performed relatively well, with the other viruses performing at an intermediate level. Of note, some viruses appeared to perform better at lower MOI; this is an artifact of performing the analysis at 72 hours, since higher MOIs produce an initial round of cell killing but allow regrowth of cells in a relatively low virus environment.

Western blot analysis of the mutant viruses on S462 (Figure 41B) demonstrate significant production of gD, a late gene product, with relative phosphorylation of eIF 2α, except in M2001, R7020; C134 as well shows these findings at 24 hpi. Interestingly, C134 shows modest activation at 12 hours, lessening at 24.

Conclusion This interesting pattern of phosphorylation of eIF 2α, combined with the replication findings above, warranted further study and thus this line was selected as one of the lines we will study in depth.

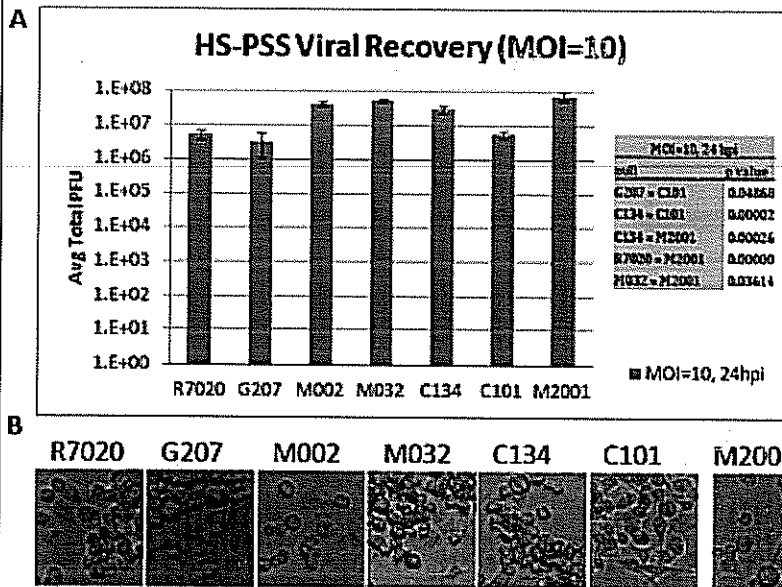


Figure 42: Composite of HS-PSS human MPNST cell line *in vitro* studies.
A. Single-step replication study using a high MOI (10 PFU/cell); viral recovery data (24hpi) shown with SD. **D.** Photomicrographs of cytopathic effect at high MOI for all oHSVs tested on HS-PSS cells (density of 1.5e5 cells/well; 100X magnification).

Summary and Analysis (HS-PSS)

Human MPNST cell line HS-PSS was in general favorable for viral replication and cytopathic effects as shown in the accompanying figures. All viruses in the single step replication assay replicated to at least 5×10^6 pfu, with M002, M032 and C134 all approaching wild-typ virus at $>5 \times 10^7$. These same 3 viruses statistically replicated superiorly to G207, C101, and R7020 (Figure 42A).

In terms of cytopathic effects (42B) all viruses produced significant cytopathic effects when high MOI was used. This cell line this was categorized as *uniformly sensitive*.

Conclusion: HS-PSS represents a human MPNST cell line that both highly supports the replication of all of our viral mutants under study and is uniformly sensitive to the virus as a cytotoxic agent. Viral mutants M002, M032, and C134 replicated particularly well in this line.

SubTask 1d. Correlate the data in the described experiments to identify oHSV-sensitive and oHSV-resistant MPNSTs and select 2 of each to study in the subsequent experiments.

Status: We have chosen representative HSV sensitive and resistant human and murine cell lines and are proceeding with studies involving these:.

Human HSV Sensitive cell lines: (S462 and NMS2PC)

Human HSV-Resistant cell lines (T265-luc and S26T-luc)

Murine HSV Sensitive cell lines (A18 and 231 Trig)

Murine HSV Resistant cell lines (A382 and A202)

Based upon our preliminary analysis summarized for Figures 5-42, we have numerous MPNST cell lines (murine and human) available that will provide us with interesting scientific questions. In the event that we experience unanticipated pitfalls with the above cell lines, we have other candidate cell lines that we can use as a substitute for future studies.

