CHARACTERIZATION OF HCMV-ENCODED CHEMOKINE RECEPTOR US28 TRANSDUCED MSU1.1 HUMAN FIBROBLAST CELLS

Danielle R. VanBeckum

Northern Michigan University, dvanbeck@nmu.edu

Follow this and additional works at: https://commons.nmu.edu/theses

Part of the Neoplasms Commons, and the Virus Diseases Commons

Recommended Citation


https://commons.nmu.edu/theses/24
CHARACTERIZATION OF HCMV-ENCODED CHEMOKINE RECEPTOR US28 TRANSDUCED MSU1.1 HUMAN FIBROBLAST CELLS

By

Danielle R. VanBeckum

THESIS

Submitted to
Northern Michigan University
In partial fulfillment of the requirements
For the degree of

MASTER OF SCIENCE

Office of Graduate Education and Research

June 2014
This thesis by Danielle R. VanBeckum is recommended for approval by the student’s Thesis Committee and Department Head in the Department of Biology and by the Assistant Provost of Graduate Education and Research.

Committee Chair: Dr. Robert J. Winn, Ph.D. Date

First Reader: Dr. Robert J. Belton, Ph.D. Date

Second Reader: Dr. Erich Ottem, Ph.D. Date

Department Head: Dr. John Rebers, Ph.D. Date

Dr. Brian D. Cherry Date
Assistant Provost of Graduate Education and Research
Human Cytomegalovirus (HCMV) encodes the G-protein coupled receptor US28. Using a mouse model system, US28 was previously found to be oncomodulatory, increasing proliferation, inducing anchorage independent growth and loss of contact inhibition. Similarly, in the human glioblastoma cell line U373, US28 activated VEGF expression. To determine if US28 is oncomodulatory in normal human cells, we engineered the human fibroblast cell line MSU1.1 to express US28 via lentivirus infection. MSU1.1 cells were transduced with pHAGE-US28-ZsGreen. Fluorescent confocal microscopy was utilized to detect the ZsGreen tag and confirmed the successful transduction of MSU1.1 cells with the US28 vector or empty pHAGE vector. MSU1.1 cells expressing US28 did not exhibit loss of contact inhibition nor anchorage independent growth in focus formation and soft agar assays, respectively. Using the AlamarBlue assay, US28-expressing cells showed a 2.303 fold increase in proliferation compared to wild type MSU1.1 cells and a 2.201 fold increase compared to mock transduced cells. US28 expression in MSU1.1 cell line increased proliferation but did not trigger a transformed phenotype. This study reveals potential cell-type and species-specific differences from previous findings assessing US28 expression in NIH-3T3 (mouse fibroblast) and U373 (malignant glioma) cell lines. The reported prevalence of US28 in GBM and confirmation of oncomodulation via increased proliferation in MSU1.1 cells makes US28 a potential target warranting further understanding of the virus’ role in GBM.
ACKNOWLEDGEMENTS

I would first like to express my appreciation to my thesis advisor Dr. Robert Winn and the Upper Michigan Brain Tumor Center. Without the support of the UMBTC, teaching assistantships, the Excellence in Education Grant and my summer research fellowship the completion of my thesis would not have been possible.

Additionally, I would like to recognize my committee members Dr. Robert Belton and Dr. Erich Ottem for being great mentors during my studies and always supporting the advancement of my success. Dr. Don Shaffer, a previous NMU graduate, also deserves a great deal of appreciation for his contributions in collaboration with the Upper Michigan Brain Tumor Center in designing the pHAGE lentiviral vector containing US28 and successfully transducing my experimental cells. I also owe my gratitude to Dr. John Lawrence, Jacob Studt, Aaron Mellesmoen, Allison Mitchell and Benjamin Carlson for their assistance in my research. Furthermore, I must thank Mandy Taisto and Rebecca Dangremond for their aid using the confocal microscope. Lastly, I want to thank my family Debbie, Steve, and Caryn VanBeckum and my wonderful girlfriend Paige Frazier for always being patient and supporting me.

This thesis follows the format described in the *Publication manual of the American Psychological Association* and Northern Michigan University’s Department of Biology.
# TABLE OF CONTENTS

List of Tables ........................................................................................................ (vi)

List of Figures ......................................................................................................... (vii)

List of Abbreviations ............................................................................................... (viii)

Introduction .............................................................................................................. 1

Chapter One: Literature Review .............................................................................. 3
  Human Cytomegalovirus ...................................................................................... 3
  HCMV in Cancer ................................................................................................. 5
  HCMV in Cancer: Cell Cycle Progression ......................................................... 7
  HCMV in Cancer: Cell Survival and Chromosome Stability ......................... 8
  HCMV in Cancer: Invasiveness ........................................................................ 10
  HCMV in Cancer: Angiogenesis ...................................................................... 11
  HCMV in Cancer: Immune Evasion ............................................................... 11
  HCMV-Encoded Chemokine Receptor US28 .............................................. 13
  Hypothesis ........................................................................................................ 16

Chapter Two: Materials and Methods ................................................................. 17
  Introduction ....................................................................................................... 17
  Expansion of pIDT-Blue-US28 Plasmid ......................................................... 17
  Recovery of pIDT-Blue-US28 Plasmid ............................................................ 19
  Digestion and Ligation of pIDT-Blue-US28 and pEYFP ............................ 20
  Kill Curve for G418 ......................................................................................... 21
  Neon Transfection of MSU1.1 Fibroblasts .................................................... 22
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalian Cell Culture</td>
<td>22</td>
</tr>
<tr>
<td>Western Blotting – US28</td>
<td>23</td>
</tr>
<tr>
<td>Confocal Microscopy</td>
<td>25</td>
</tr>
<tr>
<td>Soft Agar Assay</td>
<td>27</td>
</tr>
<tr>
<td>Focus Formation Assay</td>
<td>27</td>
</tr>
<tr>
<td>AlamarBlue® Assay</td>
<td>28</td>
</tr>
<tr>
<td>Zymography</td>
<td>29</td>
</tr>
<tr>
<td>Chapter Three: Results</td>
<td>33</td>
</tr>
<tr>
<td>Introduction</td>
<td>33</td>
</tr>
<tr>
<td>Restriction Digest of pEYFP-US28</td>
<td>33</td>
</tr>
<tr>
<td>Kill Curve and Cell Transfection</td>
<td>34</td>
</tr>
<tr>
<td>Western Blot</td>
<td>37</td>
</tr>
<tr>
<td>Soft Agar Assay</td>
<td>38</td>
</tr>
<tr>
<td>Focus Formation Assay</td>
<td>39</td>
</tr>
<tr>
<td>AlamarBlue® Assay</td>
<td>40</td>
</tr>
<tr>
<td>Zymography</td>
<td>41</td>
</tr>
<tr>
<td>Chapter Four: Summary and Conclusions</td>
<td>43</td>
</tr>
<tr>
<td>Introduction</td>
<td>43</td>
</tr>
<tr>
<td>HCMV-Encoded Chemokine Receptor US28</td>
<td>43</td>
</tr>
<tr>
<td>Generation of a US28-Expressing Cell Line</td>
<td>46</td>
</tr>
<tr>
<td>Evidence for Oncomodulatory Effects of US28</td>
<td>49</td>
</tr>
<tr>
<td>Overall Conclusions</td>
<td>52</td>
</tr>
<tr>
<td>References</td>
<td>50</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1. Neon Transfection electroporation parameters ................................................. 22
Table 2: 10% SDS-PAGE Gel Recipe ............................................................................. 30
Table 3: Zymography gel setup ....................................................................................... 30
Table 4: Nanodrop analysis of ligated pEYFP-US28 ....................................................... 33
LIST OF FIGURES

Figure 1. Ligand-dependent and constitutive signaling of US28 ................. 16
Figure 2. US28 sequence ........................................................................... 18
Figure 3. pIDT-Blue plasmid map ............................................................... 18
Figure 4. pEYFP-C1 vector map ................................................................. 20
Figure 5. DNA gel electrophoresis of ligated pEYFP-US28 ......................... 34
Figure 6. G418 kill curve .......................................................................... 35
Figure 7. pHAGE-US28-IRES-ZsGreen lentivirus map ................................. 35
Figure 8. FACS plot – MSU1.1-WT and MSU1.1-US28 ............................... 36
Figure 9. Confocal microscopy .................................................................. 36
Figure 10. Western Blot ............................................................................ 37
Figure 11. Soft Agar .................................................................................. 38
Figure 12. Focus Formation Assay ............................................................... 39
Figure 13. alamarBlue® reduction: WT vs. US28 .................................... 40
Figure 14. alamarBlue® reduction: WT vs. US28 vs. PHAGE .................. 41
Figure 15. Zymography Gel ..................................................................... 42
Figure 16. US28 signaling with specific G-protein subunits ..................... 45
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 Antagonist/Killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 Associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell Lymphoma 2 (family of proteins)</td>
</tr>
<tr>
<td>bp/kbp</td>
<td>Base Pair/Kilobase Pair</td>
</tr>
<tr>
<td>CCR1</td>
<td>Chemokine (C-C motif) Receptor 1</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Chemokine (C-X3-C motif) Receptor 1, Fractalkine Receptor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-diamidino-2-phenylindole fluorescent stain for nuclei</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled Water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s Modified Essential Medium</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>G418</td>
<td>Selective Antibiotic, also known as Geneticin®</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma Multiforme</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>gp130</td>
<td>Glycoprotein 130</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human Cytomegalovirus</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>IE1/IE2</td>
<td>Immediate Early Genes 1 and 2</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-6Rα</td>
<td>Interleukin-6 Receptor Alpha</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>JNK1</td>
<td>c-Jun N-terminal Kinase 1</td>
</tr>
<tr>
<td>JSI-124</td>
<td>JAK/STAT Inhibitor-124</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s Sarcoma-associated Virus</td>
</tr>
<tr>
<td>LB agar</td>
<td>Luria Broth agar</td>
</tr>
<tr>
<td>LSB</td>
<td>Laemmli Sample Buffer</td>
</tr>
<tr>
<td>M2</td>
<td>Alternately Activated Macrophage Phenotype</td>
</tr>
<tr>
<td>MAPK1</td>
<td>Mitogen-Activated Protein Kinase 1</td>
</tr>
<tr>
<td>MCP-1/CCL2</td>
<td>Monocyte Chemotactic Protein 1/Chemokine (C-C motif) Ligand 2</td>
</tr>
<tr>
<td>MIP-1α/CCL3</td>
<td>Macrophage Inflammatory Protein 1α/Chemokine (C-C motif) Ligand 3</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MSU1.1</td>
<td>Immortalized, but not transformed, human fibroblast cell line</td>
</tr>
<tr>
<td>NF-KB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMWL</td>
<td>Nominal Molecular Weight Limit</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>p53</td>
<td>Phosphoprotein 53</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>Platelet-Derived Growth Factor-Alpha</td>
</tr>
<tr>
<td>pEYFP</td>
<td>Plasmid with Yellow Fluorescent Protein Tag</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>pHAGE</td>
<td>Retriviral mammalian expression vector</td>
</tr>
<tr>
<td>pIDT</td>
<td>Plasmid from Integrated DNA Technologies</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>pp71</td>
<td>Phosphoprotein (71 kilodaltons)</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma Protein</td>
</tr>
<tr>
<td>RANTES/CCL5</td>
<td>Regulated on Activation, Normal T-cell Expressed and Secreted/Chemokine (C-C motif) Ligand 5</td>
</tr>
<tr>
<td>Rho-GEF</td>
<td>Rho-Guanine Exchange Factor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho Kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short Hairpin Ribonucleic Acid</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal broth with Catabolic inhibitor</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transduction 3</td>
</tr>
<tr>
<td>TE Buffer</td>
<td>Tris-EDTA (Ethylenediaminetetraacetic Acid) Buffer</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue Inhibitor of Metalloproteinases</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
</tbody>
</table>

