CHARACTERIZATION OF ONCOLYTIC HERPESVIRUSES RODRIGUES

CHARACTERIZATION OF ONCOLYTIC HERPESVIRUSES

By

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ABSTRACT

Oncolytic viruses are able to selectively replicate in tumour cells and are an attractive new avenue of cancer therapy that lacks the toxic side effects of current treatment modalities. HSV-1 mutants lacking ICP0 are promising oncolytic vectors, however, the mechanisms behind viral oncolysis remain unclear. Since PML contributes to the repression of HSV-1 and also is down-regulated in various types of cancer, but particularly in prostate cancer, PML has been implicated as a factor influencing the permissiveness of tumour cells to ICP0-null HSV-1 oncolysis. By screening a series of immortalized patient matched normal and tumour prostate epithelial cells for sensitivity to ICP0-null HSV-1 oncolysis and evaluating the levels of PML in each cell line, we were unable to establish a link between PML status and permissiveness to ICP0-null HSV-1 oncolytic vectors. Also, since a large proportion of the population possesses pre-existing immunity to HSV-1, which may hinder systemic administration of HSV-1 vectors, we sought to determine if BHV-1 could be an alternative oncolytic herpesvirus. BHV-1 was cytotoxic to various human immortalized and transformed cell lines *in vitro*, but was generally more restricted from normal human cells, suggesting that BHV-1 may have potential as an oncolytic virus. However, the sensitivity of human cells to BHV-1 infection did not correlate with type I IFN signaling, as has been demonstrated for other oncolytic viruses. Furthermore, neutralizing antibodies against HSV-1 were unable to cross-react with BHV-1 *in vitro* suggesting that pre-existing immunity to HSV-1 in humans may not hinder BHV-1 infection. It is hoped that these results will contribute to the understanding of viral mediated oncolysis and also provide some evidence that BHV-1 may be a new alternative oncolytic herpesvirus, however, *in vivo* studies are necessary to evaluate the oncolytic efficacy of BHV-1.

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ACV	-	Acyclovir
APL	-	Acute Promyelocytic Leukemia
ATCC	-	American Type Culture Collection
BHV-1	-	Bovine Herpesvirus type 1
bICP0	-	Bovine Herpesvirus type 1 Infected Cell Protein 0
BPE	-	Bovine Pituitary Extract
BVDU	-	Bromovinyldeoxyuridine
CENP	-	Centromeric Protein
CHO	-	Chinese Hamster Ovary
CML	-	Chronic Myelogenous Leukemia
CPA	-	Cyclophosphamide
CPE	-	Cytopathic Effect
CVF	-	Cobra Venom Factor
Daxx	-	Death Associated Protein 6
DMSO	-	Dimethylsulfoxide
DMEM	-	Dulbecco's Modified Eagle's Medium
DNA-PK	-	DNA-dependent Protein Kinase
DTT	-	Dithiothreitol
E	-	Early
EGF	-	Epidermal Growth Factor
eIF2α	-	Eukaryotic Translation Initiation Factor 2α
ER	-	Endoplasmic Reticulum
FBS	-	Fetal Bovine Serum
FOB	-	Fetal Osteoblast
GFP	-	Green Fluorescent Protein
HCF	-	Host Cell Factor
HDAC	-	Histone Deacetylase
HDF	-	Human Dermal Fibroblast
HEL	-	Human Embryonic Lung
HEPES	-	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HRP	-	Horseradish Peroxidase
HMBA	-	Hexamethylene Bisacetamide
HSV-1	-	Herpes Simplex Virus type 1
HVEM	-	Herpesvirus Entry Mediator
ICP0	-	Infected Cell Protein 0
IE	-	Immediate Early
IFN	-	Interferon
Ig	-	Immunoglobulin
IKK	-	IκB Kinase
IL	-	Interleukin
IRF	-	Interferon Regulatory Factor
ISG	-	Interferon Stimulated Gene

L	-	Late
MDBK	-	Madin-Darby Bovine Kidney
MEF	-	Mouse Embryonic Fibroblast
MEGM	-	Mammary Epithelial Growth Medium
α-MEM	-	α-Minimal Essential Medium
MHC	-	Major Histocompatibility Complex
MOI	-	Multiplicity of Infection
MV	-	Myxoma Virus
ND10	-	Nuclear Domain 10
NDV	-	Newcastle Disease Virus
NF-κB	-	Nuclear Factor-κB
NLS	-	Nuclear Localization Signal
Oct-1	-	Octamer binding factor-1
PBS	-	Phosphate Buffered Saline
pfu	-	Plaque Forming Units
PKR	-	Protein Kinase R
PML	-	Promyelocytic Leukemia Protein
PML-NB	-	Promyelocytic Leukemia Protein-Nuclear Body
PMSF	-	Phenylmethylsulfonyl Fluoride
PrV	-	Pseudorabies Virus
PVDF	-	Polyvinylidene Difluoride
PyMT	-	Polyoma Middle T
RAG-2	-	Recombination Activating Protein-2
RARα	-	Retinoic Acid Receptor-α
REST	-	Repressor Element Silencing Transcription
RFU	-	Relative Fluorescence Units
RING	-	Really Interesting New Gene
RPMI	-	Roswell Park Memorial Institute
SEM	-	Standard Error of the Mean
shRNA	-	Short hairpin RNA
SUMO	-	Small Ubiquitin Like Modifier
TAP	-	Transporter Associated with Antigen Presentation
TBS	-	Tris Buffered Saline
TK	-	Thymidine Kinase
TNF	-	Tumour Necrosis Factor
vhs	-	Virion Host Shutoff
VP16	-	Virion Protein 16
VSV	-	Vesicular Stomatitis Virus
WT	-	Wild-type

CHAPTER 1: INTRODUCTION

1.1 Oncolytic Viruses in Cancer Treatment

Oncolytic viruses are able to specifically replicate in cancer cells by exploiting some of the biochemical differences between normal and tumour cells. The manipulation of viruses to target and lyse cancer cells provides an attractive alternative cancer therapeutic, in that viral oncolysis is specific to tumour cells and therefore lacks the toxic side effects of many therapies currently employed. Furthermore, oncolytic viruses have the ability to lyse cancer cells that have become apoptosis-resistant and are refractory to other treatments. The safety of this treatment modality has been shown in the clinical trials that have been carried out to date, as a number of different constructs have been tested and no serious adverse effects have been reported, however, the efficacy of oncolytic virotherapy has been limited (Liu & Kirn, 2008). Furthermore, there remains a lack of understanding regarding the mechanisms underlying tumour targeting and lysis by certain viruses (Bell et al., 2003). It is important to understand the viral and host factors that contribute to specificity, as well as the factors that may make oncolytic viruses more effective at eliminating tumours, so that more successful vectors can be constructed, or existing vectors can be improved.

To this end, a number of different viral constructs are being studied, some of which are naturally oncotropic, including vesicular stomatitis virus (VSV), Newcastle disease virus (NDV), myxoma virus (MV) and reovirus. VSV, NDV and MV are non-human pathogens and are inhibited by various anti-viral signaling pathways in normal human cells, but are able to replicate in tumour cells in which these pathways are defective. Reovirus is an intrinsically benign, dsRNA virus that is inhibited by activated protein kinase R (PKR) – an anti-viral protein, which is inactivated by Ras. Consequently, this virus preferentially replicates in tumour cells with an activated Ras pathway, bypassing the anti-viral effects of PKR (Coffey et al., 1998).

A number of engineered viruses have generated a lot of interest for cancer therapy, including adenovirus, measles virus and herpes simplex virus type 1 (HSV-1). The first oncolytic virus approved for use in the clinic in conjunction with chemotherapy was an engineered oncolytic adenovirus H101 (E1B-55K and E3B-deleted), for treatment of head and neck cancer in China. This vector was a derivate of ONYX-015, which lacks E1B-55K – a protein that functions to inhibit p53-mediated apoptosis. This virus is able to replicate in tumour cells lacking p53, but recently it has been demonstrated that the absence of p53 is not an absolute marker for ONYX-015 replication (Petit et al., 2002, Rogulski et al., 2000).

Engineered HSV-1 oncolytic vectors have also generated much interest in the field. To date, Phase I and II clinical trials have been conducted with various

HSV-1 mutants, demonstrating the safety of administering various oncolytic mutants of HSV-1 in humans (Hu et al., 2006, Kemeny et al., 2006, Markert et al., 2000, Rampling et al., 2000).

1.2 Type I IFN Signaling in Cancer

One of the defects in cancer cells that is exploited by a number of different oncolytic viruses is impaired type I interferon (IFN) signaling, including IFNα/β. This family of cytokines possesses both anti-viral and anti-proliferative functions (Stark et al., 1998). In its anti-viral role, IFN is important in innate immunity. Early in infection, these cytokines are secreted by infected cells and are able to act in both an autocrine and paracrine fashion by binding to cell surface receptors. The resultant signal ultimately stimulates the expression of a wide spectrum of IFN-stimulated genes (ISGs), which serve to block viral replication (Platanias, 2005). For example, a well-characterized ISG is the dsRNA-dependent PKR. Upon activation by dsRNA, PKR mediates a number of anti-viral functions, however the most notable is the shut-off of protein synthesis by phosphorylation of the α-subunit of eukaryotic translation initiation factor 2 (eIF2α), thus inhibiting initiation of translation (Sadler & Williams, 2008). The expression of the full complement of ISGs renders the cell in an anti-viral state, meaning the cell can no longer support viral replication. By signaling to the neighbours of infected cells and inducing an anti-viral state, IFN is an important factor in rapidly inhibiting the spread of pathogens.

In addition to its role in innate immunity, IFN has many anti-proliferative properties. A number of proteins involved in IFN signaling have been implicated in tumourigenesis. In particular, a number of IFN regulatory factors (IRFs), a family of transcription factors involved in IFN signaling, have tumour suppressive functions (Takaoka et al., 2008). For example, IRF-1 has been linked to cell cycle regulation and apoptosis (Tamura et al., 2008), and the development of some human cancers has been associated with the loss or decreased expression of IRF-1, including chronic myelogenous leukemia (CML) (Tzoanopoulos et al., 2002), breast cancer (Connett et al., 2005), endometrial cancer (Giatromanolaki et al., 2004) and hepatocellular carcinoma (Moriyama et al., 2001). There is also evidence suggesting a tumour suppressive role for IRF-3, as it has been shown that this transcription factor in involved in DNA-damage induced apoptosis, and overexpression of IRF-3 can inhibit growth of tumour cells *in vivo* and *in vitro* (Duguay et al., 2002, Kim et al., 1999, Kim et al., 2003). IRF-9 (or ISGF3γ) has been linked to the IFN induced expression of the pro-apoptotic protein p53 (Takaoka et al., 2003). Additional components of IFN signaling have been implicated in tumour suppressive roles, including the transcription factor NF-κB, (Chen & Castranova, 2007) as well as the α and β-subunits of the IκB kinase

(IKK) complex (IKKα; IKKβ), which are involved in the activation of NF-κB (Lee et al., 2007, Park et al., 2007).