SubTask 1e. Correlate the expression of alternative molecules on oHSV-resistant MPNSTs with the potential to engineer oHSVs that can utilize these receptors to enter cells that resist HSV entry.

Status: Our data show that even in HSV-resistant lines, the virus is capable of entry and replication. We are currently examining (as described in Subtask 1b) if the abundance of the HSV-entry molecule (nectin 1) alters viral entry and replication. These studies will identify if viral cell entry is the principal impediment to efficient viral replication or whether other viral functions (gene expression, protein synthesis, DNA replication, virus assembly, or egress) occurring after viral entry are suppressed by cellular antiviral responses leading to lower viral replication. Preliminary results suggest that the overexpression of nectin 1 can produce a small but reproducible improvement in viral recovery in one cell line but has no effect in the other cell line tested thus far (Figure 43).

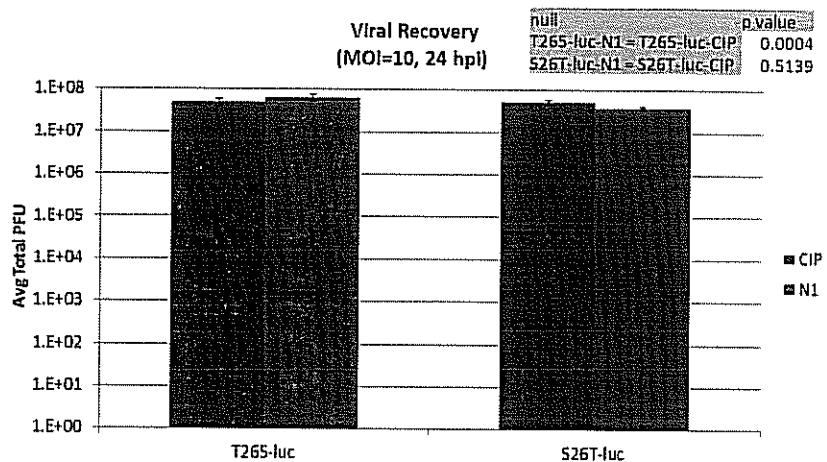


Figure 43 M2001 viral recovery following nectin-1 (N1) or mCherry (CIP) transduction in S26T-luc and T265-luc. There is a statistically significant difference in M2001 replication in the T265-luc nectin-1 overexpressing cell line. The virus recovery difference is small (state the number) and below the threshold (1/2 log) usually seen for biologically significant replication differences in in vitro replication assays. Nectin 1 overexpression did not result in a significant difference in viral recovery in the S26T cell line.

Task 2: Establish the most effective means of enhancing virus replication by modifying a HSV-resistant phenotype.

SubTask 2a. In Aim 2, we will test two different engineering solutions to enhance the expression of HSV "late genes" in both oHSV-sensitive and -resistant MPNST cell lines. We will use a combination of classical virology methods (plaque-titering at 24hr-intervals boost infection; single-step & multi-step replication assays) and FACS monitoring the extent and time course of oHSV infection based on expression of eGFP and other fluorescent markers by FACS assays

Status: These studies are commencing. We are focusing on the prototypical resistant and sensitive cell lines chosen for future studies. We are also investigating if the abundance of HSV entry receptor expression alters infection and spread in these cell lines (**Figure 44**).

SubTask 2b. Determine the ability of HSV-mediated expression of constitutively activated-MAP kinase kinase (MEK) will result in an increase in HSV late gene expression, higher HSV particle production and cytotoxicity.

