Oncolytic HSV-mediated Regulation of the Host Hypoxia Response

Undergraduate Honors Thesis

Presented in fulfillment of the requirements for a Bachelor of Science with Research Distinction in the School Health and Rehabilitation Sciences at The Ohio State University

Brian Hurwitz

Biomedical Science Major – Class of 2014
College of Health and Rehabilitation Sciences
The Ohio State University

Thesis Committee

Dr. Balveen Kaur PhD, Advisor
Dr. Deliang Guo PhD
Dr. Li Zuo PhD MS

Abstract

Glioblastoma (GBM) is the most common and deadly primary brain tumor, accounting for over 10,000 new cancer diagnoses in the United States each year. The poor prognosis for GBM patients necessitates novel biological treatments. One such approach is the use of oncolytic herpes simplex virus 1 (oHSV). Like many novel treatments oHSV therapy causes off target effects that are not yet well understood. Our lab has demonstrated that oHSV treatment increases the vascularity of brain tumors. The goal of this study is to determine the mechanism by which oHSV treatment increases the vascularity of brain tumors. Preliminary data suggested the hypoxia inducible factor-1 alpha (HIF1α) may be activated in cells infected with oHSV, even in normal oxygen conditions. HIF1α is a transcription factor, which activates a variety of genes in response to a lack of oxygen. We hypothesized that HIF1α activation may be responsible for the increased vascularity of oHSV treated brain tumors. A screen of targetscan.org for the predicted target genes of herpes simplex virus 1 (HSV-1) revealed multiple miRNAs predicted to target a protein called, factor inhibiting HIF1α (FIH). This protein functionally inhibits HIF1α activation by preventing the binding of HIF1α to DNA. We believed that FIH would be negatively regulated in GBM cells infected with oHSV, thus allowing HIF1α activation. In this study, we demonstrate that HSV-1 expresses two miRNA molecules, which target and down regulate FIH. Transfection of miRNA inhibitors (antagomirs) was able to successfully abrogate the virus' ability to down regulate FIH as demonstrated by quantitative PCR and western blot. Moreover, transfection of HSV-1 miRNA

mimics in the absence of virus was able to down regulate FIH protein levels (western blot) and activate the expression of a variety of HIF1α driven genes, including VEGF and IL-8 (quantitative PCR). However, after analyzing HIF1α promoter activity in a variety of cell lines, we determined that HIF1α was not activated during oHSV infection. The expression of VEGF and IL-8 was likely due to the activation of another transcription factor.

Acknowledgements

I would like to sincerely thank Dr. Balveen Kaur for the opportunity to work in her lab as an undergraduate. Her exceptional guidance has been a vital reason for my choice to pursue a career in biomedical research. I have had opportunities to be involved in research far surpassing what I expected to be available to an undergraduate. This experience has helped me to grow as a student and a scientist. I am grateful for the opportunity to have spent time in this lab.

I would also like to thank Dr. Ji Young Yoo for her mentorship over the past three years. Dr. Yoo has spent a tremendous amount of time and energy to train me in the skills needed to succeed in this lab. Dr. Yoo also challenged me to think through scientific problems and create my own hypotheses and plans.

I would additionally like to thank Lori Martensen and Stephen Mousetes for their time advising me throughout college. Also, thank you to Dr. Deliang Guo and Dr. Li Zuo for their time and effort on my thesis committee.

Vita

Brian Hurwitz was born in Indianapolis, Indiana. He spent the majority of his childhood in Cincinnati, Ohio and graduated in 2010 from St. Xavier High School. He enrolled that same year at The Ohio State University in the undergraduate major in Biomedical Science. He will graduate in May of 2014 and continue his education in the Tri-Institutional MD/PhD program comprised of Weill Cornell Medical College, Rockefeller University, and Memorial Sloan Kettering Cancer Center.

Field of Study

B.S. Biomedical Science in the School of Health and Rehabilitation Science

Table of Contents

List of Tables and Figures

Introduction

Background

Glioblastoma (GBM) is the most common and deadly primary brain tumor, accounting for about 10,000 new cancer diagnoses in the United States each year.[1] Although GBM is relatively rare compared to other cancers, these tumors are responsible for a disproportionately high amount of cancer deaths, and the majority of GBM patients will succumb to their disease within 12-15 months.[2] GBM tumors are especially difficult to treat due to their invasive nature and resistance to standard chemotherapies. The poor prognosis for GBM patients necessitates novel biological treatments. One such approach is the use of oncolytic herpes simplex virus 1 (oHSV).[3] These genetically altered herpes simplex 1 viruses (HSV-1) potently kill cancer cells but are unable to infect normal cells.[4] oHSV therapy offers a potentially revolutionary approach for treating highly refractory cancers such as GBM. Additionally, there are five clinical trials investigating the use of oHSV as a cancer therapy that are either actively recruiting or completed. (NCT02031965, NCT00028158, NCT01721018, NCT00931931, NCT01017185)