In addition to IFN signaling components, several ISGs have been implicated for tumour suppressive functions, including promyelocytic leukemia protein (PML) (Bernardi & Pandolfi, 2003, Salomoni & Pandolfi, 2002), ribonuclease RNaseL (Silverman, 2003) and ISG15 (Pitha-Rowe & Pitha, 2007). Consequently, it is common for tumour cells to harbour defects in their IFN signaling pathways, rendering these cells unresponsive to the effects of these cytokines (Stojdl et al., 2003). Therefore, oncolytic viruses that are inhibited by IFN are able to replicate in these types of tumour cells, but are unable to replicate in normal cells with functional IFN signaling.

1.3 Herpes Simplex Virus Type 1 Oncolytic Vectors

1.3.1 Herpesvirus Biology

HSV-1 is a prototypical member of the *Herpesviridae* family, and *Alphaherpesvirinae* subfamily. This virus is a common human pathogen that infects mucosal surfaces of the orofacial region. Productive infection results in lesions on the mucosal epithelia and viral spread to the trigeminal ganglia where the virus can establish a life-long, latent infection – a characteristic of all herpesviruses. HSV-1 is enveloped virus with a tegument coated icosahedral capsid and a large linear dsDNA genome of ~150 kb (Taylor et al., 2002).

During a lytic viral infection, virion attachment occurs through the interactions of the envelope glycoprotein C (gC) or gB with cellular binding receptors – the heparan sulfate moieties on cell surface proteoglycans. HSV-1 entry is mediated through the engagement of gD with one of the known entry receptors, which include the herpesvirus entry mediator (HVEM), a member of the tumour necrosis factor (TNF) receptor family, as well as nectin-1 and nectin-2, which are members of the immunoglobulin (Ig) superfamily (Spear, 2004). Next, membrane fusion occurs through an unknown mechanism, however, it has been shown that gD, gB and the gH/gL heterodimer are essential for this process (Taylor et al., 2002).

Upon membrane fusion, the nucleocapsid and tegument are released into the cytoplasm. The nucleocapsid and some tegument proteins, including the transcriptional activator VP16, are transported to the nucleus, where viral gene expression occurs via the host RNA polymerase (Taylor et al., 2002). Viral gene expression is categorized into three temporal cascades of immediate early (IE), early (E) and late (L) genes. In general, IE genes regulate the expression of E and L genes, whereas E genes are involved in DNA replication, and L genes are largely virion structural components.

Cellular transcription factors HCF and Oct-1 are responsible for the initiation of IE gene expression, however, the interaction between the tegument

protein VP16, Oct-1 and HCF enables more efficient IE gene expression (Katan et al., 1990, LaBoissiere & O'Hare, 2000). The IE genes infected cell protein 4 (ICP4), ICP22, ICP27 and ICP0 are responsible for regulating the expression of E genes and L genes (Taylor et al., 2002). Following E gene expression, and some L genes referred to as leaky late, DNA replication ensues in replication compartments that are adjacent to nuclear domain 10 (ND10), or PML nuclear bodies (PML-NBs), in the nucleus of infected cells (Maul et al., 1996). The replication of HSV-1 is facilitated in neurons by a number of non-essential gene products, including the ribonucleotide reductase ICP6, thymidine kinase (TK), as well as uracil-N-glycosylase. These genes can enhance the pool of available nucleotides for DNA replication in non-dividing neuronal cells. Following L gene expression and the assembly of capsids, DNA is packaged into preformed capsids in the nucleus, after which viral egress and envelopment to produce mature virions occurs (Taylor et al., 2002).

1.3.2 Modulation of the Host Cell Environment by HSV-1

Herpesviruses encode a number of proteins that function to alter the host cell environment to favour viral replication throughout the viral life cycle. For example, the tegument-derived virion host shutoff (vhs) protein is released into the cytoplasm upon infection and is responsible for the degradation of cellular and viral mRNAs early in infection, enhancing the turnover of cellular and viral mRNA (Kwong & Frenkel, 1987, Oroskar & Read, 1989). Subsequently, the activity of *de novo* vhs is inhibited by binding to VP16 (Strand & Leib, 2004).

Another avenue taken by herpesviruses to promote viral replication is the modulation of the host immune response. For example, the IE gene product ICP47 inhibits the transporter associated with antigen presentation (TAP) and as a result, antigenic peptides are not transported to the endoplasmic reticulum (ER), where they would be loaded onto MHC class I molecules for presentation to CD8+ T cells (Hill et al., 1995, York et al., 1994). ICP34.5 is also involved in suppressing host immune responses, by antagonizing the effects of PKR. ICP34.5 is able to counteract PKR activity through its action as a phosphatase accessory factor by recruiting protein phosphatase 1α to dephosphorylate eIF2α and therefore allowing protein translation to proceed (He et al., 1998).

Another example of a viral protein involved in adjusting the host cell environment to favour viral replication is ICP0 – a multi-functional protein that can modulate the host cell in a number of ways. In addition to its activity as a promiscuous transcriptional activator, ICP0 has been implicated in the inhibition of type I IFN signaling in that an ICP0-null HSV-1 is hypersensitive to the effects of IFN, and ICP0 is able to inhibit ISG induction in a proteasome-dependent fashion (Eidson et al., 2002, Mossman et al., 2000). More specifically, it has been demonstrated that ICP0 is able to inhibit ISG expression mediated by IRF-3 and IRF-7 (Lin et al., 2004).

This inhibition of IRF-3 and IRF-7 function, as well as ISG induction, has been linked to the N-terminal zinc-binding really RING finger domain of ICP0. This domain, which has E3 ubiquitin ligase activity, is responsible for mediating all the biological functions of ICP0 (Everett, 2000), with the exception of preventing rRNA degradation late in infection (Sobol & Mossman, 2006). Early in infection, ICP0 localizes to PML-NBs and subsequently promotes the proteasome-dependent degradation of PML via its E3 ubiquitin ligase activity (Boutell et al., 2003, Chelbi-Alix & de The, 1999, Maul & Everett, 1994, Maul et al., 1993). ICP0 has been shown to promote the degradation of other cellular proteins, including the catalytic subunit of the DNA dependent protein kinase (DNA-PK), centromeric proteins A (CENP-A) and CENP-C, causing the disruption of centromeres and halting mitosis (Everett et al., 1999, Lomonte & Everett, 1999, Lomonte et al., 2001, Parkinson et al., 1999). Furthermore, ICP0 interacts with Co-REST/REST/histone deacetylase-1 (HDAC-1) complex which causes the dissociation of HDAC-1, suggesting that ICP0 promotes transcription of the viral genome through the formation of euchromatin (Gu & Roizman, 2007).

Although the aforementioned genes that alter the host cell environment are valuable in promoting the replication of HSV-1, they are not absolutely essential for viral replication in certain circumstances. For example, viruses deficient in genes such as ICP34.5, and ICP0 are often able to grow in transformed cells without significant defects in viral replication. Therefore, viruses lacking genes such as these are often investigated for oncolytic virotherapy.

1.3.2 First, Second and Third Generation Oncolytic HSV-1 Vectors

HSV-1 was the first virus used to demonstrate that a genetic mutation could render a virus oncolytic (Martuza et al., 1991). This virus has generated much interest as an oncolytic virus as a result of the many advantages HSV-1 possesses for this application. The large dsDNA genome allows for the insertion of multiple transgenes, up to 30 kb (Varghese & Rabkin, 2002). Many different types of therapeutic transgenes have been explored for improved efficacy, including cytokines such as interleukin-12 (IL-12) and IL-18, soluble B7-1 as well as antiangiogenic agents, such as platelet factor 4 (Derubertis et al., 2007, Fukuhara et al., 2005, Ino et al., 2006, Liu et al., 2006). HSV-1 also has the ability to infect a broad range of cell types, as receptors are widely expressed different in human tissues. Since HSV-1 can undergo a lytic replication cycle, this virus is able to kill infected cells. HSV-1 is also advantageous in terms of safety, since this virus rarely causes severe illness in immunocompetent adults (Varghese & Rabkin, 2002). Furthermore, anti-viral drugs such as acyclovir (ACV) and ganciclovir are readily available for clinical use to control viral replication if necessary. In addition, unlike retroviral vectors, HSV-1 does not insert viral DNA into the host genome, therefore there is no risk of insertional mutagenesis (Varghese & Rabkin, 2002).

Oncolytic HSV-1 vectors have often been explored in the context of glioblastomas, capitalizing on the neurotropic nature of HSV-1. First generation oncolytic HSV-1 vectors were mutants deficient in the viral-encoded TK enzyme, or ICP6, the large subunit of the viral ribonucleotide reductase (Martuza et al., 1991, Mineta et al., 1994, Yamada et al., 1991). These mutations restricted viral replication to rapidly dividing cells, such as human gliomas cells.

Subsequently, second generation, multi-mutated vectors were designed in order to reduce the risk of the virus reverting to WT. To this end, the vector G207 was constructed with deletions in both copies of the neurovirulence gene ICP34.5, as well as a lacZ insertion in *ICP6* (Mineta et al., 1995). The use of G207 in the treatment of human gliomas has been evaluated in a phase I clinical trial, showing that intracerebral inoculation in malignant glioma patients is safe and well-tolerated (Markert et al., 2000). In order to improve the host anti-tumour immune response following viral oncolysis, G207 was modified by deleting the *ICP47* gene, yielding the third generation oncolytic vector, G47Δ (Todo et al., 2001). Given the role of ICP47 in blocking transport of antigenic peptides, G47Δ showed enhanced MHC class I presentation and was more effective than G207 at inhibiting tumour growth in both human xenografts and syngeneic murine tumour models (Todo et al., 2001).