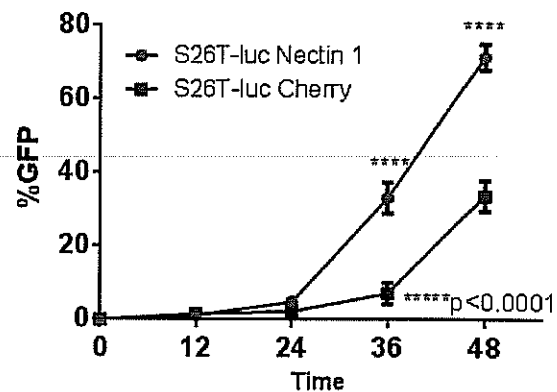
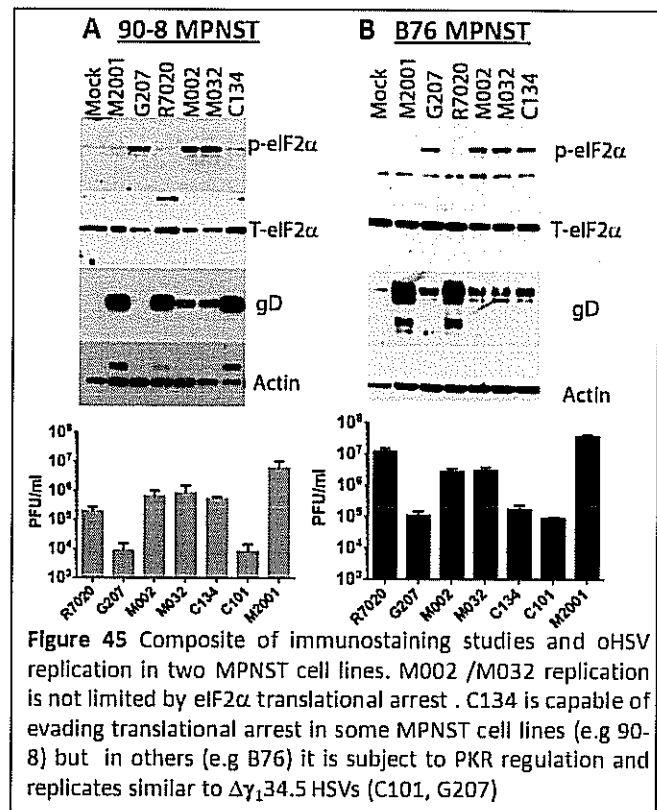


Figure 44: Summary of M2001 GFP Expression in S26T-Luc and in Nectin-1 overexpressing S26T-luc cells. These preliminary studies suggest that at low MOI the overexpression of nectin-1 may facilitate HSV spread.

Status: We have discovered two new and unexpected findings that will be pursued in follow up studies. The first novel discovery is that both of the IL-12 expressing $\Delta\gamma_134.5$ viruses, M002 and M032, are capable of late viral protein synthesis that surpasses that of other $\Delta\gamma_134.5$ viruses tested (C101 and G207) and can replicate as well as wild-type HSV in the MPNST tumor cells tested. However, unlike wild-type HSV or the two recombinants, R7020 and C134, the IL-12 expressing $\Delta\gamma_134.5$ viruses do not contain PKR-evasion genes. This is shown in **Figure 45 Panel A**, that M002 and M032 are unable to block PKR-mediated phosphorylation of eIF-2 α . (Figure 45 Panel A p-eIF2 α immunostaining panel) This suggests that in the MPNST tumor cells the M002 and M032 oHSVs encode an alternative mechanism to allow late viral protein synthesis that differs from that of the C134 and R7020. There are two possible explanations: (i) either the expression of IL-12 enhances virus translation in the infected MPNST cell lines or (ii) that the M002 and M032 recombinants contain secondary mutations that enhance viral protein translation independent of eIF-2 regulation of translation initiation. Efforts are currently underway to identify genetic differences between the M002/M032 recombinants and the parent $\Delta\gamma_134.5$ recombinant used to construct the IL-12 expressing viruses.