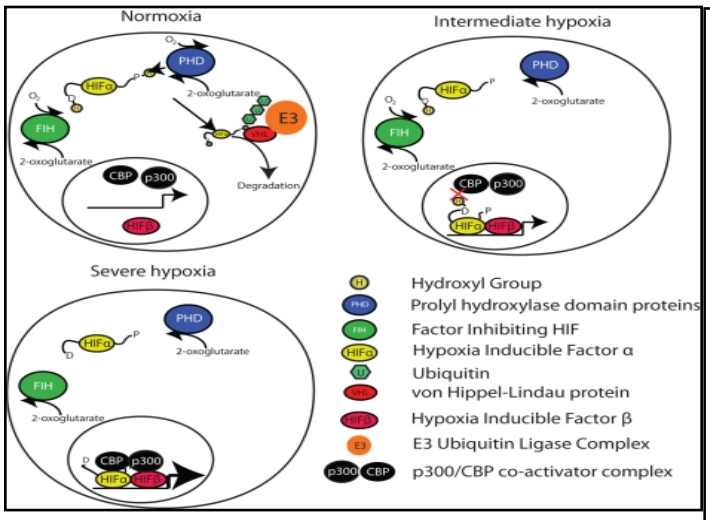
Our lab has demonstrated that oHSV treatment increases the vascularity in mouse models of glioma tumors following oHSV treatment.[5] These new blood vessels are able to sustain tumor growth and clear out infectious oHSV. We have also determined that the hypoxia inducible factor-1 alpha (HIF1α) is activated in cells infected with oHSV. HIF1α is a transcription factor that activates a variety of genes in response to a lack of oxygen.[6] Interestingly, we found that HIF1α

transcriptional activity is activated in oHSV infected cell lines even under normal oxygen conditions. A fundamental consequence of HIF1α activation is the formation of new blood vessels.[7] We believe that HIF1α activation may be responsible for the increased vascularity of oHSV treated brain tumors.

To investigate the mechanism of HIF1α activation, I explored all microRNA (miRNA) molecules expressed by HSV-1. Because our oncolytic virus is derived from HSV-1, it expresses the same miRNA profile as this virus. Using a miRNA target prediction program (targetscan.org), I screened all 26 miRNA sequences expressed by HSV-1 for potential target genes. Interestingly, I found that HSV-1 encodes for two different miRNAs, HSV-miRNA-6 and HSV-miRNA-16, each predicted to target the protein, factor inhibiting HIF1α (FIH). This protein functionally inhibits HIF1α activation by preventing the binding of HIF1α to the CBP/p300 coactivating complex, a protein complex necessary for HIF1α to bind to DNA.[8] Under normal oxygen conditions, FIH works in conjunction with a family of proteins, the prolyl hydroxylase domain (PHD) proteins to regulate HIF1α. The PHDs and FIH both add hydroxyl groups to HIF1α to target the protein for degradation and prevent DNA binding and (Fig. 1).[9]

Notably, it has previously been demonstrated that FIH is down regulated by human miRNA-31 in head & neck carcinoma, and that this activates the HIF1α mediated hypoxia response in these tumors.[10] It is possible that HSV-1 has evolved a method to mimic this human miRNA regulation to activate HIF1α, which could create a cellular environment that promotes viral replication. For the

remainder of this proposal, when I discuss HSV-1 miRNA, I am referring to the two miRNA molecules expressed by HSV-1 that were predicted to target FIH.



Mechanism of HIF1α activity. Under normal oxygen conditions, FIH and PHD hydroxylate HIF1α. While hydroxylation by PHD targets HIF1α for proteasomal degradation, hydroxylation by FIH prevents binding of HIF1α to CBP/p300 coactivation complex. Without binding to this coactivator, HIF1α cannot bind to its DNA promoter region. Under moderate hypoxia, FIH activity continues to prevent HIF1α activity. Upon severe hypoxia (1% O_2), FIH activity and PHD are both deactivated, allowing HIF1α to bind to DNA and activate a variety of genes.[9]

Significance

By determining the cause of HIF1α activation we will expand the current understanding of viral regulation of the host genome. Although there are examples of HSV-1 encoded miRNAs that regulate viral gene expression, to our knowledge no lab has demonstrated that any of these miRNAs are utilized to regulate host gene expression. Discovering that HSV-1 expresses miRNAs in order to regulate a host transcription factor would be a novel and significant discovery. Additionally, this discovery will lay down the rationale to create a next generation oHSV that lacks the ability to control the host hypoxia response and does not produce the negative effect of increased angiogenesis. Eventually, we will potentially develop a virus with mutations in the regions encoding for the miRNA molecules that target FIH. If these mutations do not compromise the

replication of the virus, this virus could be an improvement on current oHSV therapy.

Objectives

I hypothesize that herpes simplex virus 1 encodes for miRNAs that target FIH mRNA to down-regulate its translation, thus stimulating HIF1α activation. FIH is known to be regulated by human miRNA-31, it is possible that HSV-1 has evolved a similar mechanism to target FIH and activate HIF1α.[10] Additionally, we have already established that HIF1α is activated in oHSV-infected cells in normoxia, and we have found two HSV-1 miRNAs predicted to target FIH (prediction by targetscan.org).

