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The Oncolytic Potential of Tanapoxvirus to Treat Retinoblastoma

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THE ONCOLYTIC POTENTIAL OF TANAPOXVIRUS TO TREAT RETINOBLASTOMA

by

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Retinoblastoma is the leading cause of intraocular malignancy in children under 2 years of age. Current treatments, while generally effective in eliminating the tumor, have many negative side effects. Oncolytic virotherapy is the use of either wild type or recombinant viruses, to destroy tumor cells. There are currently several viruses being engineered as potential therapeutics for a wide range of cancers. They offer tumor specific alternative to current therapies with lesser toxicity. This study looked at the oncolytic efficacy of multiple tanapoxviruses (TPV) recombinants in an in vitro retinoblastoma. Here we have tested recombinants of TPV for their ability to replicate in two retinoblastoma cell lines; Y-79 and WERI-RB1. To ascertain if engineered oncolytic TPV could affect retinoblastoma cells in vitro, multiple different assays were used, testing replication competence of virus, ability to express transgenes and induce cell death. Single step replication studies using two multiplicities of infections (MOIs) of each virus and their dose dependent effect on cell viability was investigated. Our results demonstrated that retinoblastoma cells were successfully infected, transgenes were expressed, competent replication occurred at both MOIs and all viruses caused a significant decrease in the number of living cells and demonstrated that the virus lysed the tumor cells. These results show engineered TPVs and recombinants to be a potential candidate as a onocolytic virotherapy for retinoblastoma.

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John D. Christie

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INTRODUCTION

Cancer is a disease of uncontrolled cellular growth. Though evolution has provided tight controls of cellular growth and division, and molecular pathways to deal with rogue cellular growth, sometimes it is not enough to prevent oncogenesis (Casas-Selves and DeGregori 2011). Cancer in the United States represents the second leading cause of death, just behind heart disease. One in two men and one in three women will be diagnosed with cancer in their lifetimes (Xu et al 2010). As such, it represents one of the largest public health concerns facing the United States. Current treatments typically treat cancer as one disease, not as a broad class of diseases with similar features (Kesley et al 2012). In children, cancer represent one of the biggest causes of mortality and morbidity (Greenlee et al 2000). Though rare, childhood cancers represent a 1 in 300 incidence rate before the age of 20 (Hewitt et al 2003), as compared to 1 in 3 adults that will develop cancer in their lifetimes (Perkin et al 2010; Sasieni et al 2011). Even still childhood cancer still represents a major challenge to public health (Casas-Selves and DeGregori 2011). Cancers that occur during childhood can be classified as fast track cancers. This is because there is evidence that they require less genetic mutations to form tumors compared to adult cancers (Dyer and Brenner 2005; Dyer 2004). This is because they are thought to arise from cells that have more intrinsic tumor-like qualities, such as inherent resistance to apoptosis and necrosis (Chen et al 2004; Hanhan and Weinberg 2000). Retinoblastoma is one such cancer that is thought to arise from cells that are more intrinsically tumor-like (Dyer and Brenner 2005). In this study a novel potential treatment of retinoblastoma using recombinant tanapoxviruses were used in an *in vitro*

retinoblastoma model. This research was conducted under the hypothesis that oncolytic TPV would be able to replicate and destroy retinoblastoma cells. To do this four assays were used, an absorption assay, microscopic imaging, single step replication studies and finally a CCK-8 assay.

Retina

Retinoblastoma is a cancer of the retina and is the topic of this thesis. In order to understand where retinoblastoma typically occurs in the eye, one must have a basic understanding of the retina. The retina is located at the back of the eye, and is the tissue responsible for the transduction of light from the environment into electrical information for processing in the brain. The retina is made up of 5 distinct types of cells, each with a distinct function in the transduction and conveyance of light information. When light enters the retina, phototransduction occurs in the back of the retina in the photoreceptor layer. Rods react to dim light stimulus, while cones react to specific wave lengths of visible light and are responsible for detection of colors. For phototransduction to occur, light causes a change in the confirmation of the opsin protein located in the outer segment of photoreceptors. This change of confirmation causes a second messenger cascade and the cessation of glutamate release, which normally occurs when the photoreceptor is not being stimulated (Pungh Jr et al 1999). At this point, information is conducted to the next layer of the retina, the inner nuclear layer. In the inner nuclear layer there are three types of cell bodies found; horizontal cells, bipolar cells, and amacrine cells (Kolb 2012). The cessation of the release of glutamate from the photoreceptors affects the bipolar cells, because this is where the photoreceptor synapses. Depending on the type of bipolar cell,

the cessation of glutamate release can have opposite effects. On-bipolar cells are excited by the cessation of glutamate from photoreceptors and will cause the activation of a second messenger pathway. However, off-bipolar cells are inhibited and will be hyperpolarized, due to an ionotropic response and closure of sodium and potassium permeable channels (Pang et al 2012). Amacrine and horizontal cells act as inhibitory neurons that help with visual acuity. Finally, the information is passed from the inner nuclear layer to the ganglion cell layer. This layer contains the retinal ganglion cells, which are the first cells that will produce action potentials (Meister et al 1991) to send visual information to the visual cortex through the optic nerve (see supplemental figure 1 for retinal anatomy).

Retinoblastoma

Retinoblastoma is a leading cause of tumors in children under the age of two, and in early childhood, it is the leading cause of intraocular malignancy (Gogate et al., 2011). In an average year, as many as 8,000 children globally are diagnosed with retinoblastoma and as many as 4,000 children will die as a result. Historically, it also has been a leading cause of blindness in children due to the progression of the disease itself, as well as surgical procedures that are required to excise the tumor (Huston et al., 2011). The disease typically manifests in two different forms; a unilateral and bilateral form. The unilateral form is the most common form of retinoblastoma, accounting for almost 67% of cases, with a tumor in one eye while leaving the other eye unaffected. Bilateral retinoblastoma is far less common, accounting for approximately 33% of cases, and is characterized by tumor development in both eyes of the affected individual. Bilateral

retinoblastoma also manifests itself at an earlier age than the unilateral form (Huston et al., 2011). The overall incidence rate of retinoblastoma is about 1 in 17,000 births and in the developed world has a survival rate of almost 100%. In the third world, rates of survival drop drastically to under 40% (Huston et al., 2011; Ali et al., 2010).

Retinoblastoma is one of the most studied and mechanistically well-understood cancers. Most retinoblastoma tumors start with mutations leading to a dysregulation of the RB1 gene. PRB is a tumor suppressor protein important in its regulation of cell cycle progress through its binding and inhibition of the transcription factor E2F. The loss of function of the pRB can lead to genomic instability. While this mutation by itself does not lead to development of a tumor, it has been hypothesized to allow for further deleterious mutations to accrue due to increased genomic instability (van Harn et al 2010; Zheng et al 2002). Mutations to the p107 and p130 genes, which have analogous function to pRB, have also been found to be prevalent in retinoblastoma tumor cells (Laurie et al 2006). Somatic mutations of the RB1 gene can lead to sporadic, non-heritable unilateral retinoblastoma. Mutations in germline cells of RB1 can lead to bilateral and multifocal retinoblastoma (Thériault et al., 2014). However, recent research has shown that mutations of RB1 are not present in all retinoblastoma tumors. A small percentage of early onset cases of retinoblastoma has been found to be due to amplification of MYCN. MYCN is a transcription factor usually associated with neurodevelopment. Amplification of MYCN has been identified as a common cause of other aggressive neuroblastoma and usually leads to poorer outcomes when compared to other types of neuroblastomas (Rushlow et al 2013; Thériault et al 2014).

Retinoblastoma origin

The cell of origin of retinoblastoma has not yet been found, but it is known that these cells are very closely related to the retinal progenitor cells that generate all other cell types found in the retina (Dyer and Brenner 2005; Zhang et al 2004). Retinal progenitor cells are multipotent stem cells that are mitotically active (Gallie et al 2004). They are found in mammals mainly during development, though recent research has found that there are small populations of these multipotent cells still present in adulthood in some mammals (Li et al 2013). There are currently two non-mutually exclusive retinoblastoma hypotheses concerning the cell of origin (Dyer and Brenner 2005). The first is that the cells, which give rise to retinoblastoma, are in fact, the progenitor cells themselves. In this hypothesized model, the mutations to the RB gene occur early in development and lead to a large subpopulation of continually replicating progenitors. Without the RB checkpoint, new mutations can occur leading to formation of distinct, heterogeneous population of cells expressing any combination of retinal markers. Evidence for this model can be found in patient tumors, which show large variation in cell specific markers which are unique to individual cell types found in the retina. This model is only valid though if RB is the limiting step of cellular division in the progenitor cells. If it is limited by a different protein, then a second explanation is necessary.

The second hypothesis involving retinoblastoma cell origin is that the cells arise during the transitional period, after the cells are no longer progenitor cells, but are not yet terminally differentiated. These cells are post-mitotic cells, which have restricted cell fates. In these cells, it is already known that pRB is, in part, one of the proteins responsible

for the post-mitotic phenotype. For this to be true, loss of function of RB would not cause a change in the number of progenitor cells, and S and M phase cells would have to be found in areas of the retina where progenitor cells are not known to exist (Dyer and Brenner 2005; MacPherson 2008). Evidence for this hypothesized model comes from work with RB-null mice. In these mice, it was found that deactivation of RB and p107, another cell cycle modulating protein, did not increase the count of retinal progenitor cells found in the retina (Chen et al 2004; Bremner et al 2004). This study also found cells in S-phase cells in layers which retinal progenitor cells are not known to be found. Another possible transitional cell that has been hypothesized as a possible cell of origin are the photoreceptor precursor cells. Studies using fetal retinal cells with depleted RB protein and knockdown of p130 showed a retinoblastoma-like cellular proliferation (Xu et al 2014; Xu et al 2009).

Current retinoblastoma treatments

Current treatment strategies for retinoblastoma are typically limited to three options: chemotherapy, radiation and surgery. Chemotherapy is the most common treatment used to shrink intraocular tumors. Application of chemotherapeutics in retinoblastoma usually is systemic, but in advanced cases, or bilateral forms, systemic chemotherapy is combined with targeted chemotherapies. While outcomes in those who receive chemotherapy tend to be positive, there are obvious side effects and drawbacks. The side effects range from mild inflammation and loss of appetite to more severe side effects including renal toxicity, neurological side effects and development of leukemia (Chintagumpala et al 2007). Finally, there is always the risk that tumors will become

resistant to chemotherapeutics (Chintagumpala et al 2007). Another common treatment is surgical enucleation. Enucleation is the removal of the eye and part of the optic nerve, leaving orbital muscles intact. Enucleation is the preferred treatment in late stage retinoblastoma or in unresponsive cases because it limits mortality, morbidity and possible metastatic spread to other tissues (Ghassemi and Khodabande 2014). The obvious side effect of this treatment is the complete loss of sight in the treated eye. This side effect, though it does not cause a dramatic change in the quality of life for a child, has caused parents to avoid treatment or opt for chemotherapy to potentially preserve sight (Dimaras et al 2012). Finally, radiation has also been used to treat retinoblastoma. Radiation therapy can either be done by external direct beam radiation (EBR) or by brachytherapy. EBR has been shown to have up to 95% preservation rate of effected eye, which makes it an attractive treatment. This comes with an increased risk of secondary cancers manifesting later in life (Kim et al 2007; Kleinerman 2005). Brachytherapy, which is the implantation of a radioactive implant, on the other hand is much less likely to lead to secondary malignancies but comes with the risk of loss of site through side effects such as optic neuropathy, radiation induced retinopathy and development of cataracts. While these treatments have been shown to be effective in treatment of retinoblastoma, new treatments strategies must also be studied (Chintagumpala et al 2007) given the current treatment side effects.