1.3.4 ICP0-null HSV-1 Oncolytic Vectors

The oncolytic HSV-1 vectors of interest in the Mossman laboratory are a series of HSV-1 mutants possessing lesions in ICP0, including KM100 ($ICP0^{n212}VP16^{in1814}$) and KM120 ($ICP0^{7134LacZ}VHS^{UL41}$) (Lin et al., 2004, Mossman & Smiley, 1999). KM100, in addition to possessing a nonsense mutation at codon 212 (n212) in ICP0, also has an in frame insertion linker mutation in VP16, disrupting the C-terminus of the transcriptional activator such that it can no longer bind to cellular transcription factors Oct-1 and HCF, however virion assembly and structure are not affected (Ace et al., 1989, Cai et al., 1993, Cai & Schaffer, 1989). In KM120, the coding region of ICP0 has been replaced with LacZ, and vhs activity has been abolished through the deletion of 196 codons (Cai & Schaffer, 1989, Read et al., 1993). These mutant viruses are unable to reproductively infect non-transformed non-immortalized fibroblasts (Hummel et al., 2005). However, ICP0-null HSV-1 vectors were cytotoxic to a number of human and murine transformed cells lines (Hummel et al., 2005, Lin et al., 2004, Mossman & Smiley, 1999). Since ICP0 is involved in counteracting IFN signaling, it was not surprising that permissive transformed lines were also largely unresponsive to type I IFN treatment, suggesting a correlation between IFN signaling and permissiveness to ICP0-null HSV-1 vectors (Hummel et al., 2005). Furthermore, in the polyoma middle T (PyMT) murine breast adenocarcinoma model, KM100 was able to eliminate 80% of primary tumors, as well as elicit an anti-tumor immune response that was sufficient to confer resistance to tumor re-

challenge (Hummel et al., 2005). This study demonstrated that KM100 is a promising anti-cancer agent with an excellent therapeutic index. Although ICP0-null HSV-1 vectors show potential as effective oncolytic vectors, the mechanisms underlying ICP0-null HSV-1 mediated oncolysis remain to be elucidated. Recently, candidate genes that may influence the permissiveness of tumour cells to ICP0-null HSV-1 vectors have been identified, one of which is PML.

1.4 A Role for PML in Permissiveness to ICP0-null HSV-1 Oncolytic Vectors

1.4.1 The Tumour Suppressive Functions of PML

The PML gene is expressed as seven alternatively spliced mRNAs, each resulting in a distinct protein (PML I-VII), only one of which is cytoplasmic. The remaining nuclear isoforms of PML possess a nuclear localization signal (NLS), and associate with sub-nuclear structures PML-NBs (Everett & Chelbi-Alix, 2007). PML is post-translationally modified by the addition of small ubiquitin like modifier (SUMO). The presence of SUMO-modified PML is essential for the proper formation of PML-NBs (Ishov et al., 1999, Seeler & Dejean, 2001).

PML has been identified as a tumour suppressor, but because of the multiple cellular functions of PML, there are numerous mechanisms by which PML may inhibit tumourigenesis (Salomoni et al., 2008). PML was first identified as the translocation partner of retinoic acid receptor α (RARα) in acute promyelocytic leukemia (APL), thus immediately implicating a role for PML in cancer (de The et al., 1990). The resultant fusion protein PML-RARα, disrupts PML-NBs in a dominant negative manner by sequestration of PML (Daniel et al., 1993). Furthermore, a role for PML in the induction of apoptosis has been illustrated, since p53-dependent apoptosis relies on PML (Guo et al., 2000). Also, PML is required for the induction of cell death through death-associated protein 6 (Daxx) (Zhong et al., 2000). In addition to apoptosis, PML has also been linked to the cell cycle and cellular senescence. For example, PML interacts with HDACs, implicating a role in repressing transcription (Lin et al., 2006). Moreover, the overexpression of PML causes cell cycle arrest in cancer cell lines (Le et al., 1996, Mu et al., 1997).

Studies with PML knockout mice have provided more direct evidence for the involvement of PML in cancer, since PML-/- mice are more susceptible to tumourigenesis (Wang et al., 1998). Furthermore, the loss or down-regulation of PML has been demonstrated in a number of human cancers. In addition to its well characterized roll in APL, loss of PML has been associated with tumor progression in breast and central nervous system cancers, however the highest percentage of reduction has been observed in prostate adenocarcinomas (Gurrieri et al., 2004). Interestingly, the loss of PML in prostate cancer correlated with higher tumour grading (Zhang et al., 2003).

1.4.2 The Anti-Viral Functions of PML

In addition to the role of PML in tumourigenesis, PML has also been linked to cellular anti-viral responses. There is mounting evidence to support a role for PML and PML-NBs in an intrinsic anti-viral defense mechanism against both RNA and DNA viruses, and in particular, HSV-1 (Everett & Chelbi-Alix, 2007). For example PML and Sp100, another component of PML-NBs, are ISGs (Chelbi-Alix et al., 1995, Grotzinger et al., 1996, Guldner et al., 1992, Stadler et al., 1995). Also, incoming HSV-1 genomes localize to PML-NBs and ICP0 is responsible for the subsequent degradation of PML and disruption of PML-NBs, suggesting a role for PML in suppressing HSV-1 infection (Boutell et al., 2003, Chelbi-Alix & de The, 1999, Maul & Everett, 1994, Maul et al., 1993, Maul et al., 1996). Furthermore, in the absence of PML, ICP0-negative HSV-1 mutants show an increase in the efficiency of viral gene expression, as well as plaque-forming ability (Everett et al., 2006).

Recent data illustrates a role for PML and PML-NBs in influencing the permissiveness of tumour cells to ICP0-null HSV-1 oncolysis. It has been observed that cell lines such as the lymph node-derived prostate adenocarcinoma LNCaP, the osteosarcoma U2OS and the acute promyelocyte blast NB4 cell lines were susceptible to ICP0-null HSV infection. However, normal human prostate epithelial cells (RWPE-1), brain (DU-145) and bone (PC-3) derived prostate adenocarcinomas, normal human osteoblasts (FOB), histiocytic lymphoma cells (U937) and chronic myelogenous leukemia (K562) cells were resistant to ICP0-null HSV-1 oncolysis (Sobol *et al*, unpublished data). In screening for PML by immunofluorescence microscopy, it was found that permissive cell lines appeared to possess fewer and less luminescent PML-NBs compared to resistant cell lines (Sobol *et al*, unpublished data). Furthermore, the disruption of PML by expression of PML-RARα rendered the resistant PC-3 cell line sensitive to ICP0-null HSV-1 oncolysis. It was also shown that the resistance of PC-3PML-RARα cells could be attributed to the anti-viral effects of PML through the overexpression of PML, which caused the ICP0-null HSV-1 sensitive PC-3PML-RARα cells to become resistant to ICP0-null oncolysis.

1.4 Characterization of BHV-1 as an Oncolytic Virus

Although HSV-1 oncolytic vectors possess many properties that make this virus well suited for oncolytic virotherapy, there are also a number of disadvantages with this oncolytic platform. As a human pathogen, HSV-1 must be genetically manipulated in order to attenuate the virus sufficiently for HSV-1 to preferentially replicate in tumour cells. This makes production of the virus more difficult, since genetically modified viruses generally do not generate as high

titres of progeny virus as WT viruses. Furthermore, it has been estimated that 40-90% of the population has pre-existing immunity to HSV-1, which may hinder the systemic efficacy of HSV-1 vectors (Baringer & Swoveland, 1973, Corey & Spear, 1986). Although it likely will not affect intratumoural injection, it is likely that intravascular administration of oncolytic viruses will be more clinically useful, since systemic delivery may allow for treatment of multiple tumours, as well as tumours that are difficult to reach directly. For this reason, the rapid neutralization and clearance of HSV-1 before it reaches the tumour is a concern.

Consequently, the use of WT viruses that are not human pathogens as oncolytic viruses may be a better approach to cancer therapy (Parato et al., 2005). In particular, given the advantages of herpesvirus vectors such as HSV-1, we are interested in the potential of bovine herpesvirus type 1 (BHV-1) as an oncolytic virus. It is hoped that BHV-1 will retain some of the advantages of HSV-1 vectors, including a tolerance for insertion of transgenes, the ability to infect many different cell types, as well as a lytic replication cycle. But in addition, BHV-1 may also circumvent some of the problems associated with HSV-1 oncolytic vectors.

1.5.1 The Biology of BHV-1

BHV-1 is a widespread cattle pathogen that, like HSV-1, is a member of the *Alphaherpesvirinae* subfamily. Acute BHV-1 infection is also responsible for causing lesions on mucosal surfaces, followed by the establishment of life-long latency in neurons (Jones & Chowdhury, 2007). Some of the consequences of BHV-1 infection in cattle are conjunctivitis, genital disorders and abortions (Jones & Chowdhury, 2007). Furthermore, BHV-1 is involved in bovine respiratory disease complex, also known as "shipping fever," through transient immunosuppression (Jones & Chowdhury, 2007). This phenomenon is mostly associated with reactivation of the virus from latency following stress. In the absence of bacterial pneumonia seen in shipping fever, BHV-1 symptoms are typically cleared within 2-6 days (Jones & Chowdhury, 2007).

While the structure and life cycle of BHV-1 are similar to what was previously described for HSV-1 biology, there are some differences between these viruses. BHV-1 also recognizes the same attachment and entry receptors as HSV-1, including heparan sulfate and nectin-1 (Campadelli-Fiume et al., 2000). However, unlike HSV-1, BHV-1 is unable to bind to host nectin-2 and HVEM, but is capable of recognizing the poliovirus receptor CD155 (Campadelli-Fiume et al., 2000). While the genes expressed by BHV-1 are generally named for their counterpart in HSV-1, and often have similar functions, there are sometimes differences in function between these two viruses. For example, bICP0 is similar to HSV-1 ICP0 in that it is a transcriptional activator of all classes of viral genes with an N-terminal RING finger domain, which possesses E3 ubiquitin ligase activity (Everett et al., 1993, Koppel et al., 1997, Wirth et al., 1992). Similar to

ICP0, bICP0 promotes the disruption of PML-NBs (Inman et al., 2001, Parkinson & Everett, 2000). bICP0 is able to promote genome transcription through interaction with p300, a histone acetyl transferase, as well as HDAC-1 (Zhang et al., 2006, Zhang & Jones, 2001). bICP0 also inhibits IRF-3 and IRF-7, however, bICP0 differs from ICP0 in that it promotes the proteasomal-dependent degradation of IRF-3 (Henderson et al., 2005, Saira et al., 2007).

One of the interesting features of BHV-1 is the strict host range compared to other herpesviruses, such as HSV-1. Of particular interest is the inability of BHV-1 to productively infect humans or mice, despite the similarities with HSV-1 (Murata et al., 1999). This strict host range suggests that BHV-1 may have potential as an oncolytic virus in humans without genetic manipulation for attenuation. The use of a WT virus as an oncolytic vector has been demonstrated by a number of different non-human pathogens, including MV.