x
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAIL-R1</td>
<td>TNF-Related Apoptosis-Inducing Ligand Receptor 1</td>
</tr>
<tr>
<td>TSP-1</td>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>UL33</td>
<td>Unique Long region 33</td>
</tr>
<tr>
<td>UL36</td>
<td>Unique Long region 36</td>
</tr>
<tr>
<td>UL37</td>
<td>Unique Long region 37</td>
</tr>
<tr>
<td>UL38</td>
<td>Unique Long region 38</td>
</tr>
<tr>
<td>UL78</td>
<td>Unique Long region 78</td>
</tr>
<tr>
<td>UL111A</td>
<td>Unique Long region 111A</td>
</tr>
<tr>
<td>US2</td>
<td>Unique Short region 2</td>
</tr>
<tr>
<td>US3</td>
<td>Unique Short region 3</td>
</tr>
<tr>
<td>US6</td>
<td>Unique Short region 6</td>
</tr>
<tr>
<td>US11</td>
<td>Unique Short region 11</td>
</tr>
<tr>
<td>US27</td>
<td>Unique Short region 27</td>
</tr>
<tr>
<td>US28</td>
<td>Unique Short region 28</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>vICA</td>
<td>Viral Inhibitor of Caspase Activation</td>
</tr>
<tr>
<td>vMIA</td>
<td>Viral Mitochondrial Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-Related Integration Site</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
</tr>
</tbody>
</table>
INTRODUCTION

Primary brain tumors that are collectively called gliomas encompass a wide variety of glial cell origins and grades of tumors. These cells of origin share common characteristics with the tumors. Among gliomas, the most common and aggressive form is glioblastoma multiforme (GBM) which has a 5 year survival rate of less than 5%. These brain tumors are of astrocytic origin and account for nearly 65% of glioma occurrences (Ohgaki and Kleihues, 2005). The prognosis for patients diagnosed with GBM is dismal with an average overall survival time between 12 to 15 months (Boxerman et al., 2013). This fatal tumor has no definitive etiology, but in 2002 the first link between GBM and human cytomegalovirus (HCMV) infection was shown (Cobbs et al., 2002). Though there is still some controversy over the presence and role of HCMV infection in GBM, the most widely accepted theory is that of oncomodulation (Lau et al., 2005, Dziurzynski et al., 2012).

Oncomodulation, an idea first suggested by Cinatl et al. in 1996, involves HCMV’s ability to modulate the host tumor cells’ malignant properties while not directly transforming normal cells (Cinatl et al., 2003, Dziurzynski et al., 2012). This may alter the pathogenesis of GBM via a wide variety of mechanisms. In a retrospective study, the level of HCMV infection in GBM was demonstrated to be of prognostic value where the median survival time of patients with low-grade infection was 20 months longer than those with high-grade (Rahbar et al., 2013).

The genomes of various strains of HCMV contain between 165 and 252 open reading frames. This variation is accounted for in duplications and overlapping regions of
coding and regulatory DNA. Of these possible open reading frames, the majority of investigations have focused on the chemokine G-protein coupled receptor homolog US28 (Davison et al., 2003, Stern-Ginossar et al., 2012). Various studies have linked the constitutive and ligand-dependent activity of US28 to augmenting pathways leading to common tumor characteristics such as invasive potential, promotion of angiogenesis, induction of inflammation and stimulation of cell proliferation (Dziurzynski et al., 2012). Additionally, one study previously suggested that US28 may be a viral oncogene, demonstrating transforming abilities in US28-expressing cells that developed into tumors when injected into nude mice (Maussang et al., 2006).

The majority of studies involving US28 have been carried out in either non-human or established cancerous cell lines, and the use of non-transformed human cells may open new roads in characterizing the potential oncomodulatory properties of the protein (Soroceanu et al., 2011, Vomaske et al., 2009). Non-transformed human fibroblasts (MSU1.1 cells) provide a good model for observing the possible phenotypic changes caused by US28 expression without the distraction of other mutations that exist in tumor cell lines. The use of a fibroblast cell line is comparable to astrocytes—the cell of origin for GBMs—as both are supporting cells capable of migration, replication, and communication with their external environments (Ohgaki and Kleihues, 2005).
CHAPTER ONE: LITERATURE REVIEW

*Human Cytomegalovirus*

The eight known human herpes viruses can be divided into three subfamilies, α-, β- and γ-herpesvirinae. Human herpes virus-5, or human cytomegalovirus (HCMV), belongs to the β-herpesvirinae subfamily. Various strains, including the human strain, of this herpes virus were first isolated in 1956 by Weller, Smith and Rowe. In 1960 Weller and his colleagues proposed the term cytomegaly because of the resulting enlargement of infected cells (Ho, 2008, Schottstedt *et al.*, 2010). Common among all herpes viruses, HCMV has the ability to establish lifelong persistence in the human host. This persistence comes in the form of inactive latent and chronic viral shedding infections (Cannon *et al.*, 2010, Goodrum *et al.*, 2012, Sinclair, 2008). Though the mechanisms of persistence and balance of latent and chronic HCMV infections are not well characterized, it has been proposed that HCMV encodes an array of proteins involved in avoiding the immune system response. This ability to escape not only the innate but also the acquired immune system may lead to HCMV’s ability to establish latency even in the presence of a strong and healthy immune system (Goodrum *et al.*, 2012, Jackson *et al.*, 2011). This latent infection is periodically interrupted by chronic infection, even in immune-competent hosts, which results in shedding of the virus from various cell types. During the chronic shedding stage, the human host can spread primary HCMV infectious virions through contact with various body fluids (Cannon *et al.*, 2010).

While identification of HCMV markers in the blood varies among geographical regions, race and socioeconomic status, it is estimated that world-wide, the frequency of
HCMV infection ranges between 50-100% of individuals in the adult population. Interestingly, the majority of infected individuals do not experience symptoms even though seroprevalence is relatively high (Cannon et al., 2010). In a healthy individual, the chronic shedding phase of the infection may be routinely reactivated, but adverse effects are restricted through strong immune responses (Michaelis et al., 2009, Sinclair, 2008). When chronic HCMV infection is reactivated in AIDS or organ-transplant patients, whose immune system is either compromised or suppressed, the resulting uncontrolled replication and shedding of HCMV will often present serious health risks (Sinclair and Sissons, 2006). Additionally, persistent HCMV infection has been linked to the progression and acceleration of various vascular diseases such as restenosis, atherosclerosis and transplant vascular sclerosis (Streblow et al., 2008).

The genome of HCMV is approximately 240,000 base pairs in length and contains an estimated 165 to 252 open reading frames. This large range in open reading frames is further complicated by the complexity of the genome that contains numerous overlapping coding regions and regulatory regions (Stern-Ginossar et al., 2012). Only one-quarter of these open reading frames are devoted to viral replication products. The other three-quarters of the open reading frames encode products that alter the way the infected cell functions and how immune cells in the host respond. On a clinical level, these pathways can interfere with normal function or cause diseases and conditions such as vascular thrombosis, encephalitis, inflammatory bowel disease, graft rejection and various types of cancers. Many signaling pathways involved with the consequential aberrant immunopathology in HCMV-induced disease are related to cell cycle...
progression, induction of inflammation, formation of new blood vessels and evasion of the host immune response (Varani and Landini, 2011).

**HCMV in Cancer**

As previously stated, herpes viruses cause lifelong persistent infections in which the latent state can be reactivated to a chronic infection involving viral shedding during times of stress. Several other herpes viruses, such as Kaposi’s sarcoma-associated virus (KSHV) and Epstein-Barr virus (EBV) have been implicated in the causation of malignancies such as Burkitt’s lymphoma, Hodgkin’s lymphoma and Kaposi’s sarcoma (de Oliveira et al., 2010). These two viruses, belonging to the γ-herpesvirinae subfamily, are considered oncogenic viruses due to their ability to directly transform cells to a cancerous state.

In order to effectively characterize and describe the role of HCMV in cancers, researchers formulated the theory of oncomodulation. In its most simple form, oncomodulation refers to the ability of HCMV to increase the malignancy of the tumor cells or supportive stromal cells it infects without being directly responsible for malignant transformation (Dziurzynski et al., 2012, Michaelis et al., 2009). Oncomodulation of tumor-associated cells by HCMV infection can occur through various mechanisms. These may include, but are not limited to, changes in cell cycle progression, increased survival abilities, gain of invasive abilities, immune evasion, epigenetic changes, increased chromatin stability and enhanced angiogenesis in the tumor environment (Cinatl et al., 2003, Michaelis et al., 2011). Active and persistent HCMV infection has been demonstrated in various types of brain tumors, lymphomas, rhabdomyosarcoma and
cancers of the breast, prostate and colon (Soderberg-Naucler, 2006, Wolmer-Solberg et al., 2013). Interestingly, activation of the virus is only seen in the tumor-associated cells and not in the surrounding normal tissues (Cobbs et al., 2002, Michaelis et al., 2011). This expression pattern may potentially prove to be beneficial in developing therapeutic treatments for these types of cancers, especially in brain tumors where complete surgical bulk tumor resection is not always possible.

Over the past decade, the study of HCMV infections in cancers has primarily focused on brain tumors, particularly GBM (Michaelis et al., 2011). Cobbs and his colleagues were the first to demonstrate the presence of HCMV proteins and nucleic acids in both high- and low-grade GBM (2002). Since this time, several others have disputed these findings, and there has been much debate about the existence and potential role of HCMV in tumor tissues (Mitchell et al., 2007, Scheurer et al., 2008, Lau et al., 2005, Dziurzynski et al., 2012). Many proponents of the exisance of HCMV in GBM have reported specific immunohistochemistry staining techniques to be used when analyzing a specimen. These technical specifications and methods were detailed by Scheurer et al., in 2008 and are now standard procedures for detection of HCMV in GBM samples. The effects of HCMV infection on GBM in vivo may be two-fold. First, it has been suggested that HCMV could potentially promote tumor growth by infecting tumor cells to increase malignancy through their oncogenic signaling pathways. Second, HCMV may infect non-cancerous cells (i.e. stromal, immune and endothelial cells) causing changes in the tumor microenvironment through secretion of cytokines and other soluble factors (Soroceanu and Cobbs, 2011).
Many cancer cells exhibit aberrant cell cycle regulation as a result of poorly functioning regulatory checkpoints. In the presence of HCMV infection, cell cycle progression can be altered through various mechanisms (Cinatl et al., 2003, Michaelis et al., 2009). One such affected pathway involves the inactivation of the retinoblastoma protein (pRb) through hyperphosphorylation (Dziurzynski et al., 2012). HCMV proteins IE1, IE2 and pp71 bind to pRb resulting in the release of E2F transcription factors which promote the expression of genes necessary for S-phase cell cycle progression (Cinatl et al., 2003, Castillo and Kowalik, 2004).