Viral oncolytic therapy

Over the last 100 years, the use of viral oncolytic therapy as a potential treatment for cancer has been met with various degrees of scientific interest. The first observation of viral oncolytic potential was observed due to incidental infections or after vaccination (Kelly and Russell 2007). In spite of these observations, viral oncolytic therapy did not garner much interest until the 1980s, with the first studies using recombinant Herpes Simplex Virus. These studies used HSV viruses with the thymidine kinase gene ablated (Peters et al 2015). The ablation of this gene restricted the replicative ability in healthy tissues, but because of the abundance of thymine in most cancers, allowed for competent replication in these tissues. Newer studies have shown that other genes, usually immune regulatory genes, also beneficially restricted the replicative ability of virotherapies in healthy tissues. Since these first studies, many other viruses have been designed as potential oncolytic agents. These include viruses in the Adenoviridae, Picornaviridae, Reoviridae and Poxviriade (Kelly and Russell, 2007; Chan and McFadden, 2014).

Members of the Poxviridae family of viruses have shown great promise as potential oncolytic viruses. The main reason for their potential is that they are large double-stranded DNA viruses. Their genomes contain multiple genes, which are responsible for tropism, immune modulation and replication (Chan and McFadden, 2014). This allows for many different strategies of manipulation that can tailor viruses to many different cancers. The large genome also allows these viruses to accommodate multiple transgenes, which have also been shown to increase their oncolytic potential. Other poxviruses that have

studied as potential oncolytics include orthopoxvirus vacciniavirus, and leporipoxvirus myxmomavirus (Chan and McFadden, 2014).

Tanapoxvirus

Tanapoxvirus (TPV) is a member of the Yatapoxvirus genus of the Poxviridae family of viruses. Infection with TPV leads to a mild, self-limiting infection that features a febrile illness and minor skin eruptions that may last up to 6 weeks. No documented cases of human-to-human transmission have been cited during limited outbreaks of TPV (Downie et al., 1972). Recent research with TPV has shown it to be an effective candidate for viral oncolytic therapy. Studies in colorectal cancer and in melanoma have shown various degrees of success in the ability for TPV to replicate in different tumor lines. Previous research has also shown that neural derived tumor TPV, is an example of a virus that can be engineered to serve as an oncolytic agent (Conrad et al 2015). TPV has a large viral genome, and most genes code for protein with one distinct function, which allow for predictable outcomes with gene ablation. It also has only been shown to cause very mild disease in a small percentage of causes (Nazarian et al 2007; Downie et al., 1972). Finally, most people in the developed world are immunologically naïve to TPV, unlike herpesvirus and adenovirus constructs (Thomas et al 2003). Studies using TPV have investigated its efficacy in multiple *in vitro* experiments and have started to move into whole animal studies (Conrad et al 2015; Lee and Essani 2010; Kurban et al., 2015).

The genes selected for ablation and for addition in oncolytic therapies for this retinoblastoma study fall into two broad categories: Those that restrict the replicative ability of TPV and those that repress or stimulate the immune system. The first class contain viruses with ablations; specifically, the 66R thymidine kinase knockout and the 15L neuregulin knockout. The thymidine kinase knockout is a common target for ablation in large DNA viruses being engineered for oncolytic virotherapy. The basis of this is because most tumors are made up of cells undergoing constant cellular division. In order for this to occur, there must be a constant supply of thymidine triphosphate (TTP). Under normal circumstances TTP is not found in high amounts in the cell, unless the cell is in S-phase, as the triphosphate form is only necessary for DNA replication and repair (Eker 1965; Spyrou 1988). This confers an advantage, as all other triphosphorylated nitrogenous bases are necessary for RNA synthesis, energy storage, or second messenger pathways, meaning they must be abundant during all cellular phases. DNA viruses, and RNA viruses that require a DNA intermediate, require a supply of TTP in the cell for amplification of their own genomes, or genomic intermediates. Large DNA viruses, such as Pox viruses and herpes viruses have virally encoded thymidine kinase, which allows them to triphosphorylate thymine independent of host machinery (Nutter et al 1985;). The ablation of this class of gene should lead to the restriction the virus's replicative ability to only those that are undergoing active cellular division (McFadden 2005; Kim and Throne 2009). However, it is still possible that low level replication could occur in non-replicating cells, as small stores of TTP are necessary for genomic repair (Spyrou 1988).

The second gene targeted for ablation in these viral oncolytic studies fall into the category of potentially restricting viral tropism. This gene is the 15L, which is a virally encoded neuregulin. Neuregulin is a member of the epidermal growth factor (EGF) family of growth factors (Jeng et al 2013; Shneider and Wolf 2008). These growth factors bind to epidermal growth factor receptor (EGFR) family of receptors. When these receptors are activated proliferation and differentiation can occur. This happens through activation of a second messenger pathway; primarily though the JNK, Akt and MAPK pathways (Calvo et al 2011). While the precise function of the 15L virally encoded neuregulin protein is not yet known, there is evidence that it can play a non-essential role in the ability of TPV to replicate (Jeng et al 2013). In spite of this lack of knowledge, it is known that upregulation of the EGFR has been identified in multiple cancers. One study with the 15L neuregulin gene product on melanoma showed that it increased proliferation of the cancer (Esparis-Ogando et al 2002). Knocking out a gene product, that may cause further activation of this pathway, should decrease the risk of stimulating further tumor growth (Zhang et al 2016, Unpublished).

The next class of viral TPV genes manipulated in these studies involve those which impact the immune system. These genes include one that was targeted for ablation; the 2L tumor necrosis factor inhibitory protein (Brunetti et al 2003). Tumor necrosis factor (TNF) is a cytokine produced by macrophages, T-helper cells, natural killer cells, mast cells, eosinophils and neurons (Grivennikov et al 2005; Ohtori et al 2004). TNF acts a pyrogen, induces inflammation, can induce apoptosis, and acts as an inhibitor of tumorigenesis and replication of viruses. It was originally discovered and subsequently

named for its ability to reduce tumors in *in vivo* cancer models (Carswell et al 1975). Through the activation of the NF-kB pathway, it can lead to the maturation, proliferation and development of T-cells, hepatocyte cells and a few cancers (Kishimoto et al., 1992, Ledda-Columbano et al 1998; Imbert et al 2001; Wang and Lin 2013). Alternatively, it can also cause apoptosis though the JNK pathway (Liu et al 2002). The role of TNF inhibition in TPV is likely two fold, first to prevent apoptosis of infected cells while the virus is proliferating and second to slow the progression of the host immune response to the viral infection (Brunetti et al 2003). The knockout of this gene should lead to a reduced ability for the ablated virus to replicate in healthy tissues, compared to tumor tissue, as many tumors dysregulate TNF production as part of tumorigenesis. That being said, it should not be taken for granted that some cancers are able to use the proliferative properties of TNF to enhance their ability to continue divisions (Imbert et al 2001).

Some recombinant oncolytic TPV, all of which contain at least the 66R TK knockout, contain immunostimulatory transgenes. The three chosen for this study are FliC, GM-CSF, and CCL2. The first gene that was chosen for this study was the bacterial flagellum protein (FliC) from Salmonella enterica (Conrad et al 2015). FliC is an important indicator protein used by the immune system to detect bacterial infection. This detection is by activation of the toll-like receptor 5 (TLR-5) through the binding of FliC (Yoon et all 2012). TLR-5 expression is found predominantly on cells of the innate immune system such as macrophages, monocytes and dendritic cells (Salazar-Gonzalez, and McSorley 2005). Activation of the TLR-5 response, like most TLR responses, leads to activation of the Myeloid differentiation primary response gene 88 (MyD88) pathway. This activation

will lead to the final activation of two pathways, the NF-kB pathway and the JNK pathway (Takeda et al 2004). The activation of these pathways through the binding of TLR-5 in monocytes, macrophages and dendritic cells, leads to secretion of proinflammatory cytokines. It can also lead to the production of interleukin-8, which acts as a chemoattractant for neutrophils, macrophages, and mast cells, among others (Takeda et al 2004; Salazar-Gonzalez, and McSorley 2005). The attraction of cells of the innate immune system and activation of the MyD88 pathway has been shown to lead to tumor necrosis and regression (Rhee et al 2008).

The next gene chosen for this study due to its immunostimulatory effects was GM-CSF. GM-CSF stands for granulocyte-macrophage colony stimulating factor. GM-CSF is a cytokine that is secreted by a wide range of cells of both the innate and adaptive immune system, including macrophages, NK cells, mast cells, and T-cells (Gupta and Emens 2010). When secreted, it causes myeloblasts to differentiate into granulocytes and monocytes. It has also been shown as a potential chemoattractant of leukocytes, which would cause recruitment of immune cells to the site of secretion. As with the activation of the innate immune system through the TLR-5 pathway, recruitment of innate immune cells to tissue infected with GM-CSF expressing virus should increase oncolytic ability of the virus. The use of GM-CSF as a cancer therapeutic is being investigated in various ways, including oncolytic virotherapy, and as cancer vaccines that cause tumors to express the gene (Kushner and Cheung 1989; Gupta and Emens 2010).

The final gene that was selected for its immunostimulatory properties in this study was CCL2. CCL2 is a chemokine that contains a C-C motif, meaning two cysteines next to each other near the amino-terminus of the protein (Zhang et al 2010). It is a chemoattractant chemokine, which is important for recruitment of dendiric cells, monocytes and T-cells (Conti and Rollins 2004). It is usually a product of an inflammatory response. CCL2 has been found to be expressed as part of the interferon antiviral pathway and also an important in the activation of leukocytes in response to viral infection. CCL2 has also been found to act as a stimulate monocyte recruitment to sites of certain cancers, such as prostate (Loberg et al 2006)

To engineer these viruses, the p2KO vector was used. P2KO is based on the plasmid vector pBluescript II KS (+), which is a commonly used commercial vector system. Flanking sequences from desired wild type genes were inserted at specifics sites in the plasmid vector to allow for targeted ablation of desired genes (specifically 2L, 15L, and/or 66R). This also allowed for site-specific insertion of transgenes (specifically mCCL2, mGM-CSF, or FliC) and fluorescent reporters into the genome of the viruses. All genes also included the synthetic vaccinia virus early/late promoter. To achieve ablation and transgene insertions, transfection/infections were done. Both the wild type virus and the plasmid used for this study would transfect/infect cells, allowing for double crossover homologous recombination to occur. This allowed for both the genes of interested to be inserted into the viral genome while also ablating targeted genes. The presence and absence of genes were then confirmed using PCR and gel electrophoresis (Conrad et al 2015).

To assess the use of viral oncolytic therapies for retinoblastoma, these studies were designed to determine the efficacy of wild type TPV and a series of recombinant TPV using *in vitro* assays. This study was necessary because other purposed oncolytic candidates have only been shown to work in high titers and use viral constructs based on circulating viruses. Oncolytic TPV has been shown to work at much lower viral concentrations in other studies. Current viruses were generated using current knowledge based on both past experiments using TPV and other oncolytic viruses.