1.5.2 Myxoma Virus is a Natural Oncolytic Virus

The use of a non-mutated, non-human pathogen as an oncolytic vector is well illustrated by MV. MV is a rabbit-specific poxvirus, which causes lethal myxomatosis only in the European rabbit (McFadden, 2005). Since no other vertebrates were shown to be permissive to MV, it was previously thought that non-rabbit species lacked the receptor for MV (McFadden, 2005). However, it was shown that MV was able to productively infect a number of human tumour cell lines *in vitro*, indicating the block to MV infection was downstream of cellular entry (Sypula et al., 2004). Subsequently MV was shown to be an effective naturally oncolytic virus *in vivo* in a human glioma xenograft, as well as in primary and metastatic B16F10 tumours in immunocompetent mice (Lun et al., 2005, Stanford et al., 2008).

This permissiveness of cells outside of the natural host range of MV has been attributed to a number of factors. It was shown in primary human dermal fibroblasts (HDFs) and primary murine cells that sensitivity of the virus to IFN in these cells restricted viral replication (Johnston et al., 2005, Wang et al., 2004). It has also been shown that tumour cells that either possess activated Akt, or allow activation of Akt by MV through interaction with viral M-T5 protein are permissive to MV infection (Wang et al., 2006, Werden et al., 2007). Recently, it has been demonstrated that restriction of MV from primary human fibroblasts requires type I IFN in conjunction with TNF (Wang et al., 2008).

1.5.3 The Potential of BHV-1 as an Oncolytic Virus

There is evidence to suggest that BHV-1 may be an effective WT oncolytic virus, similar to what has been demonstrated for MV. BHV-1 is able to

recognize the same receptor as HSV-1, suggesting that there is a block to BHV-1 infection in human cells that is downstream of cellular entry. This is also illustrated in mice by the observation that WT mice infected with BHV-1 display no clinical symptoms, whereas mice lacking type I and type II IFN receptors, as well as *RAG-2* gene deletions, die within a few days following infection (Abril et al., 2004). This observation suggests that IFN signaling may contribute to the host range of BHV-1. Furthermore, BHV-1 has previously shown oncolytic potential, as it was shown to be cytopathic in various human transformed cell lines *in vitro*, including HeLa, KB, HEp-2 and Had-1 cell lines, as well as the melanoma cell line Mel JuSo (Hammon et al., 1963, Koppers-Lalic et al., 2003). In addition, BHV-1 was shown to inhibit tumour growth in human HeLa tumours heterotransplanted into Syrian hamster cheek pouches (Yohn et al., 1968).

While BHV-1 may be an effective WT oncolytic virus, it may also possess additional advantages over HSV-1 vectors. Despite the widespread BHV-1 infections in cattle, there have been no reports of a productive BHV-1 infection in humans, or humans seroconverting against BHV-1 (Hammon et al., 1963). This lack of immunity against BHV-1 may provide an advantage over HSV-1 as an oncolytic vector, in that BHV-1 may be more effective in immune evasion for successful systemic treatment. Furthermore, in terms of safety, while HSV-1 rarely causes severe illness in humans, BHV-1 has no pathogenic profile in humans.

1.6 Experimental Rationale

In general, given the lack of knowledge regarding the mechanisms of ICP0-null HSV-1 oncolysis, as well as the potential detriments to this oncolytic platform, this thesis explored potential mechanisms contributing to permissiveness of ICP0-null HSV-1 vectors, as well as a possible alternative to this oncolytic platform with the use of BHV-1.

More specifically, given the role of PML in repressing HSV-1 infection and the absence or down-regulation of PML in prostate cancer, this study investigated the hypothesis that the absence or down-regulation of PML often associated with prostate cancer would render prostate tumour cells more permissive to ICP0-null HSV-1 oncolysis than cells with normal levels of PML.

Furthermore, because of the possible advantages of BHV-1 as an oncolytic vector over HSV-1, this study evaluated the potential of BHV-1 as an oncolytic virus. Since viruses that are not human pathogens, such as MV, have been shown to be effective WT oncolytic vectors, we hypothesized that as a non-human pathogen, BHV-1 may also be an effective oncolytic virus. Since type I IFN has been shown to be a factor in restricting the host range of various viruses, including MV, we examined the hypothesis that IFN may contribute to the restriction of BHV-1 replication in human cells.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines

All cell types were maintained at 37°C + 5% CO_2 in media supplemented with 2 mM L-glutamine and 100 U/mL penicillin and 100 ug/mL streptomycin. Human mammary epithelial carcinoma cells MDA-MB-231 and MDA-MB-468 (American Type Culture Collection [ATCC]), as well as murine colon carcinoma CT26 cells (Dr. Yonghong Wan, McMaster University) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). Human osteosarcoma cells (U2OS; ATCC) and human embryonic lung (HEL) fibroblasts (ATCC), were maintained in DMEM with 8% FBS. Primary human adult skin fibroblasts (CCD-1140sk; ATCC) were maintained in DMEM with 12% FBS. Primate kidney epithelial cells (Vero; ATCC) were grown in DMEM with 5% FBS. Madin Darby bovine kidney (MDBK) cells were a kind gift from Dr. Vikram Misra (University of Saskatchewan) and were grown in DMEM with 10% horse serum. Human fetal osteoblast (FOB) cells (M. Subramaniam) were grown in DMEM supplemented with 10% FBS and 500 ug/mL of G418 (Sigma) at 34.9°C. Human prostate adenocarcinoma cells (PC-3P, PC-3M, PC-3M-M2, PC-3M-Pro4 and PC-3M-LN4; (Pettaway et al., 1996)) were a kind gift from Dr. Isaiah Fidler (University of Texas M.D. Anderson Cancer Center) and were maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 5 mM sodium pyruvate. Human prostate epithelial cells (RWPE-1; ATCC) were grown in serum-free keratinocyte medium (Gibco) supplemented with 25 ug/mL bovine pituitary extract (BPE) and 5 ng/mL human epidermal growth factor (EGF). Human patient matched benign and malignant prostate epithelial cells (1532-NPTX and –CP2TX, 1535-NPTX and –CP1TX, 1542-NPTX and –CP3TX (Bright et al., 1997); ATCC) were also maintained in the above medium, supplemented with 5% FBS. Human lung carcinoma cells (A549; ATCC) were grown in α-minimal essential medium (α-MEM) with 10% FBS. Human mammary epithelial cells (MCF-10A and HME-1; ATCC) were maintained in mammary epithelial growth medium (MEGM) with 10% FBS and 13 mg/mL (BPE). Human adult lung fibroblasts (Ventressca and Ronald) were a kind gift from Dr. Jack Gauldie (McMaster University) and were maintained in MEM/F15 with 15% FBS. Murine mammary polyoma middle T (PyMT) tumour cells were isolated from transgenic mice that spontaneously develop mammary adenocarcinomas and were grown in DMEM with 10% FBS. NOP32 tumour cells isolated from neu/Ova transgenic mice were grown in DMEM with 10% FBS and a 1:75 dilution of B-27 supplement (Gibco).

2.2 Viruses

HSV-1 WT strain KOS was propagated and titrated on Vero cells. HSV-1 recombinants include KM100 ($ICP0^{n212}VP16^{in1814}$) and KM120 ($ICP0^{7134LacZ}VHS^{UL41}$) and were propagated and titrated on U2OS in the presence of 3 mM hexamethylene bisacetamide (HMBA; Sigma) (Lin et al., 2004, Mossman & Smiley, 1999). BHV-1 WT strain Cooper was a kind gift from Dr. Vikram Misra (University of Saskatchewan) and was propagated and titrated on MDBK cells. VSV strain Indiana expressing GFP under the VSV genome promoter (VSV-GFP) was kindly provided by Dr. Brian Lichty (McMaster University).

All HSV-1 and BHV-1 constructs were sucrose cushion purified. Confluent cell monolayers in T150 flasks were infected with each virus at a multiplicity of infection (MOI) of 0.01 plaque forming units (pfu)/cell in 4 mL serum-free DMEM for 1 h at 37°C. Cells were maintained in DMEM with 5% FBS for Vero cells or horse serum for MDBK cells. Cells were infected for 2-3 days, after which the cell-associated virus was collected by centrifugation at 200 x *g* at 4°C for 10 min. The supernatant was collected and the cell pellet was resuspended in serum-free DMEM and frozen at -80°C. Virus was isolated from the supernatant fraction by ultracentrifugation at 25,000 rpm for 2 h at 4°C. The virus-containing pellet was resuspended in serum-free DMEM. The cell-associated fraction was processed by freeze/thawing three times, followed by homogenization with a Dounce homogenizer. Virus was collected by centrifugation at 200 x *g* at 4°C for 10 min. The supernatant was collected and combined with the virus isolated from the ultracentrifugation step. The combined samples were sonicated for 1 min. Virus was purified by ultracentrifugation through a 36% sucrose cushion at 30,000 rpm at 4°C for 2 h. Purified viral pellets were resuspended in serum-free DMEM, aliquoted and frozen at -80°C.

Viral titres were determined following one freeze/thaw cycle by serially diluting the virus from 10^{-2} to 10^{-7} in serum-free DMEM and infecting confluent monolayers of the appropriate cell line in 200 uL in a 12-well plate for 1 h. HSV-1 constructs were plaqued by maintaining the infection in DMEM with 2% human serum. BHV-1 plaques were generated by maintaining the infection in DMEM with 5% horse serum and 2% methylcellulose. Cells were fixed with methanol and stained with Giemsa (Sigma) 2-3 days post-infection and viral plaques were counted to calculate viral titre in pfu/mL.

2.3 Cytopathic Effect Assays

For all cytopathic effect (CPE) assays, 90-95% confluent cell monolayers were infected with virus at MOIs of 0.5, 1, 2.5, 5 and 10 pfu/cell MOI in serum-free DMEM. Following viral adsorption for 1 h at 37°C, cells were maintained in

DMEM with 5% FBS. Infection of RWPE-1 cells was done with serum-free keratinocyte medium and cells were maintained in the appropriate growth medium outlined previously. For CPE assays with KM100 and KM120, viral adsorption and maintenance was done in the presence of 3 mM HMBA. At 3 days post-infection, cells were fixed with methanol and stained with Giemsa to visualize CPE. Observed CPE was scored based on the effective concentration (EC_{50}) of virus required to induce CPE in ~50% of the cell monolayer and was assessed blinded.