The second novel finding is that certain MPNST cell lines restrict C134 late viral protein synthesis and replication. Preliminary studies show that in most of the MPNST cell lines, C134 is capable of PKR-evasion and replicates similar to the $\gamma_134.5$ containing viruses (R7020 and M2001). This is consistent with our prior studies in glioma tumors (Shah 2007). However, in select MPNST cell lines, C134 is unable to evade PKR-mediated translational arrest and its replication is restricted similarly to that seen with $\Delta\gamma_134.5$ virus (G207 and C101). This divergent late protein synthesis phenotype is summarized using 2 of the MPNST cell lines shown in **Figure 45**. In the Human MPNST 90-8 cell line, C134 is capable of evading translational arrest based upon decrease in eIF-2 α phosphorylation and increase in glycoprotein D expression in the C134 infected cells. In this cell line the $\Delta\gamma_134.5$ cell lines, G207, M002, and M032 induce phosphorylation of eIF-2 α and produce less glycoprotein D (a late HSV gene) than the recombinants

expressing PKR-evasion genes (M2001, R7020, and C134). This ability to synthesize late viral proteins also correlates with improved viral replication for the C134 recombinant. In contrast to the 90-8 cell line, the mouse B76 MPNST cell line activated eIF-2 α after infection by $\Delta\gamma_134.5$ oHSVs and chimeric C134, as indicated by its phosphorylation. Consonant with this, the synthesis of late gene product gD was downregulated in the $\Delta\gamma_134.5$ and C134 viruses and the recovery of infectious C134 virus assayed by plaque-forming ability was decreased to levels equivalent to that seen with the 3 $\Delta\gamma_134.5$ oHSVs. These cell lines will provide valuable tools for further characterization of the mechanism by which C134 and specifically the IRS1 gene targets PKR and the translational machinery. This work may in turn allow improved efficacy of C134 when ultimately tested in clinical trials.

SubTask 2c. Determine the ability of HSV-mediated expression of a human cytomegalovirus gene, IRS-1, that promotes late gene expression in CMV, to increase oHSV late gene expression, higher HSV particle production and MPNST cytotoxicity.

Status: These studies are near completion and as shown in Figures 5-42, We have tested if HCMV IRS1 expression improves $\Delta\gamma_134.5$ replication. The results show that in numerous MPNST cell lines (B86, 231-Trig, A18, T265-luc, 90-8, YST-1, and S462), C134 ($\Delta\gamma_134.5$, IRS1) replicates better than $\Delta\gamma_134.5$ virus or the cell line supports efficient late viral protein synthesis for all viruses. In many of these cell lines, the improved C134 replication correlates with improved late viral gene expression (e.g. gD expression) and PKR evasion (relative absence of p-eIF2 α) when compared to $\Delta\gamma_134.5$ virus. In contrast, in other cell lines (A387, A202, B91, B96, B97, B109, A292, A390, A391, B76, and S26T-luc) IRS1 expression does not benefit the virus leading to similar replication as a $\Delta\gamma_134.5$ virus.

SubTask 2d. Examine the impact of p38MAPK activation in MPNST tumors to have a positive or negative impact on the ability of these engineered oHSVs to display greater replication and oncolysis.

Status: See explanation in SubTask 2e. Based on our observations, these two tasks do not seem to be critical to the development of more effective oHSVs for treatment of MPNSTs and consequently will be deleted.

SubTask 2e. Correlate and compare the data sets obtained from the studies in oHSV-sensitive and -resistant MPNSTs using the caMEK viruses (R2660, R2636) and the IRS-1 viruses (C134, C154).

Status: One of the possible solutions to the issue of poor replication is the level of Mitogen-Activated Protein Kinase activation (phosphorylation), which is perceived as important for optimum late virus gene expression and optimum downregulation of PKR activation preventing eIF2 α activation which would shut-off protein synthesis. A strategy has been to consider exogenous expression of an upstream mediator, mitogen-activated protein kinase kinase (MEK 1/2). oHSVs expressing constitutively activated MEK (ca-MEK) or dominant negative MEK are available to study this. However, our studies show that most MPNSTs already have high levels of activated p38 MAPK and phosphorylated Erk1/Erk2, immediate downstream targets of MEK. Thus, a strategy of trying to coordinately upregulate MEK activation to achieve greater virus replication is likely not to be a worthwhile study. Based on our observations of elevated MEK with several MPNSTs, we are no longer considering that ca-MEK oHSV would be an effective strategy, unless we discover MPNSTs that would not already have phosphorylation of MEK.