Specific aims

Retinoblastoma represents the leading cause of intraocular malignancy in children. Though retinoblastoma is one the most studied cancers, current treatment paradigms focus on reducing morbidity and mortality; not on saving sight in the affected eye. Current treatments also come with a high cost later in life, which include the emergence of secondary cancers, sterility and blindness. While our understanding of the changes that drive oncogenesis has increased, so has the understanding that new treatment strategies would benefit effective treatment. Oncolytic virotherapy is one such strategy that is currently being pursued, as it offers a tissue specific, low cost and low side effect alternative to current treatment strategies. Current research in Karim Essani's laboratory of virology has been testing oncolytic tanapoxvirus as a broad spectrum oncolytic virotherapy in a number of different cancers. To continue this research, the efficacy of TPV in an *in vitro* retinoblastoma model is the focus of this thesis. It is hypothesized that

recombinant TPV can be used to infect kill retinoblastoma cell lines. To test this hypothesis, two specific aims have been designed.

1. To determine the ability of wild type tanapoxvirus (TPV) and recombinants to adsorb, infect, and express transgenes in multiple retinoblastoma cell lines.

In vitro studies in the Karim Essani lab have shown TPV and oncolytic mutants have the ability to adsorb, infect, and express transgenes in other tumor cell lines of other tissues. However, each cancer derives from distinct tissues and insights about the efficacy of TPV as an oncolytic candidate can only be understood when studied in each specific tumor cell type. Viral tropism studies have shown that TPV does not have as broad tissue tropism as other members of Poxviridae, however there is evidence that TPV is able to infect glioblastoma cell lines (Lee and Essani 2010). These results suggest that TPV may be an oncolytic candidate for retinoblastoma. Therefore, in this study, retinoblastoma cells lines were experimentally infected with wild type TPV and recombinant viruses at two concentrations. This allowed for the adsorptive ability of TPV to be determined. Following adsorption and infection of TPV and oncolytic recombinant virus studies, experiments were performed looking for evidence of transgene expression. These studies included ELISA and imaging procedures that looked for the expression of fluorescent protein reporters on the same open reading frame (ORF) as the transgene knocked into each virus (Conrad et al 2015; Kurban et al., 2015). These studies established TPV as a transgene vector which could lead to immune activation.

2. To determine the ability for wild type TPV and recombinant mutants to go through competent replication and cause cellular inviability.

To be a successful oncolytic therapy, TPV and mutants must be able to not only infect retinoblastoma cells and express transgenes, but also replicate and cause cell lysis. The mechanism for viral entry for most members of poxviriade are not well established and it has been hypothesized that the limiting factor is not the ability for members of poxviriade to gain entry into cells of different tissues, but their ability to make replication competent progeny (Moss 2006; Moss 2012; Nazarian et al 2007). To determine replicative ability, each retinoblastoma cell line was experimentally infected with each virus and incubated for predetermined amounts of time. Furthermore, even with viral replication, physical lysis of tumor cells is the ultimate goal of viral oncolytic therapies. To test this cell viability, assays were established and used on experimentally infected retinoblastoma cell line to determine if there were changes in the number of viable cells compared to controls. The results of this study have been used as preliminary data for in vivo studies in which tumors are induced in a nude mouse model and experimentally treated with wild type TPV and recombinant and transgene expressing viruses. The significance of this study is that recombinant oncolytic TPV represents a potential viral oncolytic treatment option for retinoblastoma.

MANUSCRIPT

The oncolytic potential of tanapoxvirus to treat retinoblastoma

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Abstract

Retinoblastoma is the leading cause of intraocular malignancy in children under 2 years of age. Current treatments, while generally effective in eliminating the tumor, have many negative side effects. Oncolytic virotherapy is the use of either wild type or recombinant viruses, to destroy tumor cells. There are currently several viruses being engineered as potential therapeutics for a wide range of cancers. They offer tumor specific alternative to current therapies with lesser toxicity. This study looked at the oncolytic efficacy of multiple tanapoxvirus (TPV) recombinants in an *in vitro* retinoblastoma cell culture system. Here we have tested TPV recombinants for their ability to replicate in two human retinoblastoma cell lines. Virus infected cells were tested for ability to express transgenes and induce cell death. Single step replication studies using two multiplicities of infections (MOIs) of each virus and their dose dependent effect on cell viability was investigated. Our results demonstrated that retinoblastoma cells were successfully infected, transgenes were expressed, competent replication occurred at both MOIs and all viruses caused a significant decrease in the number of living cells and demonstrated that the virus lysed the tumor cells. These results provide preliminary evidence that TPV recombinants are potential candidate for oncolytic virotherapy of retinoblastoma.

Introduction

Cancer in the United States represents the second leading cause of death, just behind the heart disease. One in two men and one in three women will be diagnosed with cancer in their lifetime [1]. As such, it represents one of the largest public health concerns facing the United States. Current treatments typically treat cancer as one disease, not as a broad class of diseases with similar features [2]. In children, cancer represent one of the biggest causes of mortality and morbidity [3].

Retinoblastoma is a leading cause of tumors in children under the age of two, and in early childhood, it is the leading cause of intraocular malignancy [4]. In an average year, as many as 8,000 children are diagnosed with retinoblastoma and as many as 4,000 children will die as a result. It also has been a leading cause of blindness in children due to the progression of the disease or surgical procedures that are required to excise the tumor [5]. The disease typically manifests in two different forms; a unilateral and bilateral form. The unilateral form is the most common form of retinoblastoma, accounting for almost 67% of cases, with a tumor in one eye while leaving the other eye unaffected. Bilateral retinoblastoma is far less common, accounting for approximately 33% of cases, and is characterized by tumor development in both eyes. Bilateral retinoblastoma also manifests itself at an earlier age than the unilateral form [5]. The overall incidence rate of retinoblastoma is about 1 in 17,000 births and in the developed world has a survival rate of almost 100%. In developing countries, rates of survival drop drastically to under 40% [5, 6].

Retinoblastoma is one of the most studied and mechanistically well understood cancers. Most retinoblastoma tumors start with mutations leading to a dysregulation of

the *RB1* gene. PRB is a tumor suppressor protein involved in the regulation of cell cycle progress through its binding and inhibition of the transcription factor elongation factor 2 (E2F). This mutation by itself does not lead to development of a tumor, but has been hypothesized to allow for further deleterious mutations to accrue. Somatic mutations of the *RB1* gene leads to sporadic, non-heritable unilateral retinoblastoma. Mutations in germline cells of *RB1* can lead to bilateral and multifocal retinoblastoma [7]. However, recent research has shown that mutations of *RB1* are not present in all retinoblastoma tumors. A small percentage of early onset cases of retinoblastoma are due to amplification of *MYCN.* MYCN is a transcription factor usually associated with neurodevelopment. Amplification of *MYCN* has been identified as a common cause of other aggressive neuroblastomas and usually leads to poorer outcomes when compared to other types of neuroblastomas [7,8].

Current treatment strategies for retinoblastoma are typically limited to three options: chemotherapy, radiation and surgery. Chemotherapy is the most common treatment used to shrink intraocular tumors. Application of chemotherapeutics in retinoblastoma usually is systemic, but in advanced cases, or bilateral forms, systemic chemotherapy is combined with targeted chemotherapies. While outcomes tend to be positive in those who receive chemotherapy, there are adverse effects and drawbacks. The adverse effects range from mild inflammation and loss of appetite to more severe side effects including renal toxicity, neurological side effects and development of leukemia [9].

Finally, there is always the risk that tumors will become resistant to chemotherapeutics [9]. Another common treatment is surgical enucleation. Enucleation

is the removal of the eye and part of the optic nerve, leaving orbital muscles intact. Enucleation is the preferred–treatment in late stage retinoblastoma or in unresponsive cases because it limits mortality, morbidity and possible metastatic spread to other tissues [10]. The obvious side effect of this treatment is the complete loss of sight in the treated eye. This side effect, though does not cause a dramatic change in the quality of life of a child, but has caused parents to avoid treatment or opt for chemotherapy to potentially preserve sight [11]. Radiation has also been used to treat retinoblastoma. Radiation therapy can either be done by external direct beam radiation (EBR) or by brachytherapy. EBR has been shown to preserve up to 95% of the effected eye, which makes it an attractive treatment. This comes with an increased risk of secondary cancers manifesting later in life [12, 13]. Brachytherapy, which is the implantation of a radioactive implant, on the other hand, is much less likely to lead to secondary malignancies but comes with the risk of loss of site through side effects such as optic neuropathy, radiation induced retinopathy and development of cataracts. While these treatments have been shown to be effective in treatment of retinoblastoma, new treatments strategies must also be studied [9] given the current treatment side effects.

Over the last 100 years, the use of viral oncolytic therapy as a potential treatment for cancer has been met with various degrees of scientific interest. The first observation of viral oncolytic potential was after incidental infections or after vaccination [14]. In spite of these observations, viral oncolytic therapy did not garner much interest until the 1980s, with the first studies using recombinant Herpes Simplex Virus (HSV). These studies used HSV with the thymidine kinase gene ablated [15]. The ablation of this gene restricted the replicative ability in healthy tissues, but allowed for competent replication in cancerous

tissues because of the abundance of thymine in most cancers. Newer studies have shown that other genes, usually immune regulatory genes, also beneficially restricted replicative ability of virotherapies in healthy tissues. Since these first studies, many other viruses have been designed as potential oncolytic agents. These include viruses in families *Adenoviridae, Picornaviridae, Reoviridae* and *Poxviriade* [14,16].

Members of the *Poxviridae* family have shown great promise as potential oncolytic viruses. The main reason for their potential is that they are large double-stranded DNA viruses. Their genomes contain multiple genes that are responsible for cell tropism, immune modulation and replication [16]. This allows for many different strategies of manipulation that can tailor viruses to many different cancers. The large genome also allows these viruses to accommodate multiple transgenes, which have also been shown to increase their oncolytic potential [16].

Tanapoxvirus (TPV) is a member of the *Yatapoxvirus* genus within the *Poxviridae* family. Infection with TPV leads to a mild, self-limiting disease in humans that features a febrile illness and minor skin eruptions that may last up to 6 weeks [17, 18]. No documented cases of human-to-human transmission have been cited during limited outbreaks of TPV [18]. Finally, global populations, except parts of Africa, are immunologically naïve to TPV, unlike herpesviruses and adenoviruses [19].

Recent research with TPV has shown it to be a good candidate for viral oncolytic therapy. Studies in colorectal cancer and in melanoma have shown various degrees of success in the ability for TPV to replicate in different tumor lines and cause tumor regression [20, 21]. TPV has a large dsDNA genome, and most genes code for protein with one distinct function, which allow for predictable outcomes with gene ablation.

Studies using TPV have investigated its efficacy in multiple *in vitro* experiments and have started to move into whole animal studies [21-23]

This study was designed to determine the efficacy of wild type TPV and a number of TPV recombinants in a series of *in vitro* assays to determine oncolytic potential. This is necessary because other purposed oncolytic candidates have only been shown to work in high titers and use viral constructs based on circulating viruses. Oncolytic TPV has been shown to work at much lower viral concentrations in other studies and is not circulating in most areas of the world. These studies tested the hypothesis that our lab derived oncolytic TPVs would serve as an effective transgene vector and viral oncolytic candidate for retinoblastoma.

Material and methods

Cells

Owl monkey kidney (OMK), Y79-HTB-18 and WERI-RBI HTB-169 retinoblastoma cells, were purchased from American Type Culture Collection (ATCC). OMKs cells were used for initial viral amplification, and titration. OMK cells were grown in Eagle's Modified Essential Medium (EMEM: Gibco 11700-077), supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamate. Following infection, the cells were maintained in EMEM supplemented with 2% (v/v) FBS. Both the Y79-HTB-18 and WERI-RB1 cells were maintained in RPMI-1640 (ATCC 30-2001) with 20% (v/v) FBS. Post infection both cell

lines were in RPMI-1640 containing 2% (v/v) FBS. All cells were grown at 37^0C with 5% CO_2.