Increased proliferation in HCMV-infected cancer cells has also been linked to amplified NF-κB transcriptional activity (Castillo and Kowalik, 2004). Cyclin D1, a key regulator for the transition from G0/G1 to S phase of mitosis, is upregulated upon activation of NF-κB (Hinz et al., 1999). NF-κB has been reported to be constitutively activated in the presence of HCMV-encoded chemokine receptor US28. This activity leads to increased expression of various oncogenes and increased cell cycle progression (Casarosa et al., 2001, Maussang et al., 2009).

The effects of HCMV on cell cycle regulation seem to be cell type specific. The proteins IE1, IE2, pp71, and US28 each elicit different effects on host cells depending upon the regulatory mechanisms innate to the cell, suggesting that there may be different effects among tumor types and stromal cells in the tumor environment. The expression of the viral proteins generally correlate with the transformed phenotype of the cells. In glioblastoma, HCMV promotes proliferation of tumor cells while inhibiting proliferation of the surrounding non-cancerous glial cells (Soroceanu and Cobbs, 2011).
**HCMV in Cancer: Cell Survival and Chromosome Stability**

In order for HCMV to produce sufficient amounts of mature virions in infected cells, it is necessary for the virus to prevent apoptosis of infected cells. These mechanisms are carried into cancerous cells and increase malignancy by causing HCMV-infected tumors to be more resistant to apoptosis (Castillo and Kowalik, 2004, Cinatl et al., 2003). Apoptosis can be induced by various stimuli—either the extrinsic method through membrane-associated death receptors or the intrinsic method via mitochondrial death, induced by various stressors (Cinatl et al., 2003). HCMV possess the ability to control apoptotic activity in infected cells through a variety of mechanisms involving interactions between viral and cellular proteins.

The immediate early gene product IE2, which demonstrates regulation of cell cycle, has also been shown to alter apoptosis in infected cells. The protein IE2 binds the tumor suppressor gene p53 and shields the host cell from p53-mediated apoptosis (Michaelis et al., 2009). In many cancers, the p53 gene contains a loss of function mutation resulting in a lower chance of formation an active tetramer. IE2 may sequester both functional and non-functional p53 in the process of mediating apoptosis. Furthermore, smooth muscle cells expressing IE2 were resistant to doxorubicin-induced apoptosis. These results suggest that even in the presence of DNA damage, IE2 is able to override apoptotic mechanisms by suppressing p53 function (Cinatl et al., 2003).

Two other HCMV immediate early gene products, UL36 and UL37, have been credited with directly influencing the apoptotic activity. UL36, also called viral inhibitor of caspase activation (vICA), has demonstrated binding capability with the pro-domain of cellular caspase-8 (Cinatl et al., 2003). In binding pro-caspase-8, vICA inhibits the
conversion to caspase-8 which halts the caspase cascade leading to apoptosis originally induced by membrane bound death receptors such as Fas, TNF-receptor-1 or TRAIL-receptors 1 and 2 (Castillo and Kowalik, 2004, Maussang et al., 2009). From the UL37 gene product, viral mitochondrial inhibitor of apoptosis, vMIA, forms a transmembrane protein that is localized to the mitochondrial membrane. At the mitochondrial membrane vMIA neutralizes Bcl-2 family proteins, such as Bax and Bak, resulting in decreased permeability of the outer mitochondrial membrane. This decrease in mitochondrial permeabilization prevents the release of cytochrome C and the downstream activation of the caspase-9 cascade leading to cell death (Castillo and Kowalik 2004, Maussang et al., 2009). This suggest that HCMV modulates apoptosis through the inhibition of both the extrinsic membrane-associated death receptor pathway and intrinsic mitochondrial pathway (Cinatl et al., 2003).

Cell survival and continued proliferation also relies heavily on the stability of the cell’s chromosomes. Telomeres are repeated sequences of DNA found at the ends of chromosomes that tend to shorten every time a cell divides. This shortening can be reversed by the activation of telomerase, an enzyme that adds DNA repeats to the ends of chromosomes for added stability (Chan and Blackburn, 2004). Normal cells exhibit strict repression of the catalytic subunit of telomerase (hTERT), but nearly 90% of cancers display active telomerase function and expression of hTERT. When hTERT is inhibited, these cancer cells often lose their oncogenic potential as their ability to proliferate and survive is dampened (Hahn et al., 1999). The activity and expression of hTERT during HCMV infection was analyzed by Strååt et al., who found that infection of normal human fibroblasts with HCMV resulted in constitutively active hTERT expression and
telomerase activity (2009). Ectopic expression of HCMV-IE1 also resulted in increased hTERT expression and telomerase activity. Additionally, increases in hTERT expression in GBM cell lines infected with HCMV were observed to correlate in a dose-dependent manner at various multiplicities of infection (Strååt et al., 2009).

HCMV in Cancer: Invasiveness

Invasive properties of human cancers vary depending upon cell type and origin. These invasive properties lead to migration and metastases from the primary tumor bed. Tumor cells adhere to the endothelium though integrin mediation during migration and invasion (Michaelis et al., 2009). HCMV infection in various cancer cell lines was found to involve or alter the functions of platelet-derived growth factor receptor-alpha (PDGFRα), extracellular matrix remodeling proteins (MMPs and TIMPs), focal adhesion kinase (FAK) and integrins (Dumortier et al., 2008, Dziurzynski et al., 2012, Michaelis et al., 2009, Soroceanu and Cobbs, 2011). By activating cellular receptor tyrosine kinases (RTKs), HCMV activates PDGFRα in tumor cells leading to downstream signaling pathways involving phosphorylated-AKT, FAK and Src. This would indicate that HCMV infection is promoting increased malignancy, especially in GBM (Soroceanu and Cobbs, 2011). Matrix metalloproteinases (MMPs) are commonly found in the extracellular matrix during angiogenesis, wound healing and cell migration. Dumortier et al. reported increased MMP expression in fibroblast cells with HCMV infection (2008). Less than a year later, Strååt et al. contested these results with their findings that HCMV infection of macrophages resulted in decreased MMP-9 expression as well as increased expression of its inhibitor TIMP-1 (2009). It is possible that the effects of HCMV on this pathway are
cell-type specific. Additionally, HCMV infection in U87 glioma cell lines resulted in an increased level of phosphorylated FAK which was correlated with an increase in migration due to extracellular matrix remodeling when compared to HCMV-infected normal glial cells (Cobbs et al., 2007).

**HCMV in Cancer: Angiogenesis**

Formation of new blood vessels to supply the tumor bed with oxygen is a crucial factor involved in cancer progression (Michaelis et al., 2009). Prior to HCMV infection, fibroblasts and GBM cells express thrombospondin-1 (TSP-1) which strongly suppresses blood vessel formation. The subsequent repression in TSP-1 activity after HCMV infection results in increased malignancy for GBM angiogenesis in vivo (Soroceanu and Cobbs, 2011). Additionally, the expression of US28 has been identified to play a role in angiogenesis in GBM. This constitutively active protein induces the expression of cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and signal transducer and activator of transduction 3 (STAT3). The combination of these three proteins drives the infected glioma cells into a highly angiogenic phenotype, which in turn greatly increases their malignancy in vivo (Dziurzynski et al., 2012, Maussang et al., 2006, Slinger et al., 2010)

**HCMV in Cancer: Immune Evasion**

Over time, HCMV’s genome has evolved to acquire an array of mechanisms to avoid the host immune response. These mechanisms not only affect the innate immune response, but also humoral and cell-mediated immunity (Loenen et al., 2001). As a first
line defense against pathogens, the innate immune response relies heavily upon chemokines and their receptors. HCMV encodes four functional chemokine receptor homologues, UL33, UL78, US27 and US28, as well as two IL-8 chemokine homologues, UL146 and UL147 (Loenen et al., 2001, Penfold et al., 1999). US28 has also been associated with a positive feedback loop involving the secretion of IL-6 from HCMV-infected cells (Slinger et al., 2010). The combination of these chemokines and their receptor homologues results in cells that can be both pro-inflammatory or anti-inflammatory depending upon cell type (Loenen et al., 2001). Additionally, IL-10 has been shown to shift monocyte differentiation toward a pro-tumor, anti-inflammatory M2 macrophage phenotype (Stout, 2009). The presence of HCMV-encoded IL-10 homologue, UL111A, has the ability to not only deactivate macrophages towards the M2 phenotype, but also has been shown to modify the expression of MHC class II on dendritic cells and subsequently decrease T-cell activation (Chang et al., 2004, Cheung et al., 2009).

Changes in MHC expression are the most critical mechanisms in which HCMV evades the adaptive immune system. When a virus infects a cell, MHC class I molecules assemble on the surface of the cell and present viral antigens for T-cells. Upon infection with HCMV, the cell’s ability to present antigen on its surface is blocked by the functions of four HCMV gene products, US2, US3, US6 and US11 (Loenen et al., 2001). These gene products alter MHC class I expression in several distinct manners—targeting for degradation, retention in the endoplasmic reticulum, interference with antigen loading, and dislocation to the cytoplasm. The end result following viral infection and expression of these proteins is decreased antigen presentation leading to reductions in T-cell

**HCMV-Encoded Chemokine Receptor US28**

One major area of focus in HCMV-related cancer research is to discover the roles of US28 in GBM pathogenesis. US28 is a seven-transmembrane G-protein coupled receptor homologue that exhibits roughly 30% homology to CCR1, human receptor for RANTES/CCL5, and 36% homology to CX3CR1, human receptor for fractalkine (Gao and Murphy, 1994, Montaner et al., 2012). The low percentage in homology to the human CCR1 and CX3CR1 receptors allows US28 to bind a much larger selection of ligands while maintaining the chemokine receptor function (Boomker et al., 2005, Gao and Murphy, 1994). Though US28 is able to bind a host of ligands (RANTES/CCL5, MCP-1/CCL2, MIP-1α/CCL3) leading to the activation of the signaling function of US28, this viral protein also exhibits constitutive activity. In 2001, Casarosa et al. described activation of NF-κB and phospholipase C (PLC) pathways of monkey fibroblast-like cells (COS-7) expressing US28. This constitutive activation occurs in a dose-dependent manner even in the absence of additional chemokines. Further studies revealed greater understanding of US28 activity through the use of various human cell lines. Together these findings suggested that constitutive activity of US28 occurred in all cells, but some significant cell-type specific changes were also observed (Miller et al., 2012).