2.4 Immunofluorescence Microscopy

Cells were seeded on cover slips in a 12-well plate so that they were 50% confluent after 24 h. Cells were fixed with 10% formalin and were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Cells were blocked in phosphate buffered saline (PBS) with 2% goat serum either overnight at 4°C, or for 1 h at room temperature. 30 uL of primary antibody (1:250 mouse anti-PML IgG [Santa Cruz], 1:750 rabbit anti-pan-Acetyl IgG [Santa Cruz]) was added to the cover slips and incubated for 45 min at room temperature (RT). Cover slips were washed 3 times with PBS before the addition of 30 uL of secondary antibody (1:1000 Alexa Fluor 488 chicken anti-mouse IgG [Molecular Probes] and Alexa Fluor 594 chicken anti-rabbit IgG [Molecular Probes]). Secondary antibody was hybridized for 45 min at RT in the dark, after which the cover slips were washed 3 times with PBS. Nuclei were stained with Hoescht (diluted 1:15000; Roche) for 5 min at RT in the dark. All antibody and Hoescht dilutions were done in PBS with 2% goat serum. Cover slips were mounted on slides with 1 uL of mounting media and stored overnight at 4°C in the dark before viewing. Images were taken and analyzed using a Leica DM IRE2 inverted microscope with Openlab software (Improvision). The mean fluorescence intensity of the nucleus of at least 100 cells over at least 3 fields of view was quantified for both PML and pan-Acetyl staining. Mean fluorescence was normalized to background fluorescence quantified from secondary antibody control samples, and the ratio of PML to pan-Acetyl mean fluorescence in the nucleus was calculated.

2.5 Interferon Responsiveness Assays

Sub-confluent monolayers of cells were treated with 1000, 100, 10, 1 and 0.1 U/mL of human IFNα (Sigma) in DMEM with 5% FBS, for 24 h at 37°C, after which the cells were infected with VSV-GFP for 1 h at 37°C. DMEM with 2% methylcellulose and 5% FBS was added to the cells following viral adsorption to promote plaque formation. The quantity of VSV-GFP used for infections varied for each cell type and was determined by titration of VSV-GFP on each cell line to determine the dilution from each VSV-GFP preparation yielding fluorescence

in a linear range for quantification. GFP fluorescence from VSV-GFP replication was quantified 24 h post-infection using a Typhoon BioAnalyzer (Amersham Biosciences). Mean GFP fluorescence for each treatment was normalized to an uninfected, untreated control for each cell line. Relative fluorescent units (RFU) of normalized samples were calculated as a percentage of untreated, infected controls.

2.6 Preparation of Cell Extracts

Whole cell extracts were prepared by scraping cells into the medium in a 6 cm dish. Cells were pelleted by centrifugation at 200 x *g* for 3 minutes at 4°C. Pellets were washed with 10 mL of cold PBS and resuspended in 100 mL of whole cell extract buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM ß-glycerophosphate, 0.2% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM dithiothreitol [DTT], 1x protease inhibitor cocktail [Sigma]) and lysed on ice for 15 minutes. Lysate was centrifuged at 13,000 x *g* for 10 minutes at 4°C and the supernatant was collected. Protein was quantified using the Bradford assay kit (Bio-Rad Laboratories).

2.7 Western Blot Analysis

To prepare whole cell extracts for Western blot analysis, samples were boiled at 100°C for 10 min in 1 x sample buffer. Whole cell extract samples were run on a 7.5% polyacrylamide gel and were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) with a semidry transfer apparatus at 400 mA for 1 h. All blots were blocked in 5% skim milk in Tris-buffered saline (TBS) at RT for 1 h. Primary antibody, rabbit anti-HSV-1 (Dako), was diluted 1:1000 in TBS-Tween (0.1%) and hybridized for 1 h at RT. Anti-rabbit secondary antibody was conjugated to horseradish peroxidase (HRP) and diluted 1:5000 in 5% skim milk in 0.1% TBS-Tween. Westerns were visualized using chemiluminescence.

2.8 Plaque Reduction Assays

For plaque reduction neutralization assays, 2-fold serial dilutions of pooled human serum in serum-free DMEM, from 1:2 to 1:1024, were incubated with 100 pfu of BHV-1 or KOS, for 1 h at 37°C. Confluent monolayers of U2OS cells in a 12-well plate were infected for 1 h. Following viral adsorption, 2% methylcellulose with DMEM and 5% FBS was added to allow for plaque formation. For inhibition with ACV, confluent monolayers of U2OS cells were

infected with 100 pfu of BHV-1 or KOS for 1 h. Cells were maintained in 2% methylcellulose with DMEM and 5% FBS, containing 2-fold dilutions of ACV ranging from 64 ug/mL to 1 ug/mL. For both assays, 100 pfu for each virus was calculated based on titres in U2OS cells. Cells were fixed with methanol and stained with Giemsa 2-3 days post-infection, and plaques were counted.

CHAPTER 3: RESULTS

3.1 A Role for PML in Permissiveness to ICP0-null HSV-1 Oncolytic Vectors

3.1.2 In Vitro Sensitivity of Normal and Tumour Prostate Cells to ICP0-null HSV-1 Oncolysis

PML has been implicated in influencing permissiveness of tumour cells to ICP0-null HSV-1 oncolytic vectors in various normal and tumour prostate, leukemic and bone cell lines (Sobol et al, unpublished data). We were interested in further investigating this observation in patient matched normal and tumour cells in order to provide a better biological comparison of normal versus tumour cells. To this end, a series of immortalized patient matched normal and tumour prostate epithelial cell lines were obtained from three patients, numbered 1532, 1535 and 1542 (Bright et al., 1997). The cell lines generated from these patients include normal prostate epithelial cells (1532-NPTX, 1535-NPTX, 1542-NPTX), as well as corresponding prostate tumour cells (1532-CP2TX, 1535-CP1TX, 1542-CP3TX) (Bright et al., 1997). In order to evaluate the role of PML in permissiveness of these cell lines to ICP0-null HSV-1 oncolytic vectors, each cell line was first investigated for sensitivity to the ICP0-null viruses KM100 and KM120 with a CPE assay.

The normal and tumour cell lines from patient 1532 were very permissive to KM100 showing almost complete CPE an MOI of 0.5 pfu/cell (Figure 1). KM120 infection of cell lines generated from patient 1532 also resulted in high cytotoxicity, with significant CPE observed at MOIs between 1 and 2.5 pfu/cell (Figure 1).

In contrast, the normal and tumour cells isolated from patient 1535 showed differential sensitivity to both ICP0-null HSV-1 vectors. The normal cell line 1535-NPTX was relatively resistant to ICP0-null HSV-1 oncolysis, with MOIs upwards of 5 pfu/cell of KM100 and KM120 required to induce CPE (Figure 1). The tumour cells isolated from patient 1535 (1535-CP1TX) were comparatively sensitive to KM100 with CPE observed at an MOI as low as 0.5

pfu/cell. Similarly, 1535-CP1TX cells were permissive to KM120 compared to the normal cell line with significant CPE observed at 2.5 pfu/cell.

The third patient matched normal and tumour pair examined for permissiveness to ICP0-null HSV-1 oncolytic vectors was from patient number 1542. Both 1542-NPTX and 1542-CP3TX were similarly permissive to KM100 with almost complete CPE at an MOI of 0.5 pfu/cell. Likewise, when assayed for permissiveness to KM120, similar CPE at MOIs of 0.5 and 1 pfu/cell was observed for both the normal and tumour cell lines (Figure 1).

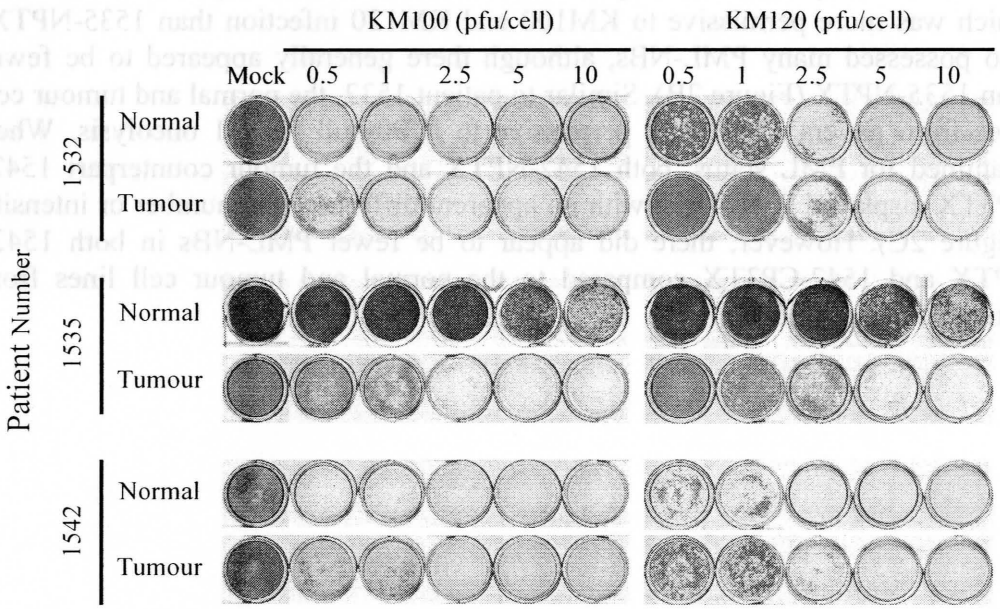


Figure 1. All patient matched normal and tumour prostate epithelial cell lines were sensitive to ICP0-null HSV-1 oncolysis, with the exception of the normal epithelial cells from patient 1535 (1535-NPTX). Cell lines were infected with MOIs ranging from 0.5 to 10 pfu/cell of KM100 or KM120 in the presence of 3mM HMBA. At 3 days post-infection, cells were fixed with methanol and stained with Giemsa to visualize CPE.