Viruses

The wild-type TPV was originally provided by Joseph Esposito. Wild type expressing green fluorescent protein (GFP) was a gift from Dr. Grant McFadden. Additional gene insertions and deletions were done in our lab and described earlier [20, 33]. Briefly genes were ablated and transgenes, including fluorescent proteins, were added using the p2KO vector. PCR and electrophoresis were used to confirm the absence of ablated genes and western blots and ELISA were used to confirm the presence and expression of transgenes as described earlier [20]. Ten viruses (Table 1) were used in this study, TPVeGFP, TPVΔ66R, TPVΔ66R/*flic,* TPVΔ66R*mCCL2,* TPVΔ66R/*mGM-CSF,* TPVΔ2L, TPVΔ66RΔ2L, TPVΔ66RΔ2L/*flic,* TPVΔ15L, and TPVΔ15L66R. All viruses were cultivated in OMK cell monolayers, which were infected at 90% confluency and collected at 240 hours post infection (hpi). Each virus was plaque purified three times and the purity of each virus was confirmed using PCR [20].

Viral concentration and titration

Infected cells were harvested and stored at -20^0C. Infected cells were then centrifuged at 1000 rpm for 20 minutes in 50 mL conical tubes to separate supernatant from cell debris. The supernatant was then carefully removed from each tube and stored at -20^0 C. To release virus from intact cells, the cell pellet was re-suspended in sterilized deionized H_2O and put through three cycles of freezing and thawing. Finally, the cell

pellets were homogenized and centrifuged at 1500 rpm and the supernatant was collected and pooled with first supernatant. The pool supernatant was then centrifuged for 90 minutes at 185,000 g using Ti-45 rotor. The virus pellet was kept in 100uL of maintenance medium overnight and then collected. The virus was re-suspended such that it resulted in 100x virus concentration. Viruses were titrated as described earlier [25].

Infection of retinoblastoma cells

Y-79 and WERI-RB1 retinoblastoma cells were first counted using a hemocytometer and $1x10^5$ cells were placed into Eppendorf tubes. Each sample was then centrifuged at 2,000 rpm for 10 minutes at room temperature. The medium was removed and each sample was re-suspended in appropriate virus (table 1) at an MOI of 0.1 or 10 in a final volume of 250 uL of maintenance medium. Viruses were allowed to adsorb and each sample was gently re-suspended every 10 minutes. At the end the adsorption period, the samples were centrifuged at 2000 rpm for 10 minutes and the supernatant was collected. Cells were re-suspended in an additional 250uL of RPMI-1640 containing 2% (v/v) FBS, centrifuged and the supernatant was collected and combined with previous supernatant. Virus was titrated as described earlier. Each experiment was independently repeated three times.

Single step TPV replication

To discern if gene knockouts or transgene knockins changed replicative ability of viruses (table 1) in retinoblastoma cells. Both retinoblastoma cell lines were grown in 20% (v/v) RPMI-1640 FBS until they were at a concentration of $1x10^6$ cells per mL. Y-79 and

WERI-RB-1 cells were then collected and separated into individual Eppendorf tubes at $1x10^5$ cells/tube. Each tube was infected with 0.1 or 10 MOI and allowed to adsorb at room temperature for one hour as described earlier. After one hour, the cells were centrifuged and washed. This was repeated three times to ensure any unadsorbed virus particles were removed. Y-79 and WERI-RB1 cells were then plated in 48 well dishes in 1 mL of maintenance medium and incubated at 37^0C at 5% CO_2. At the appropriate time points, cells were collected and virus was titrated as described earlier.

Cell viability

To assay the ability of TPV to cause cell death of infected retinoblastoma cells by either necrosis or apoptosis, a cell counting kit-8 (CCK-8) was used. The CCK assay was done over a 192-hour time course. Two MOIs were selected at 0.1 and 10. Y-79 cells were plated at 100 uL of cells at a concentration of $1x10^6$ cells per ml were plated in each well of a 96 well dishes in maintenance medium. The appropriate amount of each virus was then added to each well and plates were subsequently incubated based on their time point at 37^0C with 5% CO_2. At the end of each time point, plates were removed from the incubator, 10 uL of CCK-8 reagent was added to each well and the plate was incubated again for 1 hour at 37^0C. After one hour, the plate was read using a plate reader (Epoch) at 450nm.

Statistical analysis

All experiments were independently repeated at least 3 times. Statistical analysis was performed on all normalized data using analyses of variance (ANOVA) with Tukey post-hoc comparisons for all experiments described here. A p-value of <0.05 was considered statistically significant.

Results

Adsorption

To analyze the viral titer in infected cells, both retinoblastoma cell lines were counted and plated at $1x10^5$. Each cell line was then infected with TPVeGFP at an MOI of 10. Supernatants were collected and virus was titrated as described. At 0 minutes' post infection (mpi), all virus ($1x10^6$), was recovered from both cell lines. At 60 minutes' post infection, virus titer from both cell lines at 4^0C and 25^0C was $8x10^5$, 80% of time zero, significantly lower than 0 minutes' post infection. At 120 minutes' post infection and 180 minutes' post infection at both temperatures, virus titer was $7.5x10^5$, 75% of time zero which was significantly lower titer than at 0 minutes' post infection, but was not significantly lower than the 60 minutes' post infection time point. No significant differences were observed between cell lines or when cells were incubated in two different temperatures (Figure 1).

Visualization of infected retinoblastoma cell lines

Y-79 retinoblastoma cells were grown in a 48 well dish and experimentally infected with transgene expressing TPV. Infection was allowed to proceed for 48 hours and cells were visualized under both visible and ultraviolet light. All variants of TPV caused cells to show distinct cytopathic effect. Two fluorescent transgenes were used in the recombinant viruses, mCherry and green fluorescing protein, and were included on the same promoter as recombinant transgenes as described earlier [20, 24]. After 48 hours transgene expression was visible as shown in Figure 2, as expected [20].

Single step virus replication

Replication curves of viruses (table 1) shown in Figure 3. Y-79 and WERI-RB1 cells were infected at an MOI of 0.1 and plated into 48 well dishes at 100 uL at a concentration of $1x10^6$ cells/ml. Both retinoblastoma cell lines were then infected with 0.1 MOI of each virus, allowed to adsorb for one hour at room temperature, and then washed three times to remove any unadsorbed virus. Infected retinoblastoma cell lines were then incubated until the appropriate time point. An eclipse period of 96 hours was observed at 0.1 MOI in both Y-79 and WERI-RB1 cell lines, with no increase of virus observed between 48 and 96 hours. After 240 hours, each virus showed a significant increase, with TPVeGFP showing the largest increase from $1x10^3$ to $2x10^4$ virions. TPVΔ15L and TPVΔ2L showed the same fold increase as TPVeGFP, with no statistical difference in their replication rate at 240 hpi. The titers of viruses containing the ablated *66R* gene, TPVΔ66R, TPVΔ66R/*mCCL2*, TPVΔ66R/*mGMCSF*, TPVΔ66R/*FliC*, TPVΔ66RΔ2L and TPVΔ66RΔ2L/*FliC*, showed significant increases between 96 hours and 240 hours, an average of $1x10^3$ virions to $1.5x10^4$ virions. The same replication patterns were observed

in both cell types, Y-79 and WERI-RB1. Viruses not contain the 66R gene replicated less efficiently than those containing the intact gene, as shown in Figure 3. Figure 4 shows the replication curves of TPV by infecting Y-79 and WERI-RB1 cells in suspension at an MOI of 10. TPVeGFP, TPVΔ15L, and TPVΔ2L all showed a significant increase in the number of virions between hours 48 and 96 from an average of $5x10^4$ virions to $2.5x10^5$ virions (Figure 4). There was no significant change between 96 and 240 hpi. The viruses with the ablated thymidine kinase gene, *66R*, TPVΔ66R, TPVΔ66R/*mCCL2*, TPVΔ66R/*mGMCSF*, TPVΔ66R/*FliC*, TPVΔ66RΔ2L and TPVΔ66RΔ2L/*FliC*, showed a significant increase in virions between 48 and 96 hpi from an average of $4x10^4$ virions to an average of $1.8x10^5$ virions. No statistically significant decrease occurred 240 hpi. As with the 0.1 MOI study, viruses containing the *66R* ablation showed significantly less virions after infection than viruses containing the intact thymidine kinase gene. Also as with 0.1 MOI there were no differences in replication patterns seen between Y-79 cells and WERI-RB1 cells at the infection dose of 10 MOI.

Cell viability

To assess potential oncolytic ability of TPVeGFP and selected recombinant variants, the cell counting kit 8 assay (CCK-8), which assays the number of viable cells, was performed. Y-79 cells were plated in each well of a 96 well plate in 100 uL of maintenance medium at a density of $1x10^5$ cells per mL and were infected at either 0.1 or 10 MOI and incubated for 192 hpi. Reagents were added according to the assay instructions (96992 Cell Counting Kit – 8, Sigma-Aldrich), incubated for 2 hours and read in a plate reader at 450 nm to determine absorbance. Day 0 and 192 mock infected Y-79

cells controls were counted using a hemocytometer to verify cell number readings. Results were normalized to a 0 hours uninfected control as 100%. The 192-hour uninfected control showed no significant change compared to hour 0. TPVeGFP infected with 0.1 MOI showed a significant 51% average decrease from day 0, and a significant 72% average decrease when infected with 10 MOI. TPVΔ66R showed a 26% average decrease at an infection of 0.1 and a 66% average decrease at an infection of 10 MOI. TPVΔ2L at an infection of 0.1 MOI decreased by an average of 27% compared to day 0 control and 62% when infected with 10 MOI. TPVΔ15L decreased 42% at an infection of 0.1 MOI and 69% at an infection of 10 MOI. TPVΔ66R/*GMCSF* shows an average 63% significant reduction in viable cells at 10 MOI and an average 36% reduction at 0.1 MOI. TPVΔ66R/*mCCL2* reduced viable cells by 56% at an infection of 10 MOI and 37% at an infection of 0.1. TPV- Δ66RFliC at an infection of 10 MOI reduced viable cells by an average 68% and at an infection of 0.1 MOI by an average 43%. The double knockouts TPVΔ2LΔ66R and TPVΔ2LΔ66R/*FliC* showed an average 70% significant reduction at an infection of 10 MOI, TPVΔ2LΔ66R/*FliC* though reduced viable cells by an average 42% at an infection of 0.1 MOI whereas the non-transgene expressing virus significantly reduced viable cells by an average 32%. Finally, the TPVΔ15LΔ66R virus showed a significant reduction of 68% of viable cells at infection of 10 MOI and 43% at infection of 0.1 MOI. All viruses showed significant reduction in viable cells compared to day 0 and compared to the 196 hpi mock control. In addition, all viruses showed significant decreases with 10 MOI treatment compared to the infection at 0.1 MOI as shown in figure 5.

Discussion

Results from these experiments have supported the hypothesis that in the *in vitro* system, TPV and recombinants could be a possible candidate for oncolytic virotherapy for retinoblastoma. This determination was made using four criteria. The ability for TPV to infect, express transgenes, replicate and induce cellular death is necessary for successful treatment of tumors using virotherapy. The results from this study have demonstrated that the virus was able to attach to the cells (figure 1), infect and express transgene (figure 2), go through competent replication at both 0.1 and 10 MOIs (figure 3 and 4) and finally cause cellular death in a dose dependent manner (figure 5).