Constitutive signaling of US28 correlates with an increased angiogenic phenotype in GBM (Maussang et al., 2009, Soroceanu et al., 2011). Additionally, by altering the
host cell’s signaling pathways, both transient and stable transfection of cells with US28 alter cell proliferation, rates of apoptosis, and immune response (Dziurzynski et al., 2012, Slinger et al. 2010). The study of US28 signaling has primarily involved the use of NIH-3T3 mouse fibroblasts, smooth muscle cells from humans and rats, and various GBM cell lines (Soroceanu et al., 2011, Vomaske et al., 2009). Ligand binding for US28 may be both cell-type specific and ligand specific for the resulting downstream effects and phenotypes seen (Vomaske et al., 2009). The downstream signaling pathways affected by US28 signaling are also dependent upon the specific α and β G-protein subunits associated with the inner membrane side of the receptor (Montaner et al., 2012).

In 2010, Slinger and colleagues were the first to demonstrate the role of US28 in the promotion of proliferative signaling through the IL-6-JAK-STAT pathway (Figure 1). In NIH-3T3 mouse fibroblasts and HEK293T human embryonic kidney cells, constitutive activity of US28 was found to activate NF-KB. The transcription factor NF-KB translocated to the nucleus and induced the expression of interleukin-6 (IL-6). IL-6 is a secreted protein that can have both pro-inflammatory and anti-inflammatory effects on surrounding cells and tissues. HCMV infection in primary human hepatocytes also induced activation of the IL-6-JAK-STAT pathway leading to resistance to apoptosis and elevated cell proliferation rates (Lepiller et al., 2013). IL-6 can act in both an autocrine and paracrine fashion by binding and activating the IL-6Rα receptor and gp130 subunits on the cell surface. This results in the phosphorylation of STAT3 by JAK1 which then dimerizes and is translocated to the nucleus. The phosphorylated STAT3 dimer promotes the expression of various proliferative, survival and pro-angiogenic genes, including cyclin D1 and VEGF (Slinger et al., 2010, Sinibaldi et al., 2000). The upregulation of
VEGF has been indicated to be the primary factor resulting in increased angiogenesis seen with many tumors, specifically GBM (Plate et al., 1993). Transfection of NIH-3T3 cells with US28 resulted in a five-fold increase in VEGF expression when compared to mock-transfected cells (Maussang et al., 2006). Furthermore, knockdown of IL-6 expression by shRNA in US28-expressing cells resulted in decreased VEGF promoter activation. The use of two drugs, JSI-124 and celecoxib, which inhibit STAT3 and COX-2 respectively, also showed significant reduction in US28-induced VEGF promoter activation (Slinger et al., 2010). STAT3 drives the expression of VEGF, while COX-2 stimulates the synthesis of prostaglandin E2 (PGE2). PGE2 is exported from the cell and can act in an autocrine or paracrine manner to ultimately induce the expression of various factors including cyclin D1 and additional VEGF (Slinger et al., 2010).

US28 may have additional roles in different signaling pathways. For example, expression of US28 may alter the Wnt/β-catenin signaling pathway that is commonly deregulated in various types of cancers including GBM (Kim et al., 2012, Langemeijer et al., 2012, Zhang et al., 2012). Rather than activating β-catenin through the standard Wnt/Frizzled receptor complex, US28 activates the guanine exchange factor (Rho-GEF) and Rho kinase (ROCK) to free β-catenin from the degradation complex. This allows for US28-induced expression of a variety of Wnt target genes including cyclin D1, c-myc, and c-jun (Langemeijer et al., 2012). The activation of the Rho-ROCK pathway has also been implicated in the invasive potential of cancerous cells (Booden et al., 2005). Although US28 exhibits constitutive activity, the activation of Rho-ROCK and downstream MAP kinases requires the interaction of RANTES/CCL5 ligand to induce this increased
invasive phenotype (Billstrom et al., 1998, Zohrabian et al., 2009). Additionally, augmented invasiveness was seen in a Matrigel invasion assay where the addition of RANTES/CCL5 nearly doubled the amount of cellular invasion compared to expression of US28 alone (Soroceanu et al., 2011). Miller and colleagues have noted that the specific downstream effects of ligand binding with US28 remain largely misunderstood, and should be considered cell-type specific (2012).

**Hypothesis**

MSU1.1 human fibroblasts expressing US28 will demonstrate oncomodulatory characteristics such as loss of contact inhibition, anchorage independent growth, secretion of extracellular matrix remodelers, and increased proliferation rates.
CHAPTER TWO: MATERIALS AND METHODS

Introduction

Though fibroblasts are not present in the brain, ectopic expression of US28 in human fibroblasts (MSU1.1 cell line) provides an advantage in assessing specific oncomodulatory effects in the absence of an array of other mutations that exist in commonly used GBM cell lines such as U87, LN229 or T98. The use of a fibroblast cell line also parallels the astrocytic origin of GBMs through various similarities (Ohgaki and Kleihues, 2005). Both astrocytes and fibroblasts are capable of migration, and in this process respond to factors present in their extracellular environments. In addition, each of these cell types secrete various molecules into their environments that may affect processes in neighboring cells. Taken together, these similarities will allow for a comparative analysis of the oncomodulatory effects elicited by US28 expression in a non-transformed mammalian cell line.

Expansion of pIDT-Blue-US28 Plasmid

Novablue and JM109 competent E. coli cells were heat shock transformed with pIDT-Blue-US28 plasmid ordered from Integrated DNA Technologies with the insert sequence in Figure 2 (whole plasmid map; Figure 3). An aliquot of 20 µL of cells was removed from -80°C freezer and mixed with 1.5 µL plasmid to be chilled on ice for five minutes. The mixture was then moved to a 42°C water bath for 30 seconds and back to
ice for two
minutes. Super
optimal broth
with catabolic
inhibitor (SOC
medium) was
added to the
mixture and the
mixture was
placed on a
tabletop orbital
shaker at 37°C
and 150 rpm for 1 hour. LB agar plates containing carbenicillin (50 µg/mL) were
streaked with transformed mixture and incubated at 37°C for two days.

Bacterial colonies
containing pIDT-Blue-US28
were selected and expanded
by inoculating LB broth
containing carbenicillin (50
µg/mL) and incubating for
eight hours at 37°C and
shaking at 100 rpm on a
tabletop orbital shaker.

Figure 2. US28 sequence in reverse for cloning into pIDT-Blue vector. Shaded flanking regions indicate restriction sites for BamHI (GGATCC) and EcoRI (GAATTC) generated on either side of US28. The internal shaded region specifies the site of the start codon of US28 upon inversion and insertion into the mammalian vector pEYFP. The sequence was designed to maintain the correct reading frame while transcription occurs in transfected cells. This allows for an N-terminal tagged US28 protein.

Figure 3. pIDT-Blue plasmid construct marked with US28 sequence insertion site. Initial sequence of pIDT-Blue does not contain restriction sites for BamHI or EcoRI.
Bacterial culture was then diluted 1:500 and shaken overnight (12-16 hours) at 37°C and 300 rpm to prepare for plasmid DNA extraction. Freezing stocks of transformed Novablu and JM109 cells were prepared by centrifuging cells at 6000 x g for 15 minutes at 4°C and re-suspending the pellet in sterile 60% glycerol solution. Glycerol stocks were kept at -80°C for long term storage.

Recovery of pIDT-Blue-US28 Plasmid

Plasmid DNA was recovered from Novablu and JM109 E. coli cells using the Fast Ion Plasmid Midi Kit (IBI Scientific). Bacterial cultures were centrifuged at 6000 x g for 15 minutes at room temperature and supernatant was discarded. The pellet was suspended and lysed. This fraction was centrifuged again at 6000 x g for 20 minutes at room temperature and filtered to collect DNA by passing the supernatant through in the provided column. DNA was eluted from the column by gravity flow and collected for DNA precipitation. Precipitated DNA was centrifuged at 15,000 x g for 30 minutes at 4°C and washed with ethanol before centrifuging again for 10 minutes. Supernatant was discarded and DNA was suspended in 1 mL TE buffer.

Recovered plasmid DNA concentrations were analyzed using the Nanodrop 2000c spectrophotometer and software (Thermo Scientific). Nanodrop absorbance analysis for DNA concentrations requires 2 µL of DNA sample to perform absorbance readings at 260 nm and 280 nm wavelengths. Nanodrop software reported DNA concentrations (µg/mL) and ratios of A260 and A280 indicated purity of the DNA sample.
**Digestion and Ligation of pIDT-Blue-US28 and pEYFP**

Restriction enzymes (BamHI and EcoRI) were used to digest plasmid DNA. The US28 sequence was designed with flanking BamHI and EcoRI restriction sites to create a linear US28 DNA fragment which could be ligated into a mammalian expression vector (pEYFP, Figure 4). Both circular pIDT-Blue-US28 and pEYFP DNA (40 ng and 500 ng respectively) were digested and separated by agarose gel electrophoresis (0.7% in 1x TAE, 0.001 µg/mL ethidium bromide). Electrophoresis was completed at 75 V for 75 minutes then briefly visualized using UV light using a Molecular Imager Gel-Doc XR+

![Figure 4](image)

**Figure 4.** pEYFP-C1 vector map showing restriction sites, EYFP gene location and open reading frames. Digestion of pEYFP at BamHI and EcoRI allow for insertion of US28 fragment in the proper orientation and reading frame to continue transcription after EYFP. This results in an EYFP fluorescent tag attached to the N-terminal end of US28.
System and Image Lab software (Bio-Rad). Corresponding linear fragments were cut and collected from the gel using a single edge blade. Linear DNA was isolated from these samples using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer’s protocol then analyzed with the Nanodrop software for concentration and purity.

Using the Quick Ligation Kit (New England BioLabs), linear pEYFP and US28 were ligated. Linear pEYFP was combined with the US28 fragment and were ligated using the manufacturer’s protocol. JM109 cells were heat shock transformed (as described above) with the new pEYFP-US28 to clone the vector and glycerol stocks were prepared for long term storage at -80°C. The pEYFP-US28 vector was subsequently recovered from JM109 cells using the Fast Ion Midi Kit. A small amount of recovered pEYFP-US28 vector was digested with combinations of BamHI and EcoRI and analyzed with agarose gel electrophoresis to ensure proper ligation.