Specific Aim 1: Demonstrate that HSV-1 encoded miRNAs are capable of targeting FIH mRNA to down regulate FIH protein levels.

Specific Aim 2: Demonstrate that expression of the HSV-1 encoded miRNA is necessary to induce the HIF1α mediated hypoxia response.

Materials and Methods

Cells and Viruses

LN229 and UT51T3 glioma cells have been cultured in our lab and are maintained in Dulbecco's Modiefied Eagle Media (DMEM) supplemented with 10% fetal bovine serum, 100ug/mL penicillin, and 100ug/mL of streptomycin. To passage cells, monolayers are treated with trypsin-EDTA to dissociate cells, and one tenth of the cells are transferred to another plate.

Viruses 34.5ENVE and rHSVQ were developed previously.[11] rHSVQ has green fluorescent protein inserted within the ICP6 locus (viral rubonucleotide reductase), which both imparts its oncolytic characteristic and also allows the viral replication to be tracked under a fluorescent microscope.[12] Additionally both copies of viral ICP34.5 have been deleted. In the virus, 34.5ENVE, the ICP34.5 gene, which dampens the cells innate immune response, has been reinserted under the Nestin promoter, which is active in glioma cells. Additionally, VSTAT120 has been inserted under a Nestin promoter as well. This protein is the extracellular fragment of brain angiogenesis inhibitor-1 (BAI1), which gives this virus its anti-angiogenic properties.[13]

Western Blots

SDS-PAGE was used to solubilize cell lysates, which were transferred to polyvinylidene difluoride (PVDF) membranes. These membranes were blocked and then incubated with antibodies targeting FIH and GAPDH (Cell Signaling

Technology, Waltham, MA). The immunoreactive bands were visualized using a using enhanced chemiluminescence (ECL) (GE Healthcare, Piscataway, NJ).

Quantitative Polymerase Chain Reaction

Total RNA was purified from cellular lysates using RNeasy mini kit (Qiagen). This RNA was then converted to cDNA using the Superscript First Strand Synthesis Kit (Invitrogen). cDNA was expanded using custom primers for FIH, VEGF, IL-8. And GAPDH and the double stranded DNA product was quantified using Sybr Green Master Mix (Life-Technologies). Rather than using custom primers and Sybr Green, HSV miRNA-H6 and miRNA-H16 were quantified using taqman qPCR primers.

Viral Quantification Assay

Infectious viral particles were quantified by viral plaque assay. Cells were infected with oHSV and 72 hours after infection whole cell lysates were collected. We released oHSV from cells by three freeze-thaw cycles, and we purified oHSV by centrifuging cell lysate-virus mixtures at 4000 xG for 20 minutes and collecting the supernatant. The supernatant was diluted to concentrations of $1/30^{th}$, $1/90^{th}$, $1/270^{th}$, $1/810^{th}$, $1/2430^{th}$, and $1/7290^{th}$ by serial dilution. Diluted virus was added to 10,000 cells of Vero African Green Monkey Kidney Cells in 96 well plates, and after 14 hours human IgG was added to neutralize infectious particles in the supernatant. 72 hours following infection, viral particles were quantified by counting the viral plaques present on the vero cells.

Luciferase Assays

Cell lysates were collected with Passive Lysis Buffer (Promega), cell debris were collected by centrifugation, and the supernatant containing luciferase protein was frozen. Luciferase levels were quantified using Luciferase Assay System (Promega). 20 uL of samples were prepared and 200 uL of luciferase substrate was added to each well. The luminescence of the luciferase was measured using Fluorstar Optima plate reader. Luminescence levels were normalized to protein levels that were measured using the Reducing Agent Compatible BCA Protein Assay Kit (Pierce).

Results

Down Regulation of FIH following HSV-1 Infection

To initially investigate the relation of oHSV to the host hypoxia response, we measured at the expression of Factor Inhibiting HIF1α (FIH) following viral infection. When cells were infected with increasing doses of two oncolytic herpes viruses, 34.5ENVE and rHSVQ, the FIH protein level within a cell decreased in a dose dependent manner. Although FIH protein levels were decreasing, HIF1α levels were unaffected (Fig. 1A).

We next sought to investigate if this effect was specific to HSV-1 or a more general cellular response to viral infection. We infected cells with a panel of wild-type viruses: HSV, Adenovirus (Ad), Vesicular Stomatitis Virus (VSV), and Newcastle Disease Virus (NDV). In the cells infected with wild-type HSV-1, FIH protein was almost undetectable in the cells. In contrast FIH was up regulated in cells infected with the other types of virus. This suggested that HSV-1, as well as our oHSV vectors, have potentially developed a method to specifically target human FIH for down regulation.