Current treatment strategies for retinoblastoma, while usually successful in clearing tumors, still have life altering negative side effects [23, 26-27]. The development of newer, low side effect treatments, which still retain tumor clearance, is an active area of research currently [26]. Recent studies looking at the efficacy of oncolytic virotherapy in retinoblastoma have shown various levels of success and are a continuing area of research [26-28]. This is because oncolytic virotherapy offers multiple advantages that enucleation, radiation and chemotherapy do not [26, 29]. Unlike radiation or chemotherapy, virotherapy is tumor tissue specific, reducing the chance of systemic side effects, such as weight loss, increased susceptibility to infections, and secondary cancers [14, 30]. Unlike enucleation, which involves the removal of the entire eye, the tumor specificity associated with virotherapy should leave healthy retinal tissue intact and blindness in affected eyes should be less of a concern [26]. Oncolytic adenoviruses have

shown both *in vitro* and *in vivo* that efficacy of oncolytic virotherapies may play a role for possible future treatment. While promising, additional virotherapies are still needed to allow for serial treatment as the host immune system adapts to each virotherapy. Because of this TPV offers an attractive candidate [20].

TPV along with ablated viruses and recombinants have been studied in multiple tumor cell lines, and is currently being studied for efficacy in *in vivo* induced tumor nude mouse models [20]. TPV offers many advantages to other oncolytic virotherapies, including adenoviral therapies. For instance, humans are immunologically naïve to TPV, and it does not spread person to person [17, 20]. Past studies have shown that TPVeGFP can cause infection and replicate successful in glioblastoma cell lines, showing various degrees of successful replication [21]. As a result, it was not unreasonable to assay infective and replicative ability of TPV in retinoblastoma, which like glioblastoma, is a neural cancer and feature subpopulations of neural stem-cell like cells. To assay the ability for TPV to attach to these cells, a simple adsorption assay was used. The results of this assay showed that TPVeGFP was able to successfully attach to both WERI-RB1 and Y-79 cells and cause infection. It also showed that maximum absorption was achieved 60 mpi. Longer periods of time showed no statistical difference from 60 mpi. Other studies have shown that pox tissue tropism is usually not restricted due to intracellular factors [31, 32]. Within 48 hours after infection, irrespective of MOI or recombinant virus used, clear signs of cytopathic effects were evident in at least subpopulations of cells (shown in figure 2).

One of the most important advantages of oncolytic virotherapy is that unlike radiation or chemotherapeutics, where the initial dose decreases over time, the virus

should replicate and increase its therapeutic ability as infection spreads [33]. To assay the ability of TPV and recombinants to replicate, single step replication curves using two MOIs and three time points were done. The 0.1 MOI experiments showed a significant increase in the amount of virions after 240 hours for all viruses tested. There was no significant increase between 48 hours and 96 hours observed. This may be explained due to an eclipse phase of the virus, as initially only approximately 10% of cells were infected with virus. Within that period of time, any virus that may have been released from infected cells, through viral budding or physical lysis, goes on to infect uninfected cells. The other half of this experiment using 10 MOI, showed the biggest increase between 48 hours and 96 hours. There was no statistical difference in the number of virions after 240 hours compared to 96 hours. Viruses that did not have an ablation of the thymidine kinase showed replication that was statistically indistinguishable from the wild type virus. This is not unexpected, as the two genes ablated are either analogues of a growth factor, 15L [32], or have been implicated as part of the virus's anti-immune response, 2L. However, the viruses that contained an ablated thymidine kinase, 66R, showed statistically less robust growth compared to the wild type. Other studies using Herpes Simplex Virus (HSV) that also have an ablated thymidine kinase gene might shed light on this. In these studies, results demonstrated that thymidine kinase negative mutant HSV could infect and become latent in neurons, but could not reemerge from latency [35-37]. It is thought that low levels of intracellular host thymidine kinase lead to low level of triphosphate pyrimidines necessary for permissive viral replication [35]. In the case of retinoblastoma, as opposed to healthy neuronal tissue, there would be stores of these triphosphate pyrimidines, but these stores may not be sufficient to support the same level of replication

as observed in wild type. Viruses containing knockouts of two genes, one of which was 66R, showed the same replicative ability as the TK^- Δ66R virus. This was expected as the second genes knocked out, 2L and 15L, didn't show significant effect on replicative ability of TPV in the retinoblastoma cell lines. Transgene expression in the recombinant viruses also didn't show an overall effect on their ability to replicate compared to the non-transgene expressing Δ66R virus.

Beyond just replication of the virus and causing cellular inviability, some of the recombinant viruses used contained immune-stimulatory transgenes that activated the host immune system in different ways. The bacterial flagellar filament protein, fliC, causes activation of the TLR-5 pathway, which leads into the NF-kB pathway and eventual secretion of TNF [38]. Granulocyte macrophage colony-stimulating factor, GM-CSF, acts to stimulate the production of granulocytes and monocytes from stem cells and their terminal differentiation. GM-CSF has been investigated both as a standalone treatment and as an expressed transgene in virotherapies for possible cancer treatments [39-41]. The final transgene that was used was chemokine (c-c motif) 2 (CCL2). CCL2 has been implicated with different roles when associated with certain tumors. In prostate and breast cancers, its presence has been correlated with metastatic phenotypes [41, 42]. In lung cancer, it has been shown to increase the activation of CD8+ cells, which are an important facet of host defense against tumor growth [43]. To assay this, data from this study and previous studies were used. Fluorescent transgenes were linked to the vaccinia virus (VacV) early late promoter. Previous studies have shown that expression of fluorescent transgenes could be used as a positive indicator of immuno-stimulatory transgene expression [20]. Fluorescent microscopic imaging (shown in figure 2) shows robust

expression of the fluorescent proteins mCherry and GFP 48 hpi. This is a strong indicator of successful transgene expression by the TPV recombinants. Successful expression of transgenes *in vitro*, while not changing their ability to cause lysis or replicate, should *in vivo* allow for increased immune activity at the site of the tumor. This could allow for TPV to act as a vector for a wide range of immunostimulatory genes, not just the ones assayed in this study. The presence of these transgenes also did not cause significant decrease of replicative ability from base ablated virus, 66R, in either retinoblastoma cell line (figure 3 and 4).

The final experiment was to determine the ability to cause cellular inviability of these viruses *in vitro*. The end goal of oncolytic virotherapy is to cause physical lysis of tumor cells and subsequent regression of the tumor tissue [44]. Beyond the lysis caused by the virus itself, virally induced lysis leads to release of cellular contents and the activation of the innate immune response [45]. To assay the ability for TPV and recombinants to cause cellular lysis, the cell counting kit-8 was used. TPVeGFP and recombinants all showed a significant ability to reduce viable cells 192 hpi. Infections with 0.1 MOI of each virus showed about a 40% reduction of viable retinoblastoma tumor cells, which corresponds well with the replication data showing that the virus was still actively replicating after 240 hours. The 10 MOI infections showed decreases of viable cells of about 70% compared to time 0 controls, and showed a significant decrease compared the 0.1 treatments. These studies show that TPV and recombinants are able to significantly reduce viable tumor cells in a dose dependent manner and may become a viable future treatment for retinoblastoma.

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Figure legends

Figure 1: Y-79 and WERI-RB1 cells were infected with TPVeGFP. Virus was allowed to adsorb at 4^0C and 25^0C for 60, 120, and 180 mpi and unadsorbed was collected and titrated. Error bars represent standard error. (*= significant change compared to 0 minutes' post infection).

Figure 2: Cells were experimentally infected with TPVeGFP (A and B), TPVΔ2LΔ66R/*FliC* 48 (C and D) and TPV Kenya (E and F) and incubated for 48 hours. They were then visualized and photographed using visible light and UV fluorescence. (A) Light microscope image of TPVeGFP infected Y79 cells 48 hpi showing CPE. (B) Same image under UV fluorescence showing expression of virally encoded GFP. (C) Light microscope image of Y-79 infected with TPVΔ2LΔ66R/*FliC* 48 hpi showing CPE. (D) Same image under UV fluorescence showing expression of virally encoded mCherry. Final set shows were taken using UV fluorescence to demonstrate that cells do not autofluoresce when infected with virus lacking eGFP or mCherry (E and F).

Figure 3: Single step replication curves at 0.1 MOI. Cells were infected with 1 virion per 10 cells and incubated for 48, 96 and 240 hours. Infected cells were then recovered, lysed and viruses were titrated. A-C: Single step replication curves of all ten viruses at 0.1 MOI in a Y-79 cell line. A shows virus with single ablation. B shows Δ66R viruses with transgene. C shows viruses containing double ablations. D-F: Single step replication curve of all ten viruses at 10 MOI in a WER-RB1 cell line. D shows viruses with single ablation. E shows Δ66R viruses with transgene. F shows viruses containing double

ablations. Error bars represent standard error. (*= significant change compared to previous time point, °= change compared wild-type p=<.05).

Figure 4: Single step replication curves 10 MOI. Cells were infected with 10 virions per cell and incubated for 48, 96 and 240 hours. Infected cells were then recovered, lysed and viruses were counted using plaque counting technique. A-C: Single step replication curves of all ten viruses at 10 MOI in a Y-79 cell line. A shows virus with single ablation. B shows Δ66R viruses with transgene. C shows viruses containing double ablations. D-F: Single step replication curve of all ten viruses at 10 MOI in a WER-RB1 cell line. D shows viruses with single ablation. E shows Δ66R viruses with transgene. F shows viruses containing double ablations. Error bars represent standard error. (*= significant change compared to previous time point, °= change compared wild-type p=<.05).

Figure 5: Cell Counting Kit-8 (CCK-8) was used to assay number of living cells 192 hours post infection. Cells were counted using a hemocytometer and 100 uL were plated at a density of $1x10^6$ per mL. Cells were then infected with TPVeGFP, TPVΔ66R, TPVΔ2L, and TPVΔ15L, TPVΔ66R/*mGMCSF*, TPVΔ66R/*mCCL2*, TPVΔ66R/*FliC*, TPVΔ2LΔ66R, TPVΔ2LΔ66R/*FliC*, and TPVΔ15LΔ66R at MOIs of 0.01 and 10 and incubated for 192 hours. All treatments showed significant decreases in living cells (P<.05) compared to both day 0 uninfected cells (*). All TPV 10 MOI treatments showed significant decrease in living cells compared to 0.1 MOI (°).