**Kill Curve for G418**

G418 is an antibiotic used to select mammalian cells that express the neomycin resistance protein (Gold Biotechnology). As seen in Figure 4, pEYFP encodes a NeoR/KanR gene for resistance to G418 when inserted into mammalian cells. MSU1.1 cells were cultured in complete media containing Eagle’s Minimum Essential Medium (EMEM), 10% Fetal Bovine Serum (FBS), 1% non-essential amino acids and 1% sodium pyruvate. Using two 6-well plates MSU1.1 cells were plated at a density of 500 cells/mL in 2 mL volumes and incubated overnight to allow cells to adhere. A stock solution of G418 (50 mg/mL) was prepared in water and sterile filtered into 200 µL aliquots to be
stored at -20°C. The following day the media was then removed and replaced with media containing a range of concentrations of G418 (0, 100, 300, 500, 700 and 1000 µg/mL) with concentrations repeated in quadruplicate. The media containing G418 was replaced every three days and the cell density was observed over the course of two weeks. The lowest concentration of antibiotic to kill all of the cells in four days is the ideal concentration to use after transfection (Wallert and Provost, 2012).

*Neon Transfection of MSU1.1 Fibroblasts*

According to the manufacturers protocol 1 x 10⁷ MSU1.1 cells were combined with transfection buffers provided by the Neon Transfection system and electroporated under various conditions and parameters illustrated in Table 1. Cells were then plated in 2 mL complete media in 6-well plates and incubated at 37°C and 5% CO₂ for 24 hours before adding G-418 (500 µg/mL) to select for antibiotic resistance. Approximate cell density was observed and fluorescence was checked using the Olympus CKX41 inverted microscope, U-RFLT50 fluorescent power supply unit and cellSense Standard software.

<table>
<thead>
<tr>
<th>Voltage</th>
<th>Pulse Width</th>
<th>Pulse Number</th>
<th>Cell Density</th>
<th>Tip Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1700</td>
<td>20</td>
<td>1</td>
<td>1 x 10⁷</td>
<td>100 µL</td>
</tr>
<tr>
<td>1400</td>
<td>20</td>
<td>2</td>
<td>1 x 10⁷</td>
<td>100 µL</td>
</tr>
<tr>
<td>1500</td>
<td>30</td>
<td>1</td>
<td>1 x 10⁷</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

*Mammalian Cell Culture*

Malignant glioma cells (U87-YFP) and MSU1.1 human fibroblast cell lines transduced with US28 containing a ZS-Green tag (MSU1.1-US28) or the empty vector
(MSU1.1-pHAGE) were cultured in complete media containing EMEM, 10% FBS, 1% non-essential amino acids and 1% sodium pyruvate at 37°C and 5% CO₂. The transduced MSU1.1 cell lines were produced in collaboration with Don Shaffer at the Dana-Farber Cancer Institute at Harvard University. Additionally normal MSU1.1 (MSU1.1-WT) cells were cultured as previously described.

**Western Blotting – US28**

Cell lysate was prepared from MSU1.1-US28 cells grown to a confluent state in three 10 cm Petri dishes each. Prior to collecting lysate, complete media was replaced with serum-free media for 24 hours. The following day culture media was aspirated off of each dish and the adherent cells were washed twice in DPBS containing Mg⁺ and Ca⁺. NP-40 lysis buffer (50 mM Tris pH 8.0, 1.0% NP-40/Igepal CA-630, 150 mM NaCl, 0.1% SDS, 5 mM EDTA) was added to one dish in a 0.5 mL volume and incubated on ice for 5 minutes. Using a cell scraper and micropipetter, lysate was collected and transferred to the next pre-washed Petri dish with adherent cells. This process was repeated with each plate and total cell lysate was then transferred to a pre-chilled Eppendorf tube. Lysate was centrifuged at 13,000 rpm for 20 minutes at 4°C and the supernatant was transferred to 200 µL aliquots in fresh Eppendorf tubes to be frozen at -20°C.

Protein concentrations were determined using the Pierce™ BCA protein assay (Thermo Scientific). MSU1.1-US28 cells yielded a protein concentration of 0.748 µg/µL. Cell lysate was mixed 3:1 with 4x laemmli sample buffer and 1:1 with 2x laemmli sample buffer which diluted protein concentration to 0.561 and 0.374 µg/µL respectively.
Using two precast Bio-Rad Mini-PROTEAN® TGX™ gels, 50 µL of each diluted sample (28 µg or 18 µg) was loaded into appropriate wells along with the Precision Plus Protein Dual Color Standards (Bio-Rad). The lysate proteins were resolved using SDS polyacrylamide gel electrophoresis at a constant 200V for 30 minutes (Bio-Rad).

One gel was stained with Coomassie brilliant blue. The gel was submerged in isopropanol fixing solution (10% acetic acid, 25% isopropanol, 65% dH₂O) for 20 minutes at room temperature with gentle shaking. Fixing solution was replaced with Coomassie brilliant blue stain (10% acetic acid, 0.006% Coomassie blue dye, 90% dH₂O) and shaken gently at room temperature for 3 hours. The gel was then destained with 10% acetic acid overnight and imaged on a white background using a Molecular Imager Gel-Doc XR+ System and Image Lab software (Bio-Rad).

Proteins in the second gel were transferred to nitrocellulose membranes using the sandwich method and western transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol). The transfer was run at a constant 0.1 A for 10 hours at room temperature stirring at medium speed. Once complete the nitrocellulose membrane was removed from the transfer cassette, washed with distilled water, and stored at 4°C until probed with primary and secondary antibodies.

Membranes were probed with US28 and GAPDH primary polyclonal antibodies. Nitrocellulose membranes were removed from 4°C storage and washed briefly with TBST pH 7.4 (20 mM Tris, 150 mM NaCl and 0.1% Tween). The membrane was cut in half for one to be probed for GAPDH and the other for US28. The membranes were rocked gently at 50 rpm in western blocking buffer (5% non-fat milk in TBST) for 60 minutes. The first membrane was subsequently submerged in Anti-CMV US28 goat
polyclonal antibody (Santa Cruz Biotechnology, sc-29042), and the other membrane was submerged in Anti-GAPDH goat polyclonal antibody (Santa Cruz Biotechnology, sc-48166), diluted 1:200 and 1:2500 in 5% non-fat milk in TBST, respectively. The primary antibodies were incubated with each membrane for 90 minutes at room temperature. The membrane was washed with TBST pH 7.4 three times for 10 minutes each with gentle rocking at 50 rpm.

The membranes were incubated and rocked gently in their respective secondary antibodies for 60 minutes (Thermo Scientific, rabbit anti-goat #31402 and donkey anti-rabbit #31458). Secondary antibodies, both conjugated with horseradish peroxidase (HRP) were diluted 1:20,000 in 1% non-fat milk in TBST. The membranes were again washed three times with TBST for ten minutes each. HRP detection for each membrane was completed using the SuperSignal® West Pico Chemiluminescent Substrate according to the manufacturer’s protocol (Thermo Scientific). Luminescence was detected using a Kodak Digital Science Image Station 440CF. Exposure time for each membrane half was optimized at ten 20 second captures for GAPDH and three 20 minute captures for US28.

Confocal Microscopy

Sterile gelatin-coated coverslips were prepared by submerging in 0.1% gelatin solution (0.1 g gelatin, type A in 100 mL distilled water) and incubating at room temperature for 10 minutes. Gelatin solution was removed and the coverslips were allowed to air-dry for 15 minutes. One gelatin-coated coverslip was placed in the bottom of each well of a 6-well plate. MSU1.1-WT, and low passage number MSU1.1-US28 and
MSU1.1-pHAGE cells (P5 and P4 respectively) were seeded into the wells in 2 mL volumes at densities of $2.5 \times 10^4$ and $7.5 \times 10^4$ cells/mL. The plates were incubated at 37°C and 5% CO₂ for 48 hours before fixing. The cells were pre-fixed by adding 1 mL 4% paraformaldehyde in PBS directly to the media for 2 minutes. This was then replaced 1 mL 2% paraformaldehyde in PBS and incubated at room temperature for 20 minutes. The wells were washed again with PBS then coverslips were immersed in a wash buffer of 0.1% FBS in PBS.

For better visualization of individual cells, filamentous actin was stained using Rhodamine-phalloidin (Molecular Probes). The wash buffer was replaced with 0.1 M glycine in 1xPBS to remove excess aldehydes then the cells were permeabilized with a one minute wash in 0.1% Triton-x100 in 1xPBS. Rhodamine-phalloidin was diluted to 1 unit/mL, added to each coverslip in 2 mL volumes and incubated at room temperature for 15 minutes. Before immersing the coverslips in wash buffer again they were washed three times with 1xPBS.

To stain and mount the coverslips, each was briefly dipped in distilled water to remove salts and excess liquid was removed by capillary action using a Kimwipe. Coverslips were carefully lowered onto mounting slides with a drop of VECTASHIELD mounting medium with DAPI to prevent quenching of fluorescence and stain the nuclei (Vector Laboratories). The edges of the coverslip were subsequently sealed with clear nail polish and allowed to dry. The fixation, staining, mounting and storage of slides was completed at low light to avoid additional photo-bleaching.

Each cell specimen was visualized using the Olympus FluoView™ confocal microscope under 60x magnification. Laser excitation filters were set to 355 nm, 488 nm
and 542 nm for DAPI, ZsGreen and Rhodamine-phalloidin respectively. Nuclei stained with DAPI emitted light at 461 nm (blue), the expression vector tag ZsGreen emitted light at 506 nm (green) and f-actin stained with Rhodamine-phalloidin emitted light at 506 nm (red).

**Soft Agar Assay**

A bottom layer of 0.5% agar was prepared using equal volumes of 1.0% Fisher granulated agar in PBS mixed with 2xEMEM + 20% FBS. In the top layer 2.5x10^3 and 5x10^3 cells (MSU1.1-WT, MSU1.1-US28 and U87-YFP) were suspended in 2xEMEM + 20% FBS then mixed with and equal volume of 0.7% SeaKem® agarose. Prepared 12-well plates were incubated at 37°C and 5% CO₂ for 21 days, feeding each well with 0.5 mL EMEM + 10% FBS every 3-4 days. Photographs were taken using the Olympus CKX41 inverted microscope, U-RFLT50 fluorescent power supply unit and cellSense Standard software twice a week for three weeks for comparison of cell densities over time. Soft agar assays were repeated a total of 19 times and were done in triplicate for each cell line using all 12 wells in the plate.

**Focus Formation Assay**

MSU1.1-WT, MSU1.1-US28, MSU1.1-pHAGE and U87-YFP cells were expanded in culture as described previously. MSU1.1-WT cells were suspended and counted and added to 32 wells in two 24-well plates in 5 mL volumes at 5x10^5 cells/mL. The plates were allowed to incubate at 37°C and 5% CO₂ for 24 hours. All four cell lines were then suspended and counted to seed 250 cells on top of the monolayer of MSU1.1-
WT cells in the appropriate wells of the 24-well plates (eight repeated wells per cell line). The plates were incubated again at 37°C and 5% CO₂ and media was replaced every two days. Plates were photographed using the Olympus CKX4 inverted microscope, U-RFLT50 fluorescent power supply unit and cellSense Standard software then analyzed to determine the presence of foci. Bright field and fluorescent (excitation wavelength of 488 nm) images were captured to identify the YFP tag in U87-YFP foci and the ZsGreen tag in MSU1.1-US28 and MSU1.1-pHAGE cells.