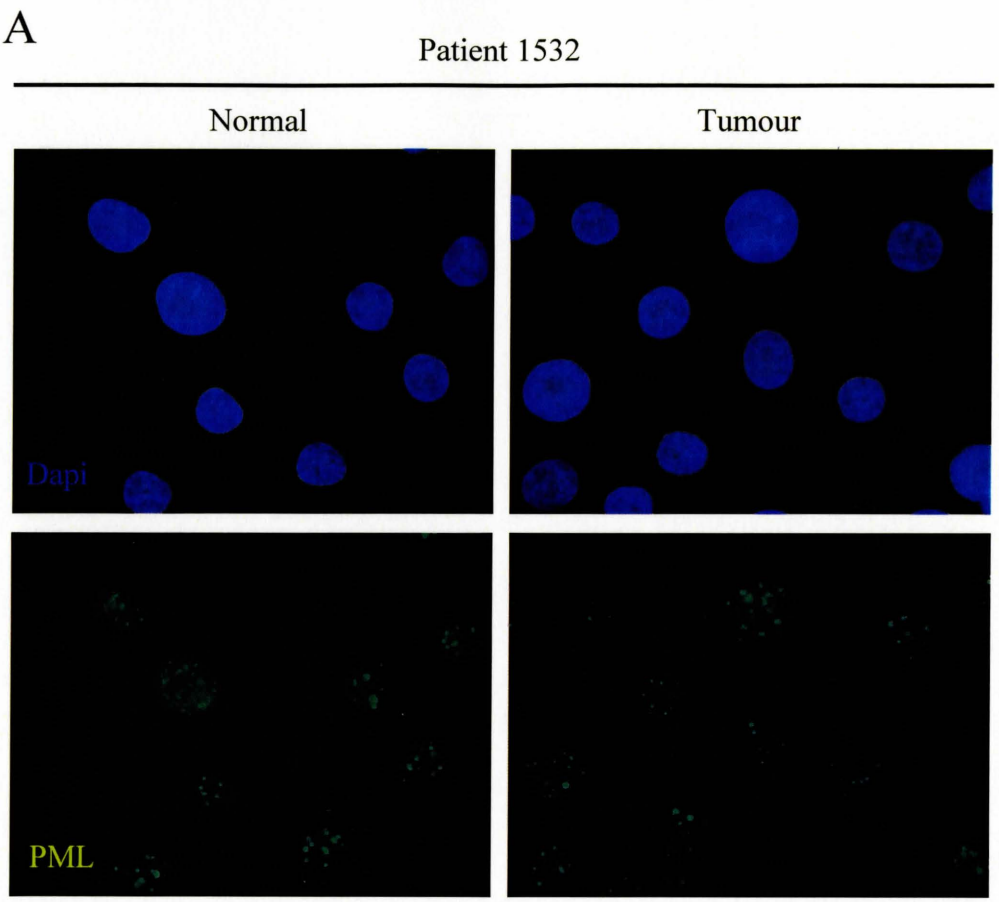
3.1.2 The State of PML in Patient Matched Normal and Tumour Cells

The absence or down-regulation of PML has been observed in a number of different cancer types, with the greatest decrease observed in prostate cancer (Gurrieri et al., 2004). Given the role of PML in suppressing HSV-1 infection, we were interested in evaluating the state of PML in the patient matched normal and tumour prostate cells to determine if there was an absence or down-regulation of

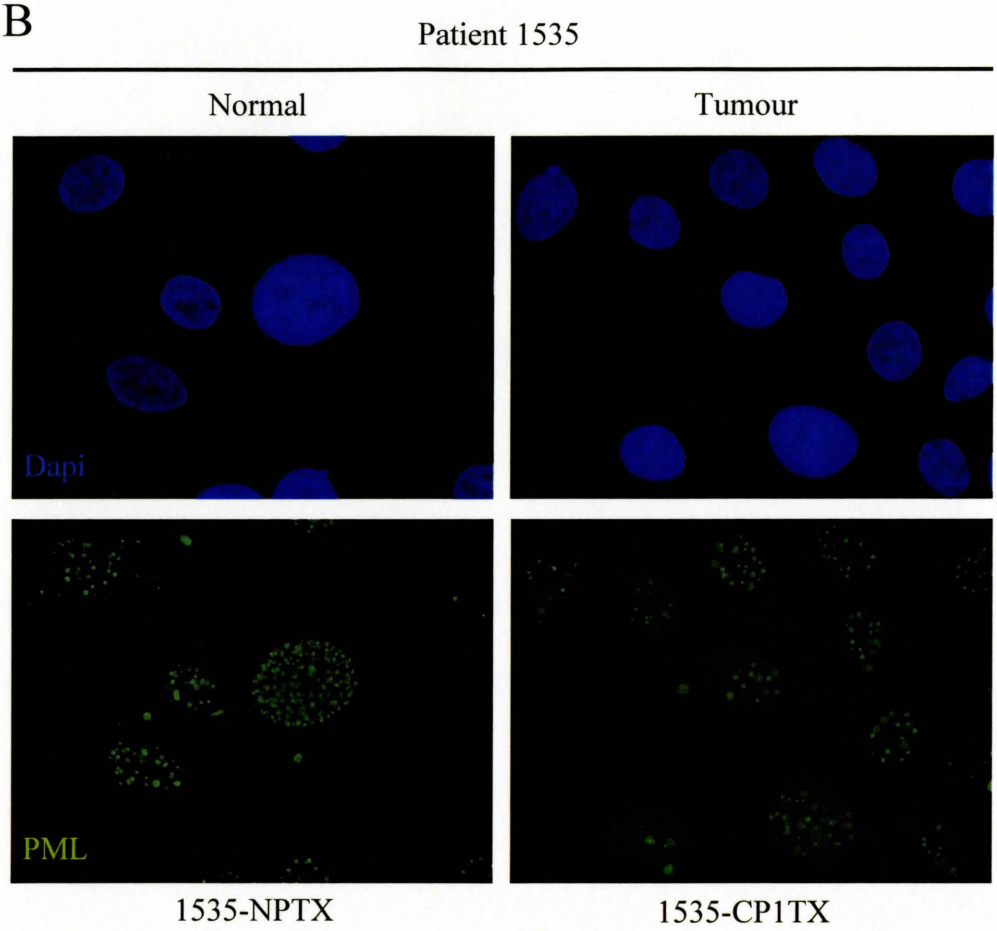
PML, and if this correlated with permissiveness to ICP0-null HSV-1 vectors. Since the presence of PML is required for the proper formation of PML-NBs, the presence of PML in each cell type was evaluated by visualizing PML-NBs with immunofluorescence microscopy.

Although both the normal and tumour cell types from patient 1532 (1532-NPTX and 1532-CP2TX) were permissive to KM100 and KM120, both cell lines possessed numerous PML-NBs (Figure 2A). The normal cell line from patient 1535 (1535-NPTX) was the only cell type that was resistant to ICP0-null HSV-1 oncolytic vectors and when stained for PML, numerous and bright PML-NBs were consistently observed (Figure 2B). The tumour counterpart 1535-CP1TX, which was more permissive to KM100 and KM120 infection than 1535-NPTX, also possessed many PML-NBs, although there generally appeared to be fewer than 1535-NPTX (Figure 2B). Similar to patient 1532, the normal and tumour cell lines from patient 1542 were permissive to ICP0-null HSV-1 oncolysis. When examined for PML status, both 1542-NPTX and the tumour counterpart 1542-CP3TX displayed PML-NBs, with no apparent differences in number or intensity (Figure 2C). However, there did appear to be fewer PML-NBs in both 1542-NPTX and 1542-CP3TX compared to the normal and tumour cell lines from patients 1532 and 1535.