Figure 1

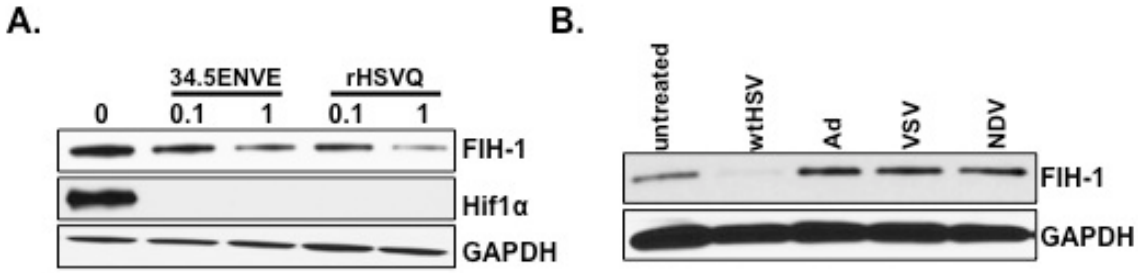


Figure 1: Down Regulation of FIH following HSV-1 infection. (A) U251T3 glioma cells were infected with a increasing multiplicities of infection (MOI) of either 34.5ENVE or rHSVQ. In both cases oHSV decreased FIH protein levels in a dose dependent manner. **(B)** Vero Cells were infected with four different virus strains: wild type HSV-1, Adenovirus, Vesicular Stomatitis Virus, or Newcastle Disease Virus. FIH was down regulated following only HSV-1 infection.

Down regulation of FIH is mediated by miRNA expressed by oHSV

We utilized the software, targetscan.org, to investigate the potential target genes of all miRNAs expressed by HSV-1. Because our oHSV vectors are derived from HSV-1, they would also express these miRNA molecules. We discovered 2 miRNAs, HSV miRNA-6 and HSV miRNA-16, that were predicted to bind to the 3'UTR of human FIH. We hypothesized that our oHSV vectors may be expressing enough of these two miRNAs to down regulate human FIH, thus activating the HIF1α hypoxia response.

We were able to confirm that these miRNA molecules were expressed by HSV-1 by using TaqMan Quantitative PCR (QPCR) on infected cells. Indeed both wild-type HSV and the oHSV, 34.5ENVE, both expressed high levels of both miRNA, as compared to two human miRNA controls (Fig. 2A). Consistent with our hypothesis, when we transfected miRNA mimics of HSV miRNA-6 and HSV miRNA-16, FIH levels were strongly down regulated as compared to the non-targeting control (Fig. 2B – FIH top row, GAPDH bottom row). These results supported our hypothesis that our oHSV vector expresses miRNA capable of specifically targeting and down-regulating FIH.

We were curious to investigate if viral miRNA transfection had a measurable effect on FIH mRNA levels. We utilized QPCR to quantify FIH mRNA levels relative to GAPDH mRNA. Although the miRNA had a strong effect on FIH protein levels, FIH mRNA was unaffected (Fig. 2C)