*AlamarBlue® Assay*

Two days after passing, MSU1.1-WT and MSU1.1-US28 cells in log-phase of growth were harvested and counted. The cells were plated at an initial density of 7.5x10⁴ cells/mL in 100 µL volumes with eight replicates per cell line and 10 µL alamarBlue® reagent was immediately added to each well in a clear-bottom 96-well plate. An additional eight wells received 100 µL media only with 10 µL alamarBlue®. Plates were shaken gently to mix then incubated at 37°C and 5% CO₂. After addition of alamarBlue®, plates were removed from the incubator at 2, 3, 4, 5 and 6 hours to measure absorbance using the Modulus Microplate Reader at 560 nm and 600 nm.

The assay was repeated and MSU1.1-WT, MSU1.1-US28 and MSU1.1-pHAGE cells were harvested while in log-phase growth. The suspended cells were diluted to 8 x 10⁴ cells/mL and seeded into a 96-well plate in 100 µL volumes (eight replicates per dilution). An additional eight wells received 100 µL of media without cells. Plating time was recorded and the plates were incubated at 37°C and 5% CO₂. After 12 hours 10 µL alamarBlue® was added to each well. This would give all of the cells time to adhere and
begin cycling through mitosis. Absorbance at 560 nm and 600 nm was analyzed every hour after adding alamarBlue® for 8 hours.

Data from each trial was analyzed and reduction of alamarBlue® was calculated with the following equation using constants and coefficients indicated in the manufacturer’s protocol.

\[
100 \times \frac{(\epsilon_{OX})_{\lambda 2} A_{\lambda 1} - (\epsilon_{OX})_{\lambda 1} A_{\lambda 2}}{(\epsilon_{RED})_{\lambda 1} A_{\lambda 2} - (\epsilon_{RED})_{\lambda 2} A_{\lambda 1}}
\]

Zymography

MSU1.1-WT, MSU1.1-US28, MSU1.1-pHAGE cells were plated in 10 cm Petri dishes and allowed to grow until approximately 70% confluent. Media was replaced with serum-free media (EMEM only) and dishes were incubated for 24 hours at 37°C and 5% CO₂. Serum-free media was collected and concentrated using 50 kDa NMWL Amicon centrifugal filter device by spinning 4 mL media at a time at 4000 rpm for 10 minutes. With each additional aliquot of media, one minute was added to the time. Samples of filtrate and concentrate were collected, transferred into Eppendorf tubes and mixed 1:1 with 2x Laemmli Sample Buffer (LSB). Adherent cells were lysed and collected using 1 mL 1% SDS/20 mM Hepes pH 7.2 per Petri dish, transferred into Eppendorf tubes and centrifuged at 14.5 x 10³ rpm for 5 minutes. Cell lysate was collected into another Eppendorf tube by pipetting, leaving the pellet behind. LSB (2x) was mixed with the lysate in a 1:1 ratio.
Polyacrylamide gels (10%) containing either gelatin or casein were prepared by combining the reagents listed below for both a resolving and stacking gel layer (Table 2).

<table>
<thead>
<tr>
<th>10% Resolving Gel - 10 mL/2 Gels</th>
<th>10% Stacking Gel - 5 mL/2 Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 mL. dH₂O</td>
<td>3.4 mL. dH₂O</td>
</tr>
<tr>
<td>1.0 mL. 1% Gelatin or Casein</td>
<td>0.83 mL. Acrylamide</td>
</tr>
<tr>
<td>3.3 mL. Acrylamide</td>
<td>0.63 mL. 1.0M Tris, pH 6.8</td>
</tr>
<tr>
<td>2.5 mL. 1.5M Tris, pH 8.8</td>
<td>0.05 mL. 10% SDS</td>
</tr>
<tr>
<td>0.1 mL. 10% SDS</td>
<td>0.05 mL. 10% APS</td>
</tr>
<tr>
<td>0.1 mL. 10% APS</td>
<td>0.005 mL. TEMED</td>
</tr>
<tr>
<td>0.004 mL. TEMED</td>
<td></td>
</tr>
</tbody>
</table>

TEMED and 10% APS were omitted until just before pouring gel into the casting frames. First the resolving layer was poured and dH₂O was added on top then allowed to set for 15 minutes. Water was removed from the top, stacking gel solution was poured into casting frame and the 1.0 mm 10-well comb was secured in place. After gel polymerized, the comb was removed and assembled in the BioRad electrophoresis holder and cell. The buffer dam and cell chamber were filled with 1x SDS Running buffer. Samples were loaded as described in Table 3. Gels were run at a constant 200V for 38 minutes then placed in renaturing buffer and shaken at 65 rpm for 30 minutes. The gelatin gels were cut at the bottom right corner to distinguish between gelatin and casein substrate gels while staining. Renaturing buffer was removed, the gels were suspended in developing buffer and shaken again at 65 rpm for 30 minutes. Fresh developing buffer was added to the gels and they were allowed to incubate and shake at room temperature for 72 hours.
The developing buffer was decanted, replaced with Coomassie brilliant blue stain and the gels were shaken at 65 rpm for 4 hours. The stain was removed and gels were destained with 50 mL/gel destaining solution for 4 hours with shaking at 65 rpm. When clear bands were visible, destaining solution was rinsed with dH$_2$O three times for 5 minutes each. Gels were submerged in gel-drying solution (35 mL/gel) for 20 minutes and the stacking gel was removed from the resolving gel using a single-edge blade.

Cellophane was immersed in gel-dry solution for 10 seconds and each gel was placed between two 5 inch x 5 inch pieces assembled in a drying frame. Gels were allowed to dry for 24 hours and excess cellophane was removed. Images were captured using a Molecular Imager Gel-Doc XR+ System and Image Lab software (Bio-Rad).

Concentration of media in 50 kDa NMWL Amicon centrifugal filter devices was later repeated using the same cell lines grown in phenol red-free DMEM as it interferes with BCA assay analysis of protein concentration. The media for MSU1.1-WT, MSU1.1-US28 and MSU1.1-pHAGE cells grown to a nearly confluent state was replaced with serum-free DMEM and incubated at 37°C and 5% CO$_2$ for 24 hours. Media was subsequently concentrated and protein concentrations were determined using the Pierce™ BCA protein assay (Thermo Scientific). The concentrated DMEM media for MSU1.1-WT, MSU1.1-US28 and MSU1.1-pHAGE cells had protein concentrations of 3.81 µg/µL, 3.67 µg/µL and 3.87 µg/µL respectively. Cell lysates were loaded into 10% SDS-PAGE gels in volumes containing 50 µg, 20 µg, 10 µg and 5µg of total protein and were resolved, renatured, developed, stained and imaged as previously described above.

Band intensity of MMP-2 and MMP-9 in both casein and gelatin zymograms were analyzed using Image J (National Institute of Health). Image J calculates the brightness
and contrast in the gels to plot band intensity with peaks along the x-axis. Relative band intensity was determined by the area under each curve for the peaks corresponding to MMP-2 (92 kDa) and MMP-9 (72 kDa).
CHAPTER THREE: RESULTS

Introduction

US28 expression in MSU1.1 cells was confirmed by confocal microscopy and Western blot. Oncogenic and oncomodulatory characteristics were analyzed through various methods. Loss of contact inhibition and anchorage independent growth were qualitatively evaluated through focus formation and soft agar assays respectively. Additionally, proliferation rates were compared by a detecting colorimetric growth indicator in the alamarBlue® assay. Remodeling of the tumor extracellular environment may indicate a metastatic advantage, thus the expression of matrix metalloproteinases was investigated using gelatin and casein zymography. Together, these assays have been completed to test the role of US28 as an oncomodulatory factor in human fibroblast cells.

Restriction Digest of pEYFP-US28

After expansion and recovery of pIDT-Blue-US28 and pEYFP, the restriction enzyme digested fragments were ligated. Concentrations and purity of pEYFP-US28 in four samples are shown in Table 4.

Proper ligation was confirmed by digesting a small

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>DNA Conc. (ng/µL)</th>
<th>A260</th>
<th>A280</th>
<th>260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYFP-US28-1</td>
<td>2529.2</td>
<td>50.585</td>
<td>25.359</td>
<td>1.99</td>
</tr>
<tr>
<td>EYFP-US28-2</td>
<td>11120.1</td>
<td>222.403</td>
<td>127.54</td>
<td>1.74</td>
</tr>
<tr>
<td>EYFP-US28-3</td>
<td>3693.6</td>
<td>73.872</td>
<td>36.402</td>
<td>2.03</td>
</tr>
<tr>
<td>EYFP-US28-4</td>
<td>12510.1</td>
<td>250.202</td>
<td>124.05</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Table 4. Nanodrop analysis of ligated pEYFP-US28 expression vector. Purity of the sample is indicated by an A260/A280 ratio near 1.80.
amount of each sample resulting in bands at ~4.7 kbp and ~1.1 kbp (Figure 5). Smearing from 300-700 bp is a result of bacterial RNA present in the sample.

![Figure 5](image)

**Figure 5.** DNA gel electrophoresis of the pEYFP-US28 samples (1-4) from Table 4. In each sample the left lane contains the undigested DNA vector. The right lane shows linear fragments of pEYFP and the US28 insert (approximately 4.7 and 1.1 kbp respectively).

**Kill Curve and Cell Transfection**

A kill curve is used to determine the appropriate concentration of antibiotic when treating cells with the antibiotic G418 for selection after transfection. After four, seven and ten days of treatment, none of the concentrations exhibited cell death (Figure 6). Therefore a concentration of 500 µg/mL G418 was selected for antibiotic resistant selection as suggested by Wallert and Provost (2012). Examination of the cells by fluorescent microscopy did not detect fluorescence of the EYFP protein tag in electroporation transfected MSU1.1 cells. After numerous trials using variations in electroporation parameters fluorescence of the EYFP protein remained undetectable (Table 1).
In collaboration with Don Shaffer at the Dana-Farber Cancer Institute – Harvard, US28 transduced (MSU1.1-US28) and empty vector transduced (MSU1.1-pHAGE) cells were received in November, 2013. These contained a retroviral mammalian expression vector (pHAGE) that expressed a fluorescent ZsGreen protein tag on the C-terminal end of US28 or a cytosolic ZsGreen protein respectively. The map of the pHAGE-US28-IRES-ZsGreen lentivirus with the locations of the US28 and ZsGreen gene are depicted in Figure 7. Successfully transduced cells were sorted using fluorescence-activated cell sorting.

**Figure 6.** G418 kill curve photographs taken at 4x bright field magnification on day 0 (before adding G418) and days 4, 7 and 10.

**Figure 7.** Map of pHAGE-US28-IRES-ZsGreen lentivirus used to transduce MSU1.1 human fibroblasts.
(FACS) to obtain >80% ZsGreen-high expressing cells (Figure 8). Fluorescence of ZsGreen was visualized using confocal microscopy with a laser excitation wavelength of 488 nm (Figure 9). MSU1.1-WT cells were also excited at 488 nm to confirm the absence of ZsGreen expression.
Western Blot

Western blot analysis of MSU1.1-US28 cells revealed a faint band of protein near 75 kDa in size indicating the expression of US28 in lentiviral transduced MSU1.1 cells (Figure 10A). GAPDH was used as a housekeeping gene to ensure proper loading of cell lysate. Specificity of antibody binding was observed between the bands’ intensities of the two amounts of protein resolved through the SDS-PAGE gel (Figure 10B).