A



B



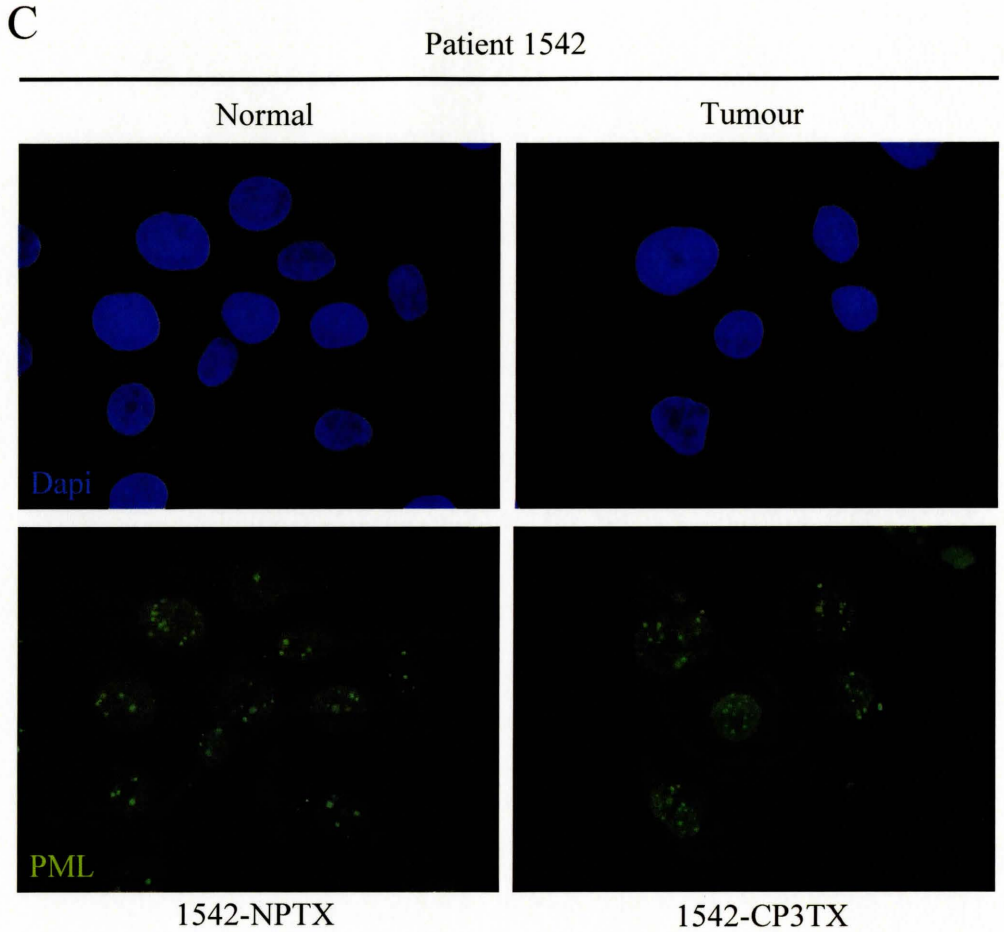


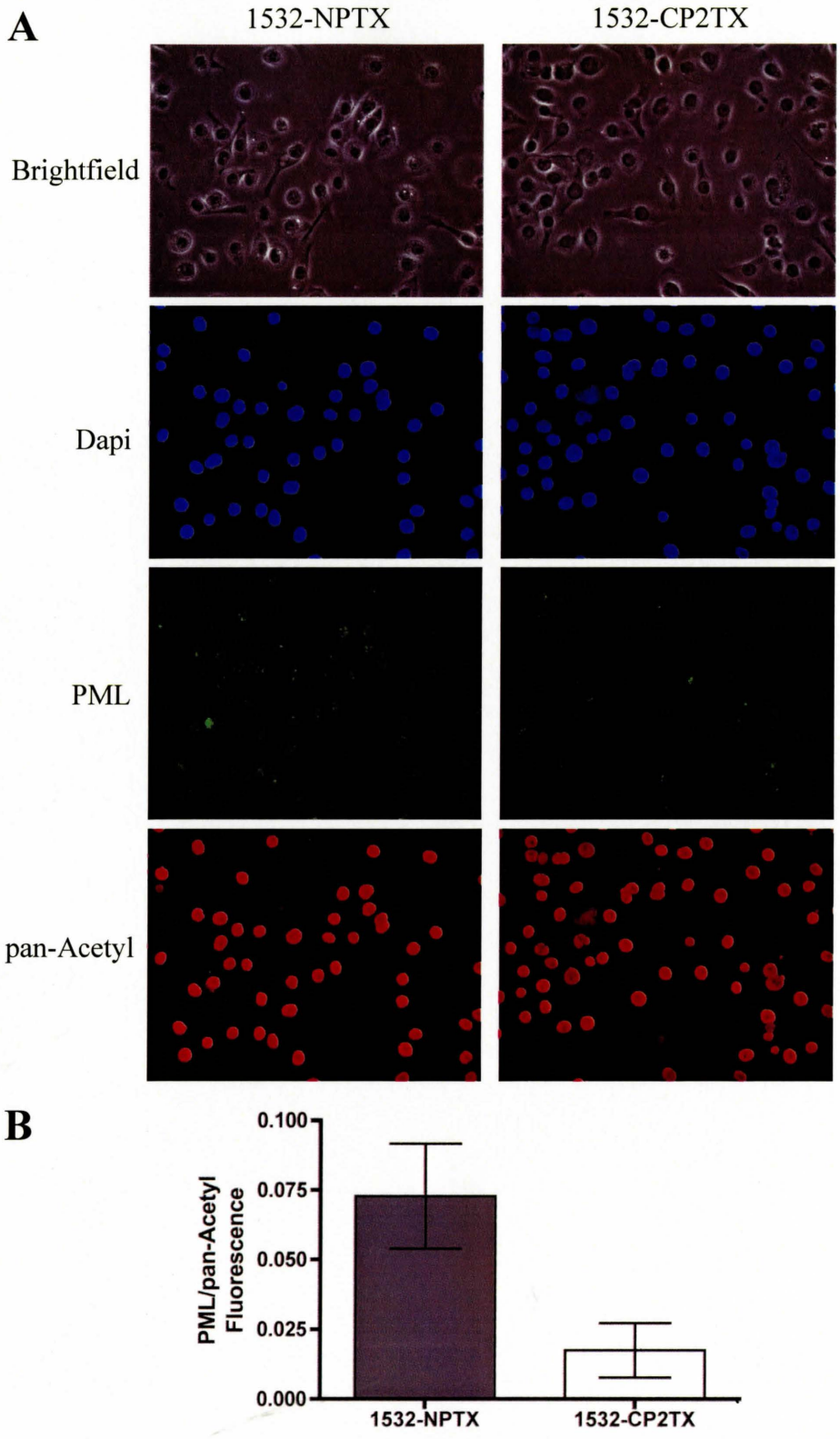
Figure 2. The state of PML in patient matched normal and tumour prostate epithelial cells. PML-NBs are shown in normal and tumour cell lines generated from patient numbers 1532 (**A**), 1535 (**B**) and 1542 (**C**). PML-NBs in each cell line were visualized by immunofluorescence microscopy and dapi staining was used to identify the nuclei. Cells were imaged under 100X magnification.

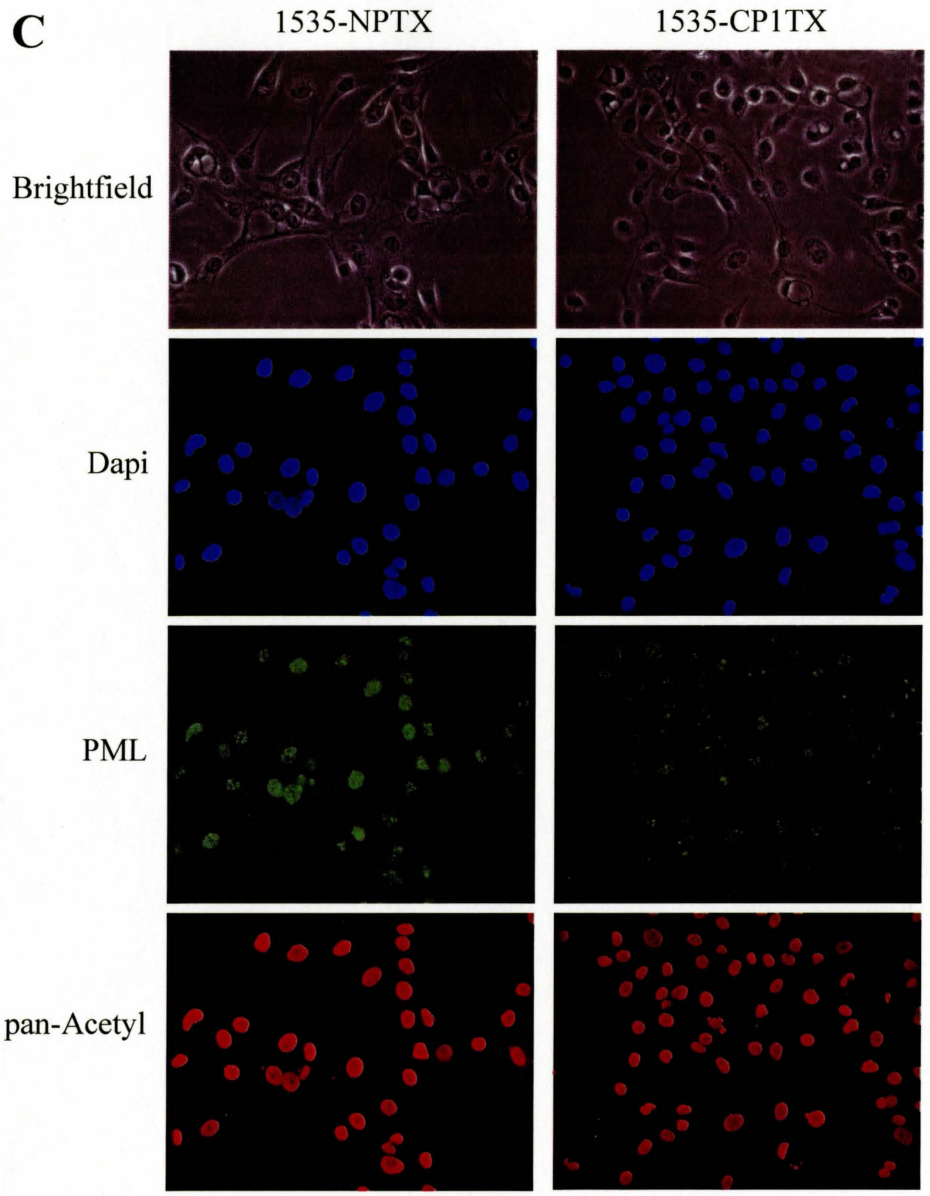
Since PML was present in all cell lines examined, PML fluorescence was quantified to determine if there were differences in PML expression between each normal and tumour cell line and if these differences correlated with ICP0-null HSV-1 oncolysis. PML levels in each cell type were estimated by obtaining the average fluorescence intensity of the nucleus in at least 100 cells, across at least 3 fields of view. Images for quantification were taken under 40x in order to obtain more cells in a given field of view (Figure 3A, C, E). Furthermore, in order to standardize for differences in fixing, permeabilization and staining between each

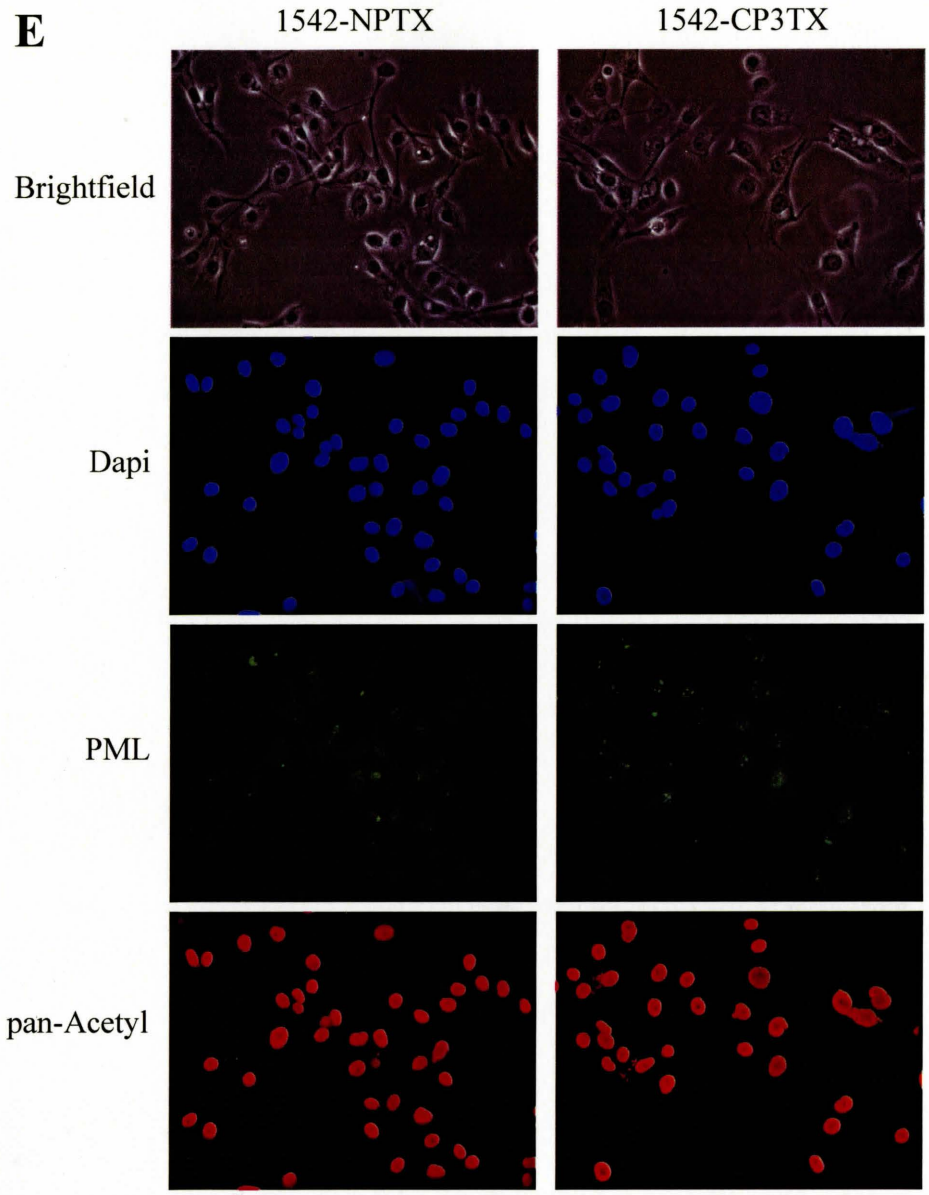
cell line, pan-Acetyl staining was used as an internal nuclear control. Finally, the ratio of PML to pan-Acetyl fluorescence was determined in order to estimate PML expression between different cell lines.