Figure 2

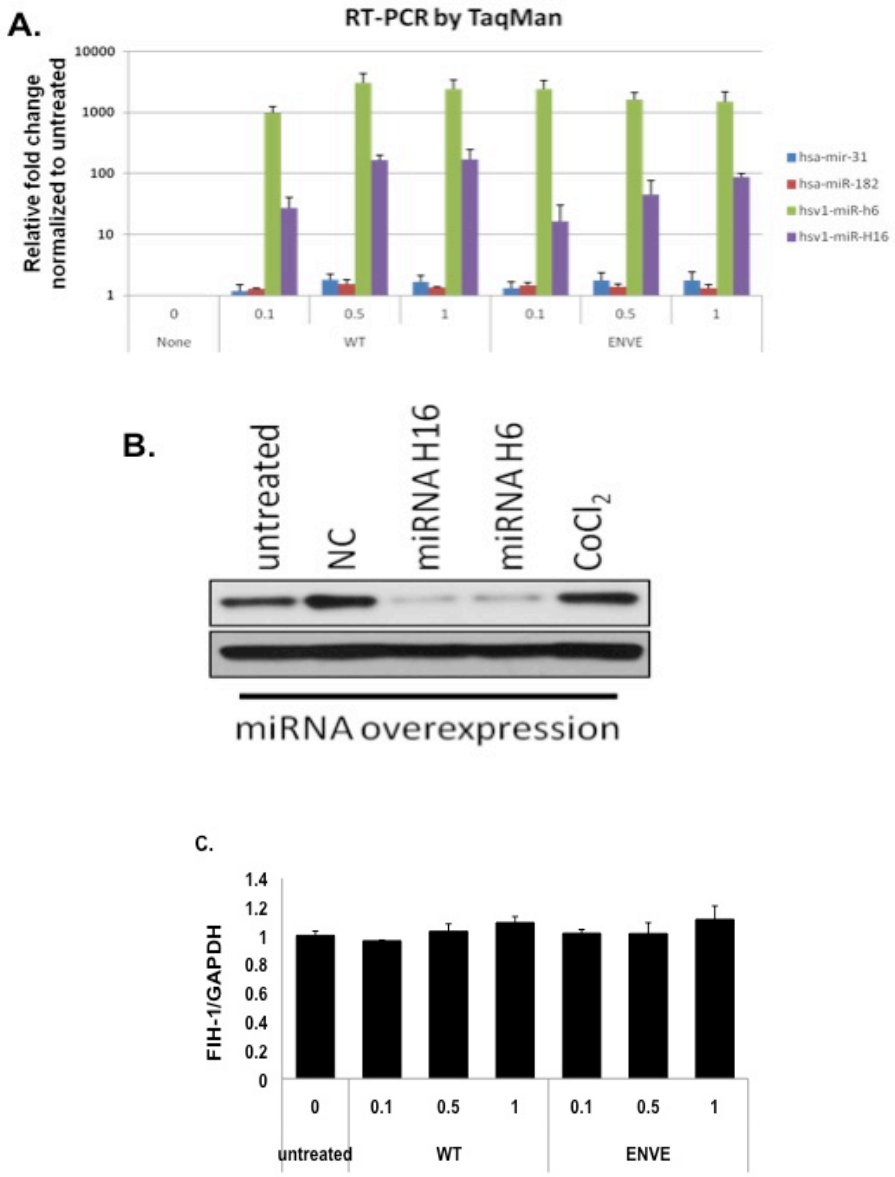


Figure 2: Down regulation of FIH is mediated by miRNA expressed by oHSV. (A) Vero cells were infected with increasing MOI of either wild type HSV-1 or 34.5ENVE. In both cases HSV miRNA-H6 and HSV miRNA-H16 were strongly induced. **(B)** U251T3 cells were transfected with non-targeting control RNA (NC), HSV miRNA-H6, HSV miRNA-H16, or $CoCl_2$ (positive control for HIF1α activation). FIH protein (top row)

decreased following HSV miRNA transfection relative to GAPDH protein (bottom row). **(C)** Infecting U251T3 cells with wild type HSV-1 or 34.5ENVE did not affect FIH mRNA levels.

FIH down-regulation is primarily dependent upon HSV miRNA-H16

From this point forward my work focused on the effect of HSV miRNA-H16. I sought to determine if viral miRNA was the primary mechanism of FIH down-regulation inside cells infected with oHSV. Although transfection with HSV miRNA-H16 was capable of down regulating FIH, transfection of miRNA mimics does not perfectly replicate the situation within oHSV infection. To confirm that miRNA was the primary cause of FIH down regulation, we pretreated cells with antagomirs prior to oHSV infection. Antagomirs selectively block the activity of miRNA molecules with specific sequences.

As expected, levels of FIH were reduced in infected cells treated with either lipofectamine (lipo) or non-targeting RNA (NC). However cells pretreated with an antagomir specific to HSV miRNA-H16 displayed higher levels of FIH apparent by western blot (Fig. 3). This data suggests that FIH is specifically targeted by viral miRNA and that HSV miRNA-H16 is the primary mechanism by which oHSV targets FIH.

Figure 3

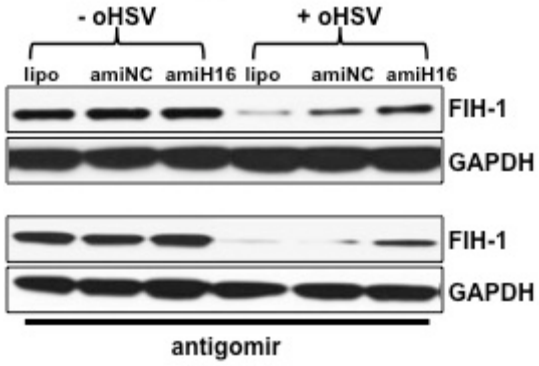


Figure 3: FIH down-regulation is primarily dependent upon HSV miRNA-H16. Prior to viral treatment, U251T3 cells were transfected with non-targeting RNA (NC), antagomir for HSV miRNA-H16 (amiH16), or simply treated with lipofectamine transfection reagent (lipo) These cells were either left uninfected or transfected with 0.1 MOI of oHSV.

Inhibition of HSV miRNA-H16 decreases viral replication

We were curious to determine if HSV miRNA-H16 was important to the viral replication cycle of oHSV. We continued to use antagomirs to investigate the importance of this miRNA. LN229 glioma cells were transfected with an antagomir specific to miRNA-H16 or non-targeting control RNA (NC). On the next day these cells were infected with GFP expressing oHSV. The GFP allowed us to visualize viral infection.