**Figure 10.** Western blot analysis for US28 and GAPDH in lentivirus transduced cells. (A) Either 28 µg and 18 µg of total protein from cell lysate was added to each well probed with the Anti-US28 primary antibody. The size of US28 is only 41 kDa but it appears as 71-76 kDa due to the ZsGreen tag (30-35 kDa) (B) Using the same amount of cell lysate protein, the membranes were probed with Anti-GAPDH to ensure proper loading of samples. GAPDH is expected to resolve through an SDS-PAGE gel to a size of 36 kDa.
**Soft Agar Assay**

Soft agar assays seeded at 5000 cells/well in 12 well plates were photographed twice a week. Weekly photographs for qualitative analysis of cell number and presence are seen in Figure 11. At day 0, MSU1.1-WT, MSU1.1-US28 and U87-YFP samples all contained a single cell suspension of cells in top layer 0.3% agar. The U87-YFP cells (positive control) were the only cells to proliferate and survive through the three week assay. The number of MSU1.1-WT and MSU1.1-US28 cells present had considerably decreased in all samples after 7 days of incubation. By three weeks, there were no cells visible in MSU1.1-WT and MSU1.1-US28 samples.

**Figure 11.** Soft Agar Assay at 0, 7, 14 and 21 days after plating cells at a density of $5 \times 10^3$ cells/well.
Focus Formation Assay

Focus formation assays incubated for 2 weeks at 37°C and 5% CO₂ had fresh media replaced every other day and were photographed using an both bright field and fluorescent microscopy. Figure 12 illustrates the growth of cells in between and on top of the mono-layer of MSU1.1-WT cells. Both MSU1.1-US28 (Figure 12: B & C) and MSU1.1-pHAGE (Figure 12: D & E) cells appear to have only grown in between the small spaces left after MSU1.1-WT cells had adhered. U87-YFP cells were shown as a positive control due to their malignant phenotype and loss of contact inhibition (Figure 11: F & G).

Figure 12. Focus Formation Assays were photographed after 2 weeks for (A) MSU1.1-WT, (B & C) MSU1.1-US28, (D & E) MSU1.1-pHAGE and (F & G) U87-YFP cell lines. Fluorescent images on the right were taken using a 488 nm blue filter to visualize ZsGreen or YFP.
**alamarBlue® Assay**

MSU1.1-WT and MSU1.1-US28 cells were plated at an initial density of 7.5 x 10^4 cells/mL in 100 µL volumes with eight replicates per cell line and 10 µL alamarBlue® reagent was added to each well in a clear-bottom 96-well plate. Plates were incubated at 37°C and 5% CO₂. After addition of alamarBlue®, plates were removed from the incubator after two hours (T0) and absorbance at 560 nm and 600 nm was measured every hour for four hours. When fitted with a trend line MSU1.1-US28 cells exhibited a 2.467 fold increase in rate of alamarBlue® reduction compared to the wild-type cells (Figure 13).

![Average % Reduction of alamarBlue®](image)

**Figure 13.** alamarBlue® reduction over 4 hours in MSU1.1-WT and MSU1.1-US28 cells plated at an initial density of 7.5 x 10^4 cells/mL. Percent reduction is shown as the means of eight replicates. Error bars indicate standard deviation and (*) designates p ≤ 0.05 between cell lines.

When this was repeated adding the MSU1.1-pHAGE cells, all at densities of 8.0 x 10^4 cells/mL, changes in alamarBlue® reduction were still observed (Figure 14).

MSU1.1-US28 cells showed a 2.303 and 2.201 fold increase in alamarBlue® reduction
compared to MSU1.1-WT and MSU1.1-pHAGE cells respectively. Means were analyzed by single factor analysis of variance where significant difference in means is indicated by $F > F_{\text{crit}}$ and $p \leq 0.05$.

**Figure 14.** alamarBlue® reduction over 6 hours in MSU1.1-WT, MSU1.1-US28 and MSU1.1-pHAGE cells plated at an initial density of 8.0 x 10⁴ cells/mL. Percent reductions are shown as the means of eight replicates for each cell line. Error bars indicate standard deviation. Data were analyzed with ANOVA single factor variance and (*) indicates that $F > F_{\text{crit}}$ and $p \leq 0.05$.

**Zymography**

Degradation of gelatin and casein substrates by MMPs in response to ectopic US28 expression was analyzed using zymography. MSU1.1-WT, MSU1.1-US28 and MSU1.1-pHAGE cells all showed similar activity levels of MMP-2 with a band of degraded gelatin substrate near 72 kDa (Figure 15 A, B & D). Interestingly, MSU1.1-WT cells tend to have lower MMP-9 activity than both MSU1.1-US28 and MSU1.1-pHAGE cells when 50 µg of total protein was added to each gel containing gelatin or casein.
substrate (Figure 15 A, C, E & F). When only 10 µg of total protein was added to the gel both MSU1.1-US28 and MSU1.1-pHAGE cells exhibited lower MMP-9 activity than the wild type cells (Figure 15 B & E). Statistical variance and significance are not shown due to a small sample number.

**Figure 15.** Casein and Gelatin Zymogram analysis of MMP activity. A and B represent the coomassie stained gelatin zymograms with either 50 or 10 µg total protein respectively. C depicts the casein substrate zymogram with 50 µg total protein. Image J software from the National Institutes of Health was used to analyze MMP-2 (~72 kDa) and MMP-9 (~92 kDa) activity in each of the gels—these data are shown in D-F.
CHAPTER FOUR: SUMMARY AND CONCLUSIONS

Introduction

Despite many recent studies and current treatments, GBM remains the most aggressive malignant brain tumor in adults with an overall survival of only 12 to 15 months (Ohgaki and Kleihues, 2005, Boxerman et al., 2013). The etiology for these tumors of glial origin is still unclear, but numerous studies have suggested a link between GBM and HCMV (Cobbs et al., 2002, Scheurer et al., 2008, Michaelis et al., 2009). First described by Cinatl et al. in 1996, this link has been assigned the term oncomodulation, which accounts for the ability of HCMV to alter tumor cell signaling pathways in the infected tissues (Cinatl et al., 2003, Dziurzynski et al., 2012). Changes in cell signaling pathways caused by HCMV gene expression may modify cell cycle progression, survival, invasiveness, evasion of the immune system, angiogenesis and modification of host gene expression through epigenetic remodeling of chromatin (Cinatl et al., 2003, Michaelis et al., 2011).

HCMV-Encoded Chemokine Receptor US28

HCMV possesses between 165 and 252 open reading frames, one of which encodes the chemokine receptor protein US28 (Stern-Ginossar et al., 2012). This protein has been shown to exhibit about 30% homology to the RANTES/CCL5 receptor and 36% homology to the CX3CL1 receptor in humans (Montaner et al., 2012). This homology gives US28 the ability to bind and become activated by a variety of chemokine ligands which elicit various downstream signaling effects in the host cell. In addition of binding
RANTES/CCL5 and CX3CL1 ligands, US28 may also bind MCP-1/CCL2 and MIP-1α/CCL3 (Gao and Murphy, 1994). Many cells in our bodies require stimulation by inflammatory cytokines TNF-1α and IL-1 to induce the expression of the ligand RANTES (Rathanaswami et al., 1993). To further understand the production of RANTES associated with HCMV infection, Michelson et al. infected human fibroblasts with two different laboratory strains of HCMV. Expression levels of RANTES were recorded at various times, noting marked increase in RANTES expression only eight hours after infection (1997). This dramatic increase in expression of RANTES was found to be independent of TNF-α and IL-1 activity. Uninfected human fibroblasts do not produce RANTES unless stimulated by exogenous factors like HCMV-encoded proteins. In a mechanism that is not well understood, viral particles have been shown to be necessary for RANTES upregulation in HCMV-infected tissues (Billstrom Schroeder and Worthen, 2001). The previous data suggest that in normal human fibroblasts, RANTES/CCL5 ligand expression is negligible due to the absence of HCMV viral particles.

Unlike the human homolog receptors for RANTES/CCL5 and CX3CL1, US28 also demonstrates constitutive activity when coupled with specific G-protein subunits Gβ/Gγ and Gαq or Gα11 (Figure 16). In the absence of ligand, US28 is able to maintain constant activity in Gαq or Gα11 G-protein subunits, while the augmented activation of Gα12, Gα16, and Gai is only induced through ligand-dependent signaling of US28. The constitutive activity of US28 causes activation of multiple signaling pathways including PLC, NF-KB, CREB, NFAT and MAPK. In HCMV-infected cells PLC activity eventually results in increased expression of the oncogenes c-myc, c-fos, and c-jun which induce the expression of immediate early genes IE1 and IE2. Transcriptional regulation
by NF-KB leads to upregulation of genes involved in cell proliferation and resistance to apoptosis, while CREB activates the transcription of genes involved in cell growth after exposure to growth factors and hormones. Interestingly, stimulation of NFAT is associated with increased angiogenesis and changes in immune response through expression of various cytokines and membrane proteins (Boomker et al., 2005). Taken together the activation of these signaling molecules and transcription factors may play a role in the oncomodulatory and oncogenic effects observed in US28-expressing cells.
Generation of a US28-Expressing Cell Line

US28 expression in mammalian cells can be studied by transfecting or transducing cells with an expression vector containing the US28 gene or through infection of cells with whole virus. During the latter, a virus containing a mutated or inactive form of US28 has been previously used for comparison in investigation of the specific roles of US28 activity in HCMV-infected cells. To study the effects of the single gene product on normal cellular function, it is beneficial to use the expression vector method to induce ectopic production of US28. This prevents the possible interactions between host and viral particles that result in the expression of RANTES/CCL5 as well as other possible cellular signaling factors. The effects of US28 expression, either ectopically or during HCMV infection, are highly dependent upon the cellular environment. This allows US28 to adapt its functions to a specific cell type and environment in a way that is the most advantageous for viral infection (Boomker et al., 2005).

To analyze the oncomodulatory constitutive activity of US28 in normal human cells, human foreskin fibroblasts (MSU1.1) were chosen as a model system (Morgan et al., 1991). By using this non-transformed cell line, interference with cancer-associated oncogenes could be minimized. Fibroblasts may also parallel the biological function of astrocytes, the cell of origin for GBM (Ohgaki and Kleihues, 2005). Both astrocytes and fibroblasts serve as supporting cells in their respective tissues in the brain and connective tissues, respectively. Fibroblasts play a crucial role in wound healing in the periphery through various steps including proliferation, remodeling of the extracellular matrix and migration. Through remodeling the area of tissue damage and the release of cytokines and chemokines, fibroblasts provide a framework for migrating cells to adhere and
proliferate (Darby and Hewitson, 2007). Similarly, astrocytes in the central nervous system respond to brain injury through enhanced proliferation within a few hours of damage. This proliferation results in migration of macrophages and other glial cells to the area of damage. Additionally, astrocytes have shown the ability to express matrix metalloproteinases for extra cellular matrix remodeling in response to tissue damage (Chen and Swanson, 2003). These similarities in biological functions make fibroblasts a good model system for investigating the transforming potential of US28 in brain tumor formation.