Table 1
List of viruses used

Recombinant virus name	Deleted genes	Transgene
TPVeGFP	n/a	Enhanced-GFP
TPVΔ66R	Viral thymidine kinase	mCherry
TPV-Δ2L	Virus encoded TNF-Alpha inhibitor	mCherry
TPV-Δ15L	Virus encoded neuroregulin	Enhanced-GFP
TPV-Δ66R*Flic*	Viral thymidine kinase and flagellin protein	flagella protein, Enhanced-GFP
TPV-Δ66R*GM-CSF*	Viral thymidine kinase	Mouse Granulocyte-macrophage colony-stimulating factor, mCherry
TPV-Δ66R*mCCL2*	Viral thymidine kinase	C-C motif chemokine 2
TPV-Δ66RΔ2L	Viral thymidine kinase, Virus encoded tumor necrosis factor-alpha (TNF-Alpha) inhibitor	Enhanced-GFP, mCherry
TPV-Δ66R15L	Viral thymidine kinase, Virus encoded neuroregulin	Enhanced-GFP, mCherry
TPV-Δ66RΔ2L*flic*	Viral thymidine kinase, Virus encoded TNF-Alpha inhibitor	flagella protein, Enhanced-GFP, mCherry

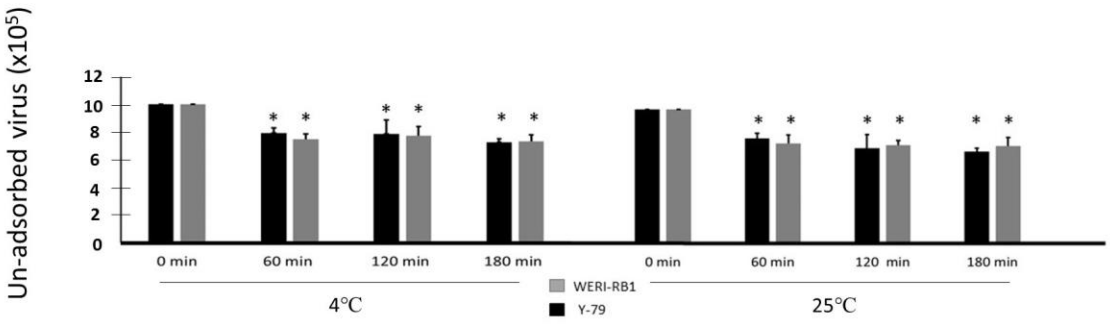

Figure 1
Adsorption assay

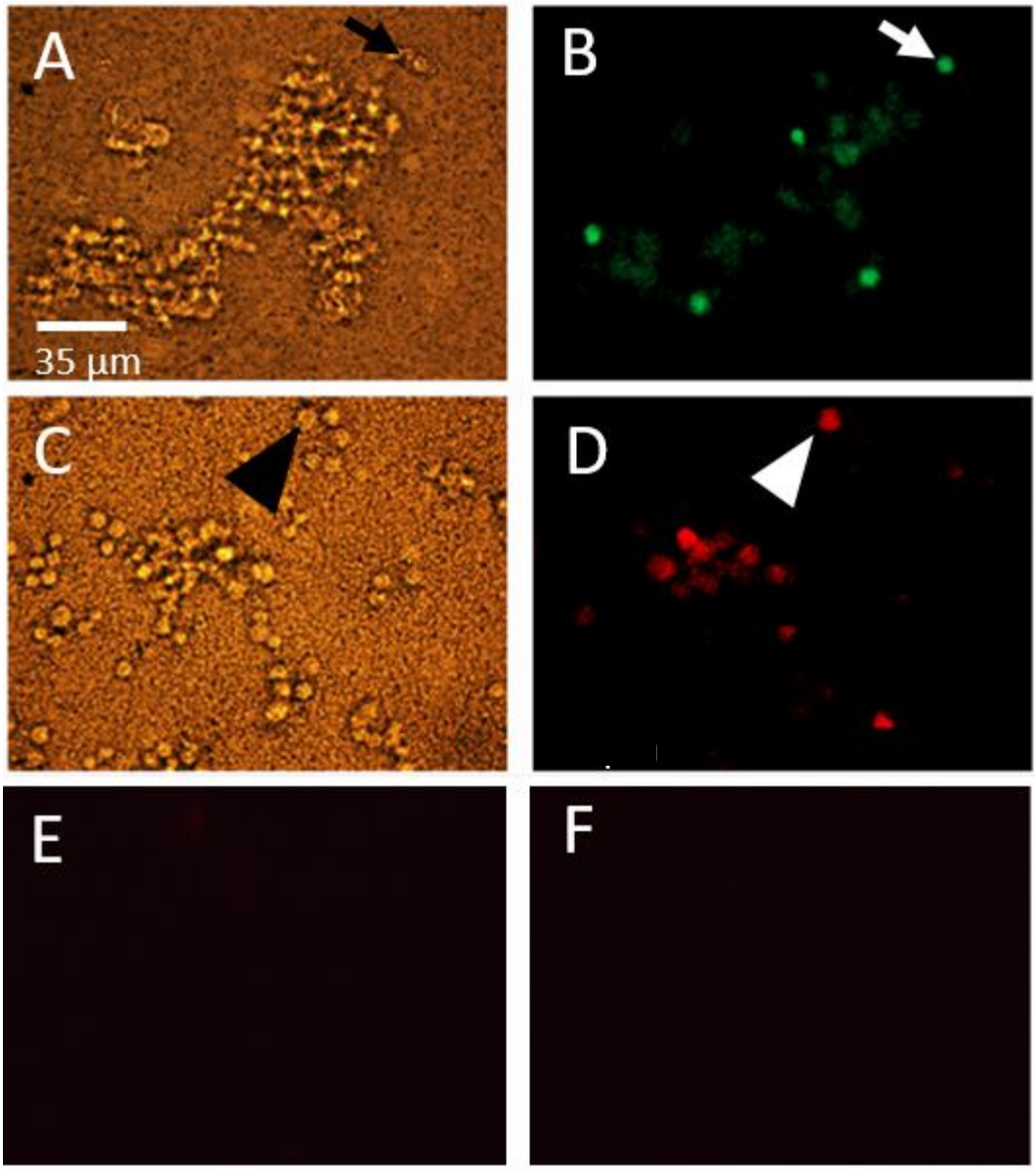

Figure 2
Visible light and fluorescent light images

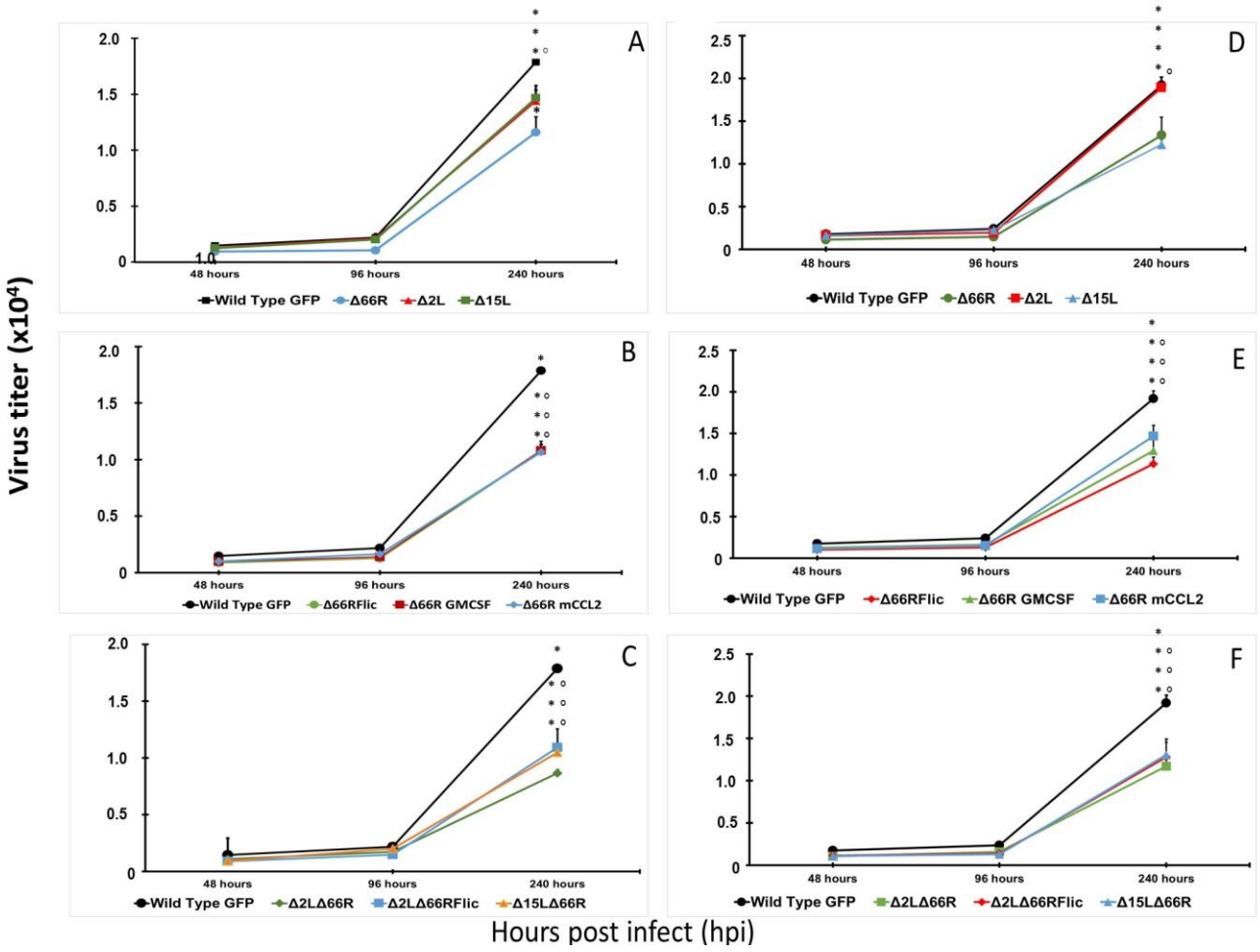

Figure 3
0.1 MOI replication

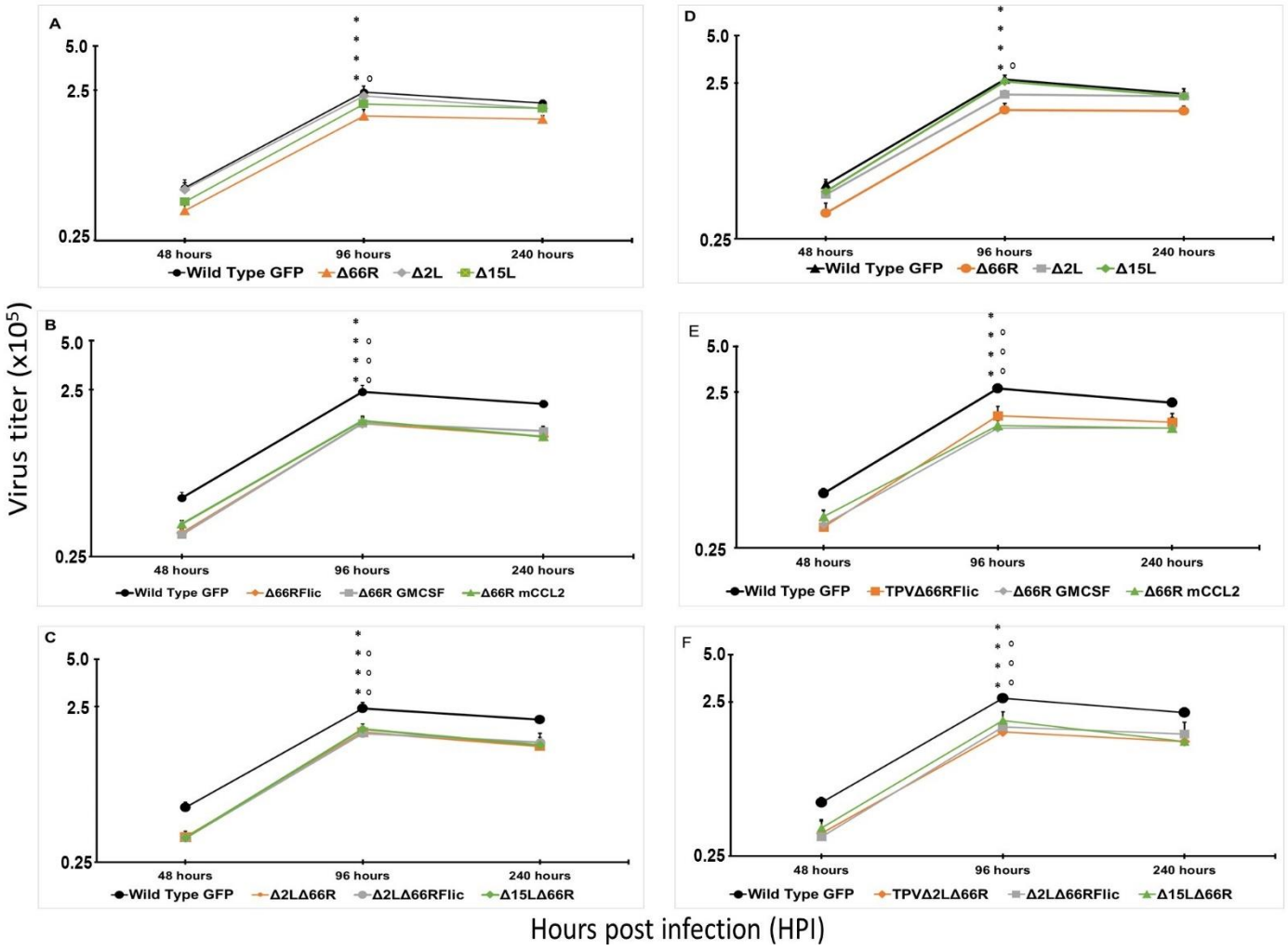

Figure 4
10 MOI virus replication

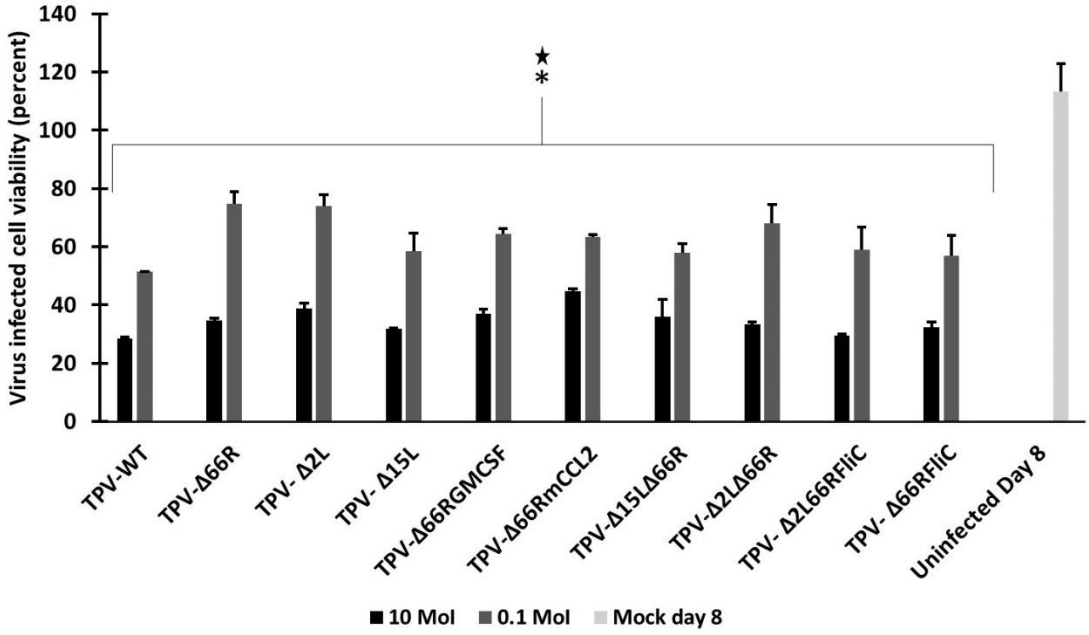

Figure 5

Cell viability assay

DISCUSSION

Current treatment strategies for retinoblastoma, while usually successful in clearing tumors, still have life altering negative side effects (Abramson and Schefler, 2004; Byrne et al 1995; Song et al 2010). The development of newer, low side effect treatments, which still retain tumor clearance, is an active area of research currently (Song et al 2010). Recent studies looking at the efficacy of oncolytic virotherapy in retinoblastoma have shown various levels of success and are a continuing area of research (Song et al 2010; Ji et al 2008; Wang et al 2013). This is because oncolytic virotherapy offers multiple advantages that enucleation, radiation and chemotherapy do not (Song et al 2010; Vähä-Koskela et al 2007). Unlike radiation or chemotherapy, virotherapy is tumor tissue specific, reducing the chance of systemic side effects, such as weight loss, increased susceptibility to infections, and increasing the chance of secondary cancers (Kelley and Russell 2006; Schwartz 1999). Unlike enucleation, which involves the removal of the entire eye, the tumor specificity associated with virotherapy should leave healthy retinal tissue intact and blindness in affected eye should be less of a concern (Song et al 2010). Current research using oncolytic adenoviruses have shown both in vitro and in vivo efficacy of oncolytic virotherapies may play a role for possible future treatment. While promising, additional virotherapies are still needed to allow for serial treatment as the host immune system adapts to each virotherapy. Because of this TPV offers an attractive candidate (Conrad et al 2015). The results of this study has given new insights into how oncolytic TPV fits into the greater scheme of potential retinoblastoma treatments.

The results from these experiments show that in the in vitro system, TPV and recombinants are a possible candidate for oncolytic virotherapy for retinoblastoma. This determination was made using four criteria, based upon two outline specific aims. The first specific aim determined the ability for TPV to absorb, infect, and express transgenes, in retinoblastoma cells and the second specific aim determined the ability of TPV to replicate new virions and cause cellular inviability. These four criteria are necessary for successful treatment of tumors using virotherapy. The results show that the virus is able to attach to the cells (figure 1), infect and express transgene (figure 2), go through competent replication at both 0.1 and 10 MOIs (figure 3 and 4) and finally cause cellular death in a dose dependent manner (figure 5). Based on the criteria outline, oncolytic TPV is potential candidate for virotherapy in retinoblastoma. This lends good evidence both hypothesizes that were purposed are valid. The way in which results satisfy the specific aims of this study will be discussed.

Specific aim one: To determine the ability of wild type TPV and recombinants to adsorb, infect, and express transgenes in multiple retinoblastoma cell lines.

Tanapoxvirus along with ablated viruses and recombinants have been studied in multiple tumor cell lines, and is currently being studied for efficacy in in vivo induced tumor nude mouse models (Conrad et al 2015; Zhang et al 2016). TPV offers many advantages to other oncolytic virotherapies, including adenoviral therapies. For instance, humans are immunologically naïve to TPV, and it does not spread person to person (Nazarian et al