In these cells, inhibiting HSV miRNA-H16 dramatically reduced the replication of 34.5ENVE. When we visualized the GFP expressed in cells infected with virus, the cells pretreated with antagomir displayed much lower GFP fluorescence (Fig. 4A). Consistent with this observation were decreased

viral particles quantified by viral plaque assay. Our oHSV virus replicated to a significantly lower extent in cells pretreated with antagomir for HSV miRNA-H16 (Fig. 4B)

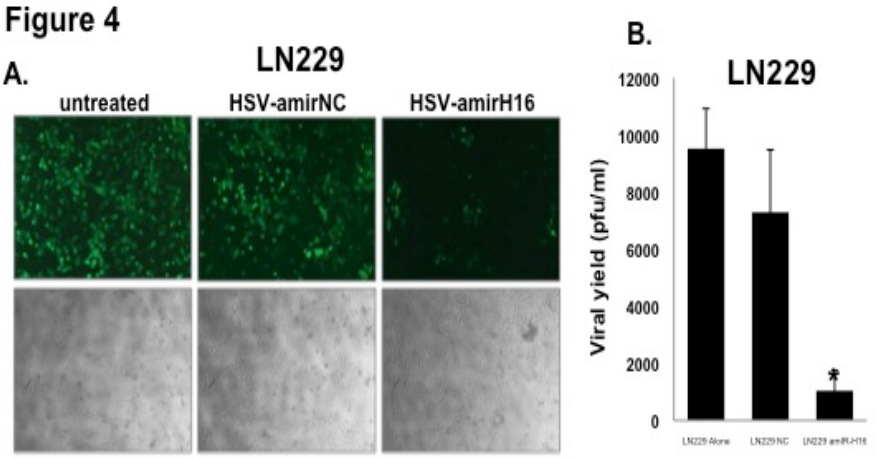


Figure 4: Inhibition of HSV miRNA-H16 decreases viral replication. LN229 glioma cells were pretreated with either non-targeting RNA (HSV-amirNC), or an antagomir for HSV miRNA-H16 (HSV-amirH16). These cells were then infected with 0.01 MOI of 34.5ENVE. (A) 72 hours after infection we visualized GFP expressed by the oHSV, which revealed that HSV-amirH16treatment decreased viral GFP transduction. (B) We used viral plaque assay to confirm that HSV-amirH16 transfection significantly decreased viral replication.

HSV miRNA-H16 increases the expression of HIF1α target genes

To investigate the effect of viral miRNA on the expression of HIF1α target genes, we utilized QPCR to measure the mRNA of VEGF and IL-8. We had hypothesized that the down-regulation of FIH may allow for increased activation of the HIF1α transcription factor. VEGF and IL-8 are two genes that are positively regulated by HIF1α activation.[14]

Consistent with our hypothesis, VEGF expression was up regulated about 10-fold fold compared to cells treated with non-targeting miRNA (NC – Fig. 5a). Additionally, mRNA levels of the cytokine IL-8 was increased about 25-fold following transfection with HSV miRNA-H16 mimics.

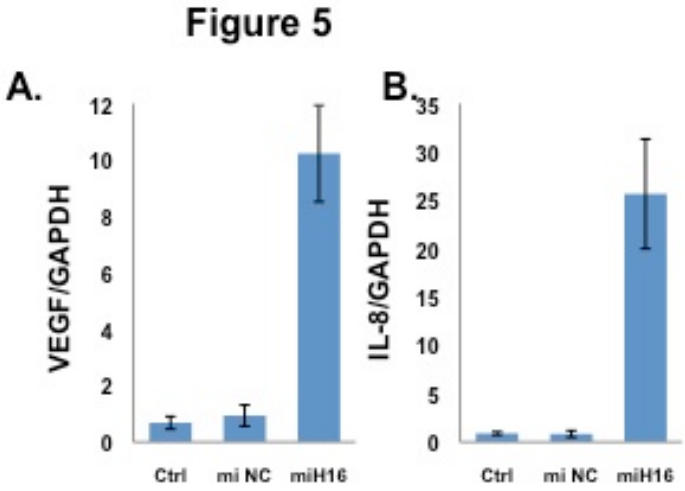


Figure 5: HSV miRNA-H16 increases the expression of two HIF1α target genes. Cells were transfected with non-targeting RNA (NC) or mimic for HSV miRNA-H16 (miH16). **(A-B)** We then performed PCR on VEGF and IL-8. This revealed that HSV miRNA-H16 increased expression of both HIF1α target genes.

HIF1α is not activated following oHSV infection

The final aim of this project was to find out if oHSV activation indeed activated the host hypoxia response mediated by HIF1α. To investigate the potential HIF1α activation we created two luciferase vectors driven by the minimal CMV promoter (mCMV). The DNA sequence for the hypoxia response element (HRE) was inserted to the control luciferase vector to measure HIF1α transcriptional activity.

Cells were transfected with either the control luciferase vector or the HRE-luciferase vector, and then cells were infected with increasing MOI of oHSV or left uninfected. In LN229 cells infected with 0.01 MOI of oHSV, HRE activity was slightly elevated relative to uninfected cells (Fig. 6 A-B). This effect was not replicated in U251T3 cells (Fig. 6 C-D). Due to the higher basal luciferase activity in the HRE vector, the trend was difficult to measure in both cell lines. Additionally, oHSV infection increased luciferase expression in the control vector. These complications made us uncertain that oHSV actually induces HIF1α activity. Additionally even when oHSV increased HRE expression relative to the amount that it increased luciferase in the control vector, the induction was very slight. This data makes us believe that oHSV does not regulate HIF1α activation and that HSV miRNA-H16 likely induces an inflammatory phenotype through another pathway.