To generate a US28-expressing MSU1.1 cell line for this study, human fibroblasts were initially transfected with pEYFP-US28 by electroporation. This mammalian expression vector was designed to express the US28 protein with an N-terminal EYFP fluorescent tag (Figure 3, Figure 4). The N-terminus of US28 contains the ligand binding site on the extracellular surface of the cell. This construct was chosen versus the intracellular C-terminal end due to the G-protein coupling domain located in this region. Disruption of the G-protein coupling domain could potentially result in inactivation of downstream pathways if the G-protein subunits cannot associate with US28. After numerous attempts with electroporation transfection of MSU1.1 cells, fluorescence was not detected when cells were imaged using inverted fluorescent microscopy. It was determined that the EYFP protein was designed to function inside the cell versus outside which may have contributed to the absence of fluorescent detection. Furthermore, EYFP proteins form a dimer when fluorescing and may have experience steric hindrance in their spatial orientation as membrane bound US28 proteins would need to become close to one another.
In a collaboration with Don Shaffer at the Dana-Farber Cancer Institute and Harvard University, a US28-expressing MSU1.1 cell line was created using lentiviral transduction. A small amount of pIDTBlue-US28 plasmid was used to expand then isolate the US28 sequence for insertion into a pHAGE-IRES-ZsGreen lentivirus (Figure 7). The pHAGE-IRES-US28-ZsGreen lentivirus used to transduce the MSU1.1 cells results in constitutive expression of the US28 protein with a C-terminal ZsGreen fluorescent tag. Successfully transduced cells were sorted using FACS for ZsGreen fluorescence (Figure 8). ZsGreen, which belongs to the green fluorescent protein family, is derived from Zoanthus species of reef coral and has a robust fluorescence emission spectrum containing a peak at 506 nm (Matz et al., 1999).

Fluorescent signals from ZsGreen were imaged by confocal microscopy (Figure 9). Though differences in signal intensity between MSU1.1-US28 and MSU1.1-pHAGE cells are difficult to detect due to laser optimization, MSU1.1-US28 cells exhibited a much weaker fluorescent signal than MSU1.1-pHAGE. These differing intensities in fluorescent signal were observed using inverted fluorescent microscopy in the focus formation assay (Figure 12). Variations in intensity may be due to ZsGreen’s necessity to form a tetramer to elicit a fluorescent signal (Clontech). Cytosolic ZsGreen in the mock transduced cells likely forms a tetramer more easily compared to the tag form of ZsGreen on the membrane-bound US28 protein.

Additionally, western blot analysis, which is commonly used to detect specific protein expression in cells, confirmed the presence of ZsGreen tagged US28 protein in transduced MSU1.1 fibroblasts (Figure 10). Though a clear and intense signal was not observed, banding at approximately 71-76 kDa indicates the presence of US28 containing
the ZsGreen tag in MSU1.1-US28 cells (US28 = 41 kDa, ZsGreen = 30-35 kDa). This faint detection may be a result of low protein concentrations, possible antibody binding interference due to the ZsGreen tag and efficacy issues with the chosen Anti-CMV US28 primary antibody.

Evidence for Oncomodulatory Effects of US28

In order to classify US28 as an oncogene, several growth properties assigned to cancer cells must be elicited upon ectopic expression in normal cells. To assess anchorage independent growth, MSU1.1 cells (WT, US28 and pHAGE) and U87-YFP malignant glioma cells were suspended in a layer of 0.3% agar and cell viability was monitored for three weeks. Untransformed cells require a solid matrix for attachment, such as the bottom of a cell culture flask, while transformed cells remain viable regardless of attachment (Booden et al., 2005). In a qualitative analysis of growth and survival, the viability of MSU1.1-WT, MSU1.1-US28 and MSU1.1-pHAGE cells all decreased rapidly after suspension in a semi-solid matrix. Conversely, the U87-YFP cells were used as a positive control to exhibit cell viability despite the absence of a solid matrix (Figure 11). MSU1.1 cells do not exhibit a fully transformed phenotype with and without US28 expression due to their inability to survive and proliferate in a soft agar. Interestingly, constitutive, ligand-independent activity of many oncogenic G-protein coupled receptors usually elicits a signal too weak to allow anchorage independent growth (Booden et al., 2005). Future analysis of soft agar assays that include treating MSU1.1-US28 cells with RANTES may be warranted.
Loss of contact inhibition, another growth characteristic caused by oncogenes, was previously demonstrated in response to ectopic expression of US28 in NIH-3T3 mouse fibroblast cells (Slinger et al., 2010). Cells that exhibit loss of contact inhibition will grow on top of one another rather than changing the direction of growth upon physical interaction with another cell. When MSU1.1 cells were transduced with US28, loss of contact inhibition was not observed (Figure 12). As a positive control, U87-YFP malignant glioma cells were able to form foci on top of a monolayer of MSU1.1-WT cells. This indicates that, despite the loss of contact inhibition observed in mouse fibroblasts, ectopic US28 expression human fibroblasts does not induce a transformed phenotype. Boomker et al. also suggests that G-protein coupled receptors may require ligand binding activity in focus formation assays (2005). Prospective studies on MSU1.1-US28 cells may also include RANTES treatment in the cell culture media to determine the effects of ligand binding activity on loss of contact inhibition in human fibroblasts.

Through synchronization of NIH-3T3 mouse fibroblasts transfected with wild-type and inactive mutated US28, Maussang et al. showed that increased cell cycle progression was dependent upon US28 activity (2006). The proliferation index, indicated by the ratio of cells in S or G2/M phases to cells in G0/G1 phases, in wild-type US28 expressing mouse fibroblasts was an average of two times higher than mock-transfected and untransfected cells (Maussang et al., 2006). Previous studies have also shown that metabolic activity of cells directly correlates to the proliferative activity in vitro (Kanemura et al., 2002). By utilizing the alamarBlue® reagent for cell viability, metabolic activity of MSU1.1 cells was analyzed in a colorimetric assay based on the reduction of resazurin to resorufin. This results in a color change from blue to red in
metabolically active cells that was quantified using absorbance readings at 560 nm and 600 nm wavelengths. The rate of reduction of alamarBlue® in MSU1.1-US28 cells was found to be 2.303 and 2.201 times higher than in MSU1.1-WT and MSU1.1-pHAGE cells respectively (Figure 14). These results are consistent with previous findings analyzing the effects of US28 activity on proliferation in mouse fibroblasts. The observed increased metabolic activity correlates with an increased rate of proliferation induced by the constitutive activity of US28, suggesting that cell growth and survival in the tumor environment is promoted by US28 activity.

In order to grow and expand, a tumor must make room for itself by remodeling the extracellular environment. Various studies have explored the invasive and migratory potential of cells expressing US28 through chemokine dependent activation of pathways previously shown to induce MMPs activity such as FAK, Src, ERK1/2, Pyk2 and RhoA (Vomaske et al., 2009). MMPs that are released from the cell break down collagen and other extracellular matrix components which aids in cell motility. This study sought to find a relationship between the constitutive activity of US28 and MMP activity in human fibroblasts through gelatin and casein zymography. By separating secreted proteins from concentrated conditioned media by SDS-PAGE, activity of specific extracellular matrix remodeling proteins (MMP-2 and MMP-9) were analyzed. The constitutive activity of US28 in MSU1.1 cells appeared to have no effect on MMP-2 activity while MMP-9 activity seemed to increase in both US28-transduced and mock-transduced cells compared to wild-type (Figure 15). In one instance, with only 10 µg of total protein added to the gel MMP-9 activity appeared to decrease slightly in both the US28-transduced and mock-transduced cells. Because of a small sample size, statistical variance
and significance were not calculated. Further analysis of MMP activity in MSU1.1 cells may expose a potential connection to the expression of constitutively active US28 though it may not be likely due to the tendency for mock-transduced cells to mimic this behavior in US28 expressing cells.

**Overall Conclusions**

This project ruled out a number of roles for the constitutive activity of US28 in oncomodulation of human cells. Previous studies examining the constitutive activity of US28 and other virally-encoded G-protein coupled receptors in a mouse model have revealed a significant role in the survival of the virus and host invasion. Additionally, it appears that in some cases US28 hijacks the specific signaling cascades involved in oncogenesis and cardiovascular disease through ligand-independent pathways (Kralj et al., 2013). The data presented in this study are consistent with previous statements regarding the apparent cell-type dependence and specificity of US28 activity (Vomaske et al., 2009). Although previous studies have demonstrated the transforming capabilities of ectopic US28 expression in mouse fibroblasts, this study revealed no substantial changes in cell invasive potential, anchorage independence or loss of contact inhibition when US28 was introduced into MSU1.1 human fibroblasts (Maussang et al., 2006, Maussang et al., 2009, Soroceanu et al., 2011, Streblow et al., 1999). However, these data do suggest a role for constitutive US28 signaling in increased metabolic activity and proliferative potential in human fibroblasts. The major pathways involved in this remain unclear, but STAT3, NF-KB, β-catenin and cyclin D1 have been implicated in a proliferative phenotype observed in US28-expressing cells (Slinger et al., 2010,
Maussang et al., 2006, Langemeijer et al., 2012, Vomaske et al., 2009). Analysis of alamarBlue® reduction by cells expressing US28 revealed a 2.303 fold and 2.201 fold increase from both MSU1.1-WT and MSU1.1-pHAGE cells respectively. This confirms that the constitutive activity of US28 increases the metabolic activity and proliferative phenotype in human fibroblasts. Additional analysis of the specific proteins involved may improve the understanding of US28’s ability to promote cell cycle progression in a ligand-independent manner.

Together these data provide a better understanding of US28’s oncomodulatory effects due to the constitutive activity in non-transformed human fibroblasts. By using human fibroblasts as a model, this study also attempted to parallel the biological functions of astrocytes, the suggested cell of origin for GBM. Further investigations may be warranted to explore the ligand-independent properties of US28 expression in both non-cancerous astrocytic and malignant glioma cell lines. The Upper Michigan Brain Tumor Center also has a US28-expressing malignant glioma cell line, U87-US28. Despite the inability of ectopic US28 expression to induce transformation of MSU1.1 fibroblasts, US28 may still prove to be a potential target in cancer therapy due to its reported prevalence in GBM and previous confirmations of oncomodulation.
REFERENCES


Induction of p21 WAF1/CIP1 and Cyclin D1 Expression by the Src Oncoprotein in Mouse Fibroblasts: Role of Activated STAT3 Signaling. *Oncogene*. 19, 5419-5427.