2007; Conrad et al 2015). Past studies have shown that TPV-WTeGFP can cause infection and replicate successful in glioblastoma cell lines, showing various degrees of successful replication (Lee and Essani 2010). As a result, it was not unreasonable to assay infective and replicative ability of TPV in retinoblastoma, which like glioblastoma, is a neural cancer and feature subpopulations of neural stem-cell like cells. To assay the ability for TPV to attach to these cells, a simple adsorption assay was used. The results of this assay showed that TPV-WTeGFP was able to successfully attach to the both the WERI-RB1 and Y-79 cells and cause infection. It also showed that maximum absorption was achieved 60 minutes' post infection. Longer periods of time showed no statistical difference from 60 mpi. The upper limit of absorption may not have been found due to techniques used for infection, as these cells were in suspension and constant agitation of the cells was required to keep them in suspense and centrifugation for washing of virus. This may have caused sheering of the virus from the receptor and limited the number of viral particles that could cause infection. Determining the exact number of virions that could infect retinoblastoma cells though was outside the purview of this study, as only ability for the virus to absorb and to cause infection was assayed.

Beyond achieving oncolytic effect through only replication of the virus and causing cellular inviability, some of the recombinant viruses used contained immune-stimulatory transgenes. The three transgenes which were used in the recombinant TPV target activation of the host immune system in different ways. The bacterial flagellar filament protein, fliC, causes activation of the the TLR-5 pathway, which leads into the NF-kB pathway and eventually the secretion of TNF (Yoon et al 2013). Granulocyte macrophage

colony-stimulating factor, GM-CSF, acts to stimulate the production of granulocytes and monocytes from stem cells and their terminal differentiation. GM-CSF has been investigated both as a standalone treatment and as an expressed transgene in virotherapies for possible cancer treatments (Kim et al 2006; Buschmann et al 2001). The final transgene that was used was chemokine (c-c motif) 2 (CCL2). CCL2 has been implicated with different roles when associated with certain tumors. In prostate and breast cancers, its presence has been correlated with metastatic phenotypes (Mizutani et al 2009; Qian et al 2011). In lung cancer, it has been shown to increase the activation of CD8+ cells, which are an important facet of host defense against tumor growth (Fridlender et al 2011). To assay this, data from this study and previous studies were used. Fluorescent transgenes were linked to the Vaccinia Virus (VacV) early late promoter. Previous studies have shown that expression of fluorescent transgenes could be used as a positive indicator of immuno-stimulatory transgene expression (Conrad et al 2015). Fluorescent microscopic imaging (shown in figure 2) shows robust expression of the fluorescent proteins mCherry and GFP 48 hours post infection. This is a strong indicator of successful transgene expression by the TPV recombinants. This is because both of the fluorescent transgenes and the immunostimulatory genes are linked to the same VacV early late promoter. Successful expression of transgenes in vitro, while not changing their ability to cause lysis or replicate, should in vivo allow for increased immune activity at the site of the tumor. This could allow for TPV to act as a vector for a wide range of immunostimulatory genes, not just the ones assayed in this study. The presence of these transgenes also did not cause significant decrease of replicative ability from base ablated virus, 66R, in either retinoblastoma cell line (figure 3 and 4).

The expression of transgenes didn't show any effect, positive or negative, on the ability of the virus to replicate or decrease viability of retinoblastoma in vitro. This is not an unexpected result. These genes are relatively small, so they shouldn't slow down or deflect too many resources from the virus while it is replicating. The genes also do not activate any pathways that will affect cellular proliferation in vitro, but were selected for their effects on the innate immune system.

Specific aim two. To determine the ability for wild type TPV and recombinants to go through competent replication and cause cellular inviability.