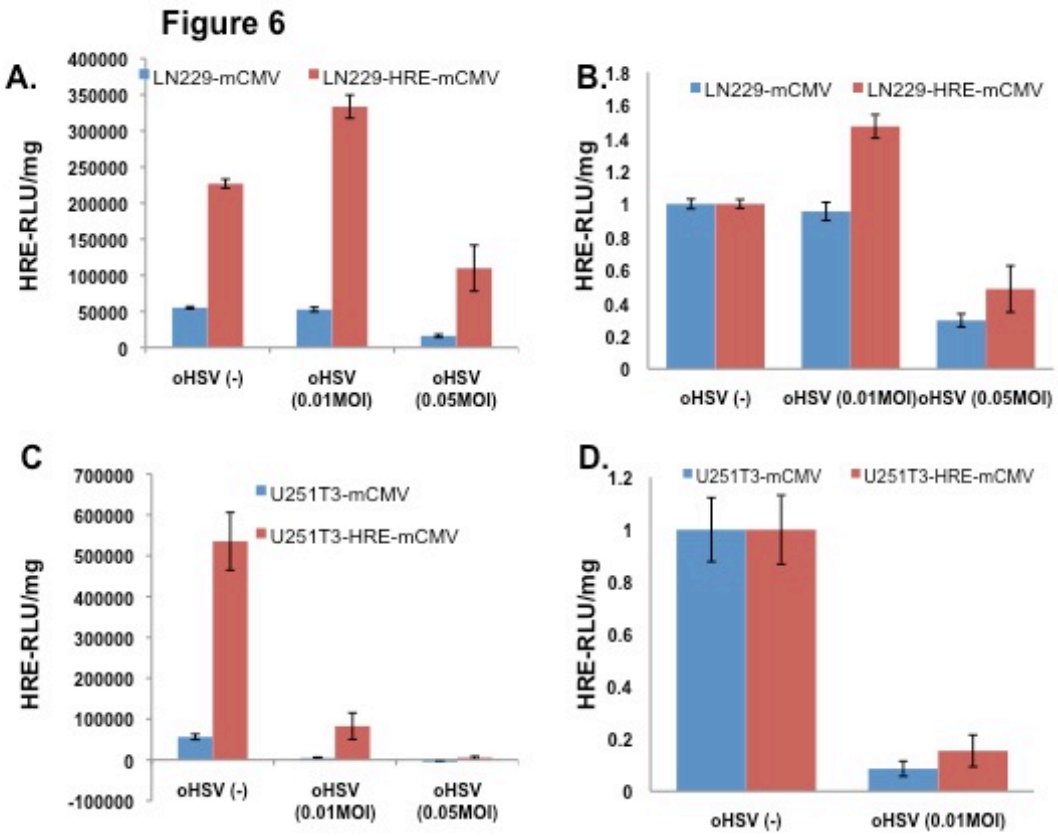


Figure 6: HIF1α is not activated following oHSV infection. (A-B) LN229 cells were transfected with either an HRE luciferase reporter vector or a control luciferase vector. Cells were then infected with 0.01 MOI or 0.1 MOI of 34.5ENVE and compared to uninfected cells. The HRE luciferase vector expressed higher basal levels of luciferase, but was induced to even higher expression levels following treatment with 0.01 MOI of oHSV (A). When luciferase levels of each vector were normalized to their untreated control we found that 0.01 MOI of oHSV induced luciferase expression by a significant but small amount (B). **(C-D)** We then repeated this test in U251T3 cells but saw no effect.

Conclusions and Discussion

We sought to investigate the interplay between miRNA expressed by oHSV and genes involved with host hypoxia response. The novel discovery of this project is that HSV-1 expresses miRNA capable of directly targeting and inhibiting the expression of FIH. Importantly, the down-regulation of FIH was primarily dependent upon viral miRNA as opposed to another mechanism of protein degradation. To our knowledge, this is the first study to demonstrate that HSV-1 expresses a miRNA that is utilized to specifically down-regulate a host gene. Interestingly, HSV miRNA-H16 decreased FIH protein levels without affecting FIH mRNA levels. This is consistent with a mechanism of translational repression due to miRNA.[15]

It was interesting to find that expression of miRNA-H16 was vital to HSV replication within LN229 cells. When cells were pretreated with miRNA-H16 antagomir prior to infection, the virus displayed much lower expression. This suggests that down-regulation of FIH is important for the viral replication cycle to be completed efficiently. It is important to note that this experiment does not necessarily suggest that down regulation of FIH is the reason that viral replication was inhibited. It is possible that HSV miRNA-H16 targets multiple genes simultaneously, which means that this miRNA could be important to viral miRNA for multiple reasons.