SubTask 2f. Select the most appropriate (set of) oHSV virus(es) to advance to preclinical *in vivo* studies with human and mouse MPNSTs.

Status: Several studies remain to be completed before the candidate oHSVs can be appropriately selected. Preliminarily, we believe both M032 and C134 will prove to be the most attractive candidates. Once we have completed our evaluation of our panels of MPNSTs with regard to virus infectivity, virus replication and capacity of our experimental and clinical candidate HSVs to produce an oncolytic effect *in*

vitro, we will be able to select the most appropriate MPNST lines to use in our heterotopic and orthotopic models to evaluate the *in vivo* effects. In essence, the xenogeneic models with human tumors in immunocompromised mice will permit evaluation of the 3 clinical candidate viruses. In our syngeneic mouse models in immunocompetent mice, we will also be able to assess anti-tumor efficacy as it is affected by the host immune response. These studies are the basis of Milestone 4.

Task 3: Validate the ability of selected oHSV to produce an oncolytic anti-MPNST effect in established tumors in mouse models and quantify the capacity of a low dose of radiation to enhance this anti-tumor effect.

Status: None of the animal studies have been initiated since selection of the appropriate prototypic model tumors for these studies is still pending. We need a much more complete picture that was to be provided by the first 3 tasks to make an informed decision regarding the design of these animal studies. With a significant portion of the data in hand, we expect that we will be able to begin the studies associated with the Milestone during the second year of funding.

SubTask 3a. Tumor cells growing *in vivo* often display significant biologic differences from those growing *in vitro*. The first subtask will be to establish a baseline of the ability of oHSVs to infect and kill human or mouse MPNST cell lines transplanted into appropriate host mouse strains. The ability of generic $\Delta\gamma_{134.5}$ HSV (G207, NV1070) to produce an antitumor effect as observed in Task 1c *in vitro* will be determined by direct injection of bioluminescence-enabled human or mouse MPNSTs placed in an orthotopic location (sciatic nerve). Both oHSV-sensitive and oHSV-resistant MPNSTs will be compared.

Status: We have not initiated these experiments

SubTask 3b. Compare the abilities of selected oHSVs (e.g., M002, C134, R2660, etc) from previous studies to produce an enhanced anti-MPNST effect compared to that of the generic viruses. Oncolysis of orthotopically-placed oHSV-sensitive and oHSV-resistant MPNSTs will be compared.

Status: We have not initiated these experiments.

SubTask 3c. Determine whether or not a single low dose of radiation (2-5Gy) delivered to the tumor within 24 hrs of injection of selected oHSVs enhances the replication and spread of the virus yielding an enhanced anti-MPNST effect. Irradiation has a more pronounced and sometimes paradoxical effect *in vivo* than it does *in vitro* and thus, irradiation effects will not be explored *in vitro*.

Status: We have not initiated these experiments.

SubTask 3d. Compare and correlate the findings from these sub-tasks to select the most likely combination of oHSV and adjunctive therapy that will be most effective oncolytic, anti-MPNST modality for human or mouse MPNSTs transplanted orthotopically and test this combination in the P₀-GGF β 3 x Elux mouse against MPNST tumors that arise sporadically and spontaneously.

Status: We have not obtained sufficient data to be able to complete this subtask.

SubTask 3e. Review the entire data set to design studies that will be able to validate the selected oHSV with or without adjunctive therapy that can be advanced to a Phase I/II clinical trial to test the safety, identify unanticipated toxicities and establish preliminary evidence of efficacy in patients with MPNST.

Status: We have not obtained sufficient data to be able to complete this subtask.