One of the most important advantages of oncolytic virotherapy is that unlike radiation or chemotherapeutics, where the initial dose decreases over time, the viruses should replicate and increase its therapeutic ability as infection spreads (Liu et al 2007). To assay the ability of TPV and recombinants to undergo competent replication, single step replications curves using two MOIs and three time points were generated. The experiments using 0.1 MOI showed a significant increase in the amount of virions after 240 hrs for all viruses tested. There was no significant increase between 48 hours and 96 hours observed. This may be explained due to an eclipse phase of the virus, as initially only approximately 10% of cells were infected with virus. Within that period of time, any virus that may have been released from infected cells, through viral budding or physical lysis, would go on to infect uninfected cells. The other half of the experiment using 10

MOI, showed the biggest increase between 48 hours and 96 hours. There was no statistical difference in the number of virions after 240 hours compared to 96 hours. Viruses that did not have an ablation of the thymidine kinase showed replication that was statistically indistinguishable from the wild type virus. This was expected as the two genes ablated were analogues of a growth factor, 15L (Jeng et al 2013), or have been implicated as part of the virus's anti-immune response, 2L. However, the viruses that contained an ablated thymidine kinase, 66R, showed statistically less robust growth compared to the wild type. Other studies using Herpes Simplex Virus (HSV) that also have an ablated thymidine kinase gene, might shed light on this. In these previous studies, results demonstrated that thymidine kinase negative mutant HSV could infect and become latent in neurons, but could not reemerge from latency (Coen et al 1989; Tenser et al 1979; Price and Khan 1981). It is thought that low levels of intracellular host thymidine kinase lead to low level of triphosphate pyrimidines necessary for permissive viral replication (Coen et al 1989). In the case of retinoblastoma, as opposed to healthy neuronal tissue, there would be stores of these triphosphate pyrimidines, but these stores may not be sufficient to support the same level of replication as observed in wild type. Viruses containing knockouts of two genes, one of which was 66R, showed the same replicative ability as the TK- Δ66R virus. This was expected as the second genes knocked out, 2L and 15L, did not show significant effect on replicative ability of TPV in the retinoblastoma cell lines. Transgene expression in the recombinant viruses also did not show an overall effect on their ability to replicate compared to the non-transgene expressing Δ66R virus.

Other studies have shown that pox tissue tropism is usually restricted by intracellular factors, not by the absence of receptors (Moss 2006). Within 48 hours after infection, irrespective of the MOI or the recombinant virus used, clear signs of cytopathic effects were evident in at least subpopulations of cells (shown in figure 2). Cells infected at 10 MOI showed aggregation of cells near the walls of the wells that were incubated for 48 hours. At the lower infection concentration, 0.1 MOI, aggregation was not seen until 5 days' post infection. This observed aggregation may represent a mechanism of viral movement from infected cells to uninfected cells.

While the TK knockout Δ66R virus does not replicate as well as virus with intact 66R due to low levels of endemic thymidine kinase, it should not disqualify the virus as a good candidate for treatment. One of the main goals of oncolytic virotherapy is to engineer candidate viruses that will infect and proliferate only tumor cells, while leaving healthy tissue alive. Studies using herpes simplex virus with an ablated thymidine kinase gene demonstrated that without the viral ability to produce TTP, the virus was not able to undergo competent replication in the retina. Studies in lower vertebrates and cell culture have shown that levels of thymidine kinase drop significantly after development, to levels that are well under what is found in other tissues (Al-Ubaidi et al 1996; Tesoriere et al 1983). Because retinoblastoma, like all cancers, is actively undergoing cellular divisions, the cellular stores of TTP will very likely be higher than non-cancerous cells. However, the significant drop of replication compared to those viruses with an intact 66R gene would suggest that those stores are not large enough to restore wild type function to the virus. As such, the exceedingly low levels of thymidine kinase found in healthy retinal tissues

and the marked increase found in proliferating retinoblastoma cells mean that it is possible that TPV-Δ66R is could be oncoselective in the retina. This would be important because destruction of adult retinal tissue cannot currently be treated effectively and can lead to significant loss of sight. If the virus is replication competent in tumor tissue only, then this is no longer a consideration.

Conversely, just because the Δ2L TNF inhibitory protein knockout virus showed significant replication does not necessarily mean that it is a good candidate for virotherapy. For the TNF-inhibitor knockout to work, the cancer must be sensitive to tumor necrosis factor. Some cancers are not sensitive to the effects of TNF, and others use it to simulate proliferation. Studies of retinoblastoma cells, especially Y-79 cells, have shown that a common mutation in retinoblastoma is an amplification of chromosomal segments containing TNF genes (Imbert et al 2001). TNF was induced by viral infection among other things (Kawai and Akira 2006). To overcome this, many poxviruses have developed the ability to interrupt TNF production or activation ofTNFRs. Tanapoxvirus is an example of this and has a highly specialized protein to perform this function (Brunetti et al 2002). In many cancers, the presence of TNF causes tumor regression, so it was logical to target this gene for ablation. However retinoblastoma is an exception to this rule. As such, the increased levels of TNF that would be induced by viral infection of TPV-Δ2L could actually lead to increased proliferation of retinoblastoma instead of regression (Imbert et al 2001; Want and Lin 2008).

In general, replication that was observed in this study was orders of magnitude lower than what has been observed in OMK cells and some of the other cancers that have been assayed in the past (Lee and Essani 2010; Conrad et al 2015). On the other hand, replication was significantly better than some other cancers. (Lee and Essani 2010) The low level replication could be caused by many different mechanisms. First, retinoblastoma is considered to be a fast track cancer. This means that compared to adult cancers, it requires less mutations for tumorigenesis to occur. These mutations represent a mechanism that allows for oncolytic virotherapies to retain, or gain tropism in tumor cells (Kaufman et al 2015). A second possible contributing cause of relatively low level replication can be attributed to the relatively small size of the retinoblastoma cells. Retinoblastoma cells are about 5 to 7 micrometers in diameter. This is much smaller than cells such as HeLa and OMK which are 15-30 micrometers or larger (Zhao et al 2007). This drastically small size means that it is likely that Y-79 and WERI-RB1 have significantly less cell volume. To further complicate the issue, these cells also have a large nucleus to cytoplasm ratio (Busch et al 2014). Other studies have shown that most of the Y-79 and WERI-RB1 cell is taken up by the nucleus leaving very little cytoplasm (Busch et al 2014). Poxviruses are relatively large in size, around 300-400 nanometers in length and replicate in the cytoplasm of the cell (Johnson et al 2006; Moyer and Graves 1981). The combination of the small size of the cell and large size of the nucleus of the retinoblastoma cell gives very little room for viral growth to occur.

The final experiment was to determine the ability of these viruses to cause cellular inviability *in vitro*. The end goal of oncolytic virotherapy is to cause lysis of tumor cells and

subsequent regression of the tumor tissue (Guo et al 2008). Beyond lysis caused by the virus itself, virally induced lysis leads to dumping of cellular contents and the activation of the innate immune response (Woller et al 2015). To assay the ability for TPV and recombinants to cause cellular lysis, a cell counting kit-8 was used. TPV-WT-eGFP, ablated viruses and transgene expressing recombinants all showed a significant ability to reduce viable cells 192 hours post infection. Infections with 0.1 MOI of each virus showed about a 40% reduction of viable retinoblastoma tumor cells, which corresponds well with the replication data demonstrating that the virus continued to replicate after 240 hours. The 10 MOI infections showed a significant decrease of viable cells, of about 70%, compared to time 0 controls, and showed a significant decrease compared to 0.1 treatments. The results of the CCK-8 studies demonstrated that TPV and recombinants were able to significantly reduce viable tumor cells in a dose-dependent manner. Individual viruses did not show statistically significant differences in ability to cause cellular inviability. This was not an unexpected result, as the transgenes were not large enough to significantly alter replication, and none of the transgene expressing viruses were shown to increase inviability of retinoblastoma cells. This is likely because they were expressed independent of the innate mammalian immune system.

The ability to reduce the viability of cells of TPV in retinoblastoma is an important indicator of its potential as an ocolytic virotherapy. This ability of TPV to decrease cellular viability by as much as 70% after 8 days in vitro is a very significant result, especially with relatively low level of replication. The reason for this was not explored in these studies, and was outside its scope. However, one possible mechanism that could explain this

could be the fact that retinoblastoma cells require fewer mutations to become cancerous compared to cancers that affect adults (Knudson 1971). Some of the pathways implicated as cellular anti-viral pathways are also pathways that are important in preventing cellular proliferation associated with cancer. If these pathways are still partially intact, they may impede viral replication, and eventually cause the cell to undergo apoptosis or necrosis (Kaufman et al 2015). As a result, high dose treatments of oncolytic TPV would likely be needed to be a tenable potential treatment. Other researchers have assayed the use of MOIs of as high as 1000 to treat retinoblastoma. The results of this study show large decreases, in a dose dependent manner, when cells are infected at a high (10) MOI. In vivo, this may overcome the low level infection seen, especially if the Δ66R TK virus is used.

Future direction:

TPV, as shown in these studies, can be a candidate for further studies to assay potential oncolytic effects. When going forward with these studies, three different avenues of experiments should be taken. The first studies should develop new versions of oncolytic Tanapoxvirus. The first way to do this is to create new ablations. A possible candidate for testing would be the Δ66RΔ136 virus. The proposed virus would contain an ablation of the interferon inhibitory protein gene as well as the 66R thymidine kinase knockout. This would likely further restrict tropism of the virus beyond that of the TK knockout. This virus, as with the rest, would need to be assayed for its ability to competently replicate in retinoblastoma cells and to demonstrate the virus's ability to cause cellular inviability.

Future studies could also create new retinoblastoma specific recombinants. Current recombinant TPVs have been created using genes that are implicated as important in eliciting a general innate immune response. Past studies have shown that interferon-gamma treated retinoblastoma (Cullinan and Brandt 2004) cells cause apoptosis. Other studies conducted using other candidate viruses assayed the ability of non-immuno-stimulatory genes. These oncolytic viruses carried a HSV-tk gene that converted ganciclovir into a form that can be incorporated into the DNA of infected cells; leading to chain terminations and interfering with replication (Ji et al 2008). This would increase cellular inviability, which could be assayed in vitro using the CCK-8 procedure used with other viruses. The one drawback to this virus could be that it would increase viral tropism for healthy retinal cells, as the HSV-TK should function an analogous manner as the 66R gene in making TTP. The second purposed transgene, interleukin 2 (IL-2), is a cytokine involved in generating a Th1 response (Mossmann and Sad 1996). A recent study looking at the response of dendritic cells to the supernatant from retinoblastoma cells found that they produce higher levels of IL-10, which suppresses IL-2 production, and increases evasion by the tumor from the immune system (Ma et al 2014). By inducing IL-2 production as part of the viral life cycle, it could increase Th1 cell proliferation and reduce the ability of retinoblastoma to evade the immune system. Conversely, a current study assaying the replicative ability of TPV-Δ66R-mIL-2 has shown that it reduces the ability of TPV to replicate. As a result, single step replication studies could be conducted to determine if TPV-Δ66R-mIL-2 can replicate successfully in retinoblastoma or if it will hamper replication to the point that it is no longer a candidate (Zhang et al 2016).

Another set of experiments designed for future studies would be to examine the effects of interferon on tanapoxvirus tropism. To study this, an established procedure to assay the effects of interferon on TPV tropism could be used. Other studies using pox viruses have used an immunologically intact mouse models to study the role of the adaptive immune system during treatments with their virotherapies. If the inability for TPV to downregulate host interferon is responsible for tropism limits, then it would be possible to replicate these studies using genetic mouse models and gain better insights into the role of the adaptive immune system in reducing tumor size after treatment with oncolytic TPV. One possible way to assay this would be to use immortalized mouse fibroblasts, which have broken INF-alpha/beta pathways (Wang et al 2009).

These results can also be used as preliminary data for an in vivo study using an induced tumor nude mouse model. These studies would assay the ability of each virus to reduce tumor size, change innate immune cell profiles, and cause changes in tumor morphology and histology compared to mock tumors. A past study has shown that oncolytic TPV recombinant containing fliC was able to reduce induced colorectal tumors in a nude mouse model (Conrad et al 2015). Current *in vivo* studies have shown that oncolytic TPV can also successfully reduce tumors in melanoma, triple negative breast cancer and ovarian cancer (Zhang et al 2016 and Unpublished results). Being able to show that the oncolytic TPV are able to not only show positive results in vitro but also in a living system is an important next step before considering clinical trials.

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