After this conclusion we finally sought to see if oHSV caused activation of the host hypoxia response. Although we found increased expression of HIF1α target genes VEGF and IL-8 following miRNA-H16 transfection, we did not

observe consistent HIF1α activation following oHSV infection. At this point we conclude that oHSV is not likely to activate the hypoxia response. The consistency of these results was likely affected by the use of the mCMV promoter. It is possible that the oHSV replication cycle increased luciferase activity in the control vector. In the future we will try to find a promoter compatible with this luciferase vector that is unaffected by viral infection. We checked promoter activity with a few other promoters (HSV-TK, HSP68, and SV40), but unfortunately all of these promoters were activated by oHSV infection as well (data not shown).

It is interesting to speculate how oHSV has developed a mechanism to down-regulate FIH. It is possible that HSV-1 picked up its miRNA sequences from related miRNA genes in the human genome. It would also be interesting to understand the evolutionary pressure on HSV-1 to down-regulate FIH. It is possible that HIF1α target genes such as VEGF are beneficial to viral infection. FIH may also associate with other proteins within the cell that interact with HSV-1, which would explain the lack of consistency with the HIF1α activation assay.

Moving forward we plan to investigate the effect of HIF1α on viral replication. We can develop cells that stably express HIF1α shRNA and see if these cells display lower oHSV replication. Additionally we plan to further elucidate the importance of FIH down-regulation on viral replication. Finally, we plan to investigate the relevance of miRNA-H16 *in vivo*. It would be important to see how the role of this miRNA changes inside oHSV infection within a brain tumor.

References

1) Cloughesy, T. F., W. K. Cavenee, and P. S. Mischel. "Glioblastoma: From Molecular Pathology to Targeted Treatment." *Annual review of pathology* 9 (2014): 1-25. Print.
2) Quick, A., et al. "Current Therapeutic Paradigms in Glioblastoma." *Reviews on recent clinical trials* 5.1 (2010): 14-27. Print.
3) Grandi, P., et al. "Design and Application of Oncolytic HSV Vectors for Glioblastoma Therapy." *Expert review of neurotherapeutics* 9.4 (2009): 505-17. Print.
4) Kaur, B., E. A. Chiocca, and T. P. Cripe. "Oncolytic HSV-1 Virotherapy: Clinical Experience and Opportunities for Progress." *Current Pharmaceutical Biotechnology* 13.9 (2012): 1842-51. Print.
5) Kurozumi, K., et al. "Effect of Tumor Microenvironment Modulation on the Efficacy of Oncolytic Virus Therapy." *Journal of the National Cancer Institute* 99.23 (2007): 1768-81. Print.
6) Semenza, G. L. "HIF-1 Mediates Metabolic Responses to Intratumoral Hypoxia and Oncogenic Mutations." *The Journal of clinical investigation* 123.9 (2013): 3664-71. Print.
7) Ben Lassoued, A., et al. "Hypoxia-Inducible Factor-1alpha as Prognostic Marker." *Expert opinion on medical diagnostics* 7.1 (2013): 53-70. Print.

8) Coleman, M. L., and P. J. Ratcliffe. "Signalling Cross Talk of the HIF System: Involvement of the FIH Protein." *Current pharmaceutical design* 15.33 (2009): 3904-7. Print.

9) Virtue, S., and A. Vidal-Puig. "Nothing Iffy about HIF in the Hypothalamus." *PLoS biology* 9.7 (2011): e1001116. Print.

10) Chen, T., et al. "MicroRNA-31 Contributes to Colorectal Cancer Development by Targeting Factor Inhibiting HIF-1alpha (FIH-1)." *Cancer biology & therapy* 15.5 (2014)Print.

11) Yoo, J. Y., et al. "Antitumor Efficacy of 34.5ENVE: A Transcriptionally Retargeted and "Vstat120"-Expressing Oncolytic Virus." *Molecular therapy : the journal of the American Society of Gene Therapy* 20.2 (2012): 287-97. Print.

12) Kanai, R., et al. "Effect of gamma34.5 Deletions on Oncolytic Herpes Simplex Virus Activity in Brain Tumors." *Journal of virology* 86.8 (2012): 4420-31. Print.

13) Kaur, B., et al. "Brain Angiogenesis Inhibitor 1 is Differentially Expressed in Normal Brain and Glioblastoma Independently of p53 Expression." *The American journal of pathology* 162.1 (2003): 19-27. Print.

14) Rong, Y., et al. "'Pseudopalisading' Necrosis in Glioblastoma: A Familiar Morphologic Feature that Links Vascular Pathology, Hypoxia, and Angiogenesis." *Journal of neuropathology and experimental neurology* 65.6 (2006): 529-39. Print.

15) Huntzinger, E., and E. Izaurralde. "Gene Silencing by microRNAs: Contributions of Translational Repression and mRNA Decay." *Nature reviews.Genetics* 12.2 (2011): 99-110. Print.