The normal cell line 1532-NPTX had an average 4-fold increase in the PML to pan-Acetyl ratio over the tumour cell line 1532-CP2TX, although this difference was not significant (P=0.12; student's t-test; Figure 5A,B). This result correlated with Western blot analysis, showing similar levels of PML in both cell lines from patient 1532 (P. Sobol, unpublished data, Appendix 1). In contrast, the normal and tumour cell lines from patient 1535 showed a significant difference in the PML to pan-Acetyl ratio, with an average 1.8-fold increase in PML in the normal compared to the tumour cell line (P=0.03; student's t-test; Figure 5C,D). Similar results were observed by Western blot, with relatively higher levels of PML in 1535-NPTX compared to 1535-CP1TX (P. Sobol, unpublished data, Appendix 1). In the cell lines established from patient 1542, there was not a significant difference in PML expression between the normal and the tumour cell lines, as both had similar average PML to pan-Acetyl ratios (P=0.72; student's t-test; Figure 5E,F).

The levels of PML in the normal and tumour cells from patients 1535 and 1542 appeared to correlate with sensitivity to ICP0-null HSV-1 oncolysis. 1535-NPTX, which was relatively resistant to KM100 and KM120 oncolysis, showed a high PML to pan-Acetyl ratio, as well as relatively high protein levels compared to the patient matched tumour cell line 1535-CP1TX. In addition to having lower levels of PML compared to 1535-NPTX, 1535-CP1TX was also more permissive to ICP0-null HSV-1 oncolysis. The cell lines from patient 1542 also appeared to show a correlation. While both the normal and tumour cell lines from patient 1542 were sensitive to KM100 and KM120, both cell lines also showed a low PML to pan-Acetyl ratio, suggesting lower levels of PML in these cell lines compared to the other cell lines examined. In contrast, the high sensitivity of both the normal and tumour cell lines from patient 1532 to ICP0-null HSV-1 oncolysis did not appear to correlate with PML expression, given that PML was present in both cell lines at levels similar to 1535-NPTX, which was relatively resistant to KM100 and KM120.


A
1532-NPTX
1532-CP2TX
Brightfield
Dapi
PML
pan-Acetyl
B
PML/pan-Acetyl Fluorescence
0.100
0.075
0.050
0.025
0.000
1532-NPTX
1532-CP2TX


C
1535-NPTX
1535-CP1TX
Brightfield
Dapi
PML
pan-Acetyl
D
PML/pan-Acetyl Fluorescence
0.100
0.075
0.050
0.025
0.000
1535-NPTX
1535-CP1TX


E
1542-NPTX
1542-CP3TX
Brightfield
Dapi
PML
pan-Acetyl

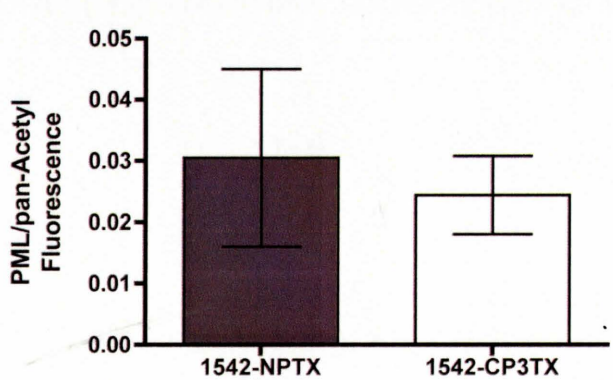

F
PML/pan-Acetyl Fluorescence
0.05
0.04
0.03
0.02
0.01
0.00
1542-NPTX
1542-CP3TX

Figure 3. PML expression in normal prostate epithelial cells compared to patient matched tumour cells. PML was quantitated by obtaining the average fluorescence intensity of the nucleus for at least 100 cells from at least three fields of view. Pan-Acetyl fluorescence was used as an internal nuclear control to standardize differences in fixing, permeabilization and staining between cell lines. The ratio of PML to pan-acetyl fluorescence was calculated in order to compare PML expression between cell lines. Representative immunofluorescence images used for quantitation are shown for patient number 1532 (**A**), 1535 (**C**) and 1542 (**E**). The PML to pan-Acetyl ratio was not significantly different between 1532-NPTX and 1532-CP2TX (P=0.12) (**B**). Patient 1535 showed a significantly reduced PML to pan-Acetyl ratio in 1535-CP1TX compared to 1535-NPTX (P=0.03) (**D**). The PML to pan-Acetyl ratio was not significantly different between 1542-NPTX and 1542-CP3TX (P=0.72) (**F**). The ratios calculated were averaged from 2 independent experiments from patient 1532, and 3 independent experiments from patients 1535 and 1542. Data are expressed as means ± SEM and analyzed with a student's t-test.

3.2 Characterization of BHV-1 as an Oncolytic Virus

3.2.1 Permissiveness of Normal and Tumour Cells to BHV-1

BHV-1 has generally been considered to be species specific, but has been shown to infect human tumour cells *in vitro*, and has shown some oncolytic potential in a hamster tumour model (Hammon et al., 1963, Koppers-Lalic et al., 2003, Yohn et al., 1968). In order to further investigate the oncolytic capacity of BHV-1, we assayed a broad panel of normal and tumour cell lines for sensitivity to BHV-1 *in vitro*. The panel examined included primary normal cells (non-immortalized, non-transformed), phenotypically normal but immortalized cells, and transformed cells from multiple histological origins, including human lung, mammary, bone, prostate and skin cells. In addition, murine cells were examined. Each cell line was assayed for viral induced CPE to evaluate the permissiveness of each cell line. In order to compare the relative permissiveness of each cell line to BHV-1, the observed CPE was scored based on the effective concentration (EC_{50}) of virus required to induce approximately 50% of cell death, with representative images of CPE and the corresponding scores outlined in Figure 4.

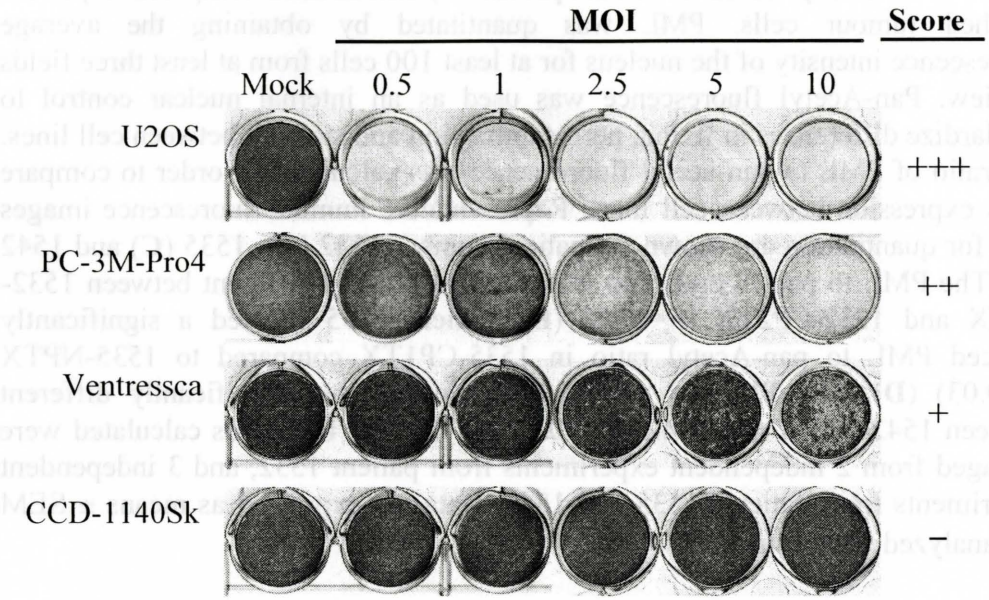


Figure 4. The varying degrees of CPE observed from BHV-1 infection from different cell normal and tumour cell types. Three days post-infection cell monolayers were stained with Giemsa to observe and score CPE: +++ indicates very permissive cells, such as U2OS osteosarcoma cells, in which 50% CPE was seen at an MOI <1; ++ indicates a moderately permissive cell type, such as PC-3M-Pro4 prostate tumour cells, in which 50% CPE was seen between MOIs of 1 and 2.5; + indicates a cell type that is not very permissive, such as Ventressca lung fibroblasts, showing 50% CPE at an MOI >2.5; – indicates a non-permissive cell type in which no CPE is observed at MOIs between 0.5 and 10, such as CCD-1140Sk skin fibroblasts.

All human and murine cell types assayed were sensitive to KOS, with the exception of B16F10 murine melanoma cells (Table 1). This confirmed the presence of the appropriate receptor for both HSV-1 constructs and BHV-1, since it has been demonstrated that both human and murine variants of nectin-1 can mediate entry of both viruses (Geraghty et al., 1998, Menotti et al., 2000, Shukla et al., 2000). CPE as a result of infection with the ICP0-null HSV-1 oncolytic vectors, KM100 and KM120, was determined for comparison to BHV-1, as these viruses have already been characterized for oncolytic effects (Hummel et al., 2005). A number of human lung cells were assayed for permissiveness to BHV-1, including three normal primary lung fibroblasts HEL, Ronald and Ventressca, as well as the lung carcinoma cell line A549. The CPE observed in human lung cells from infection with BHV-1 showed a very similar pattern to that seen for KM100

and KM120. Infection of the normal cells with KM100, KM120 and BHV-1 resulted in some CPE at high MOIs, however these viruses were very cytotoxic to the tumour cell line A549 (Table 1).

Human mammary cells, including two immortalized mammary epithelial cell lines MCF-10A and HME-1 and two transformed cell lines MDA-MB-468 and MDA-MB-231 were all permissive to KM100 and KM120, regardless of normal or tumour phenotype (Table 1). Similarly, BHV-1 did not show preferential cytotoxicity in the tumour cell lines compared to the immortalized cells. With the exception of MDA-MB-231 cells, which were not very permissive to BHV-1, all mammary cell lines assayed were generally sensitive to BHV-1 (Table 1).

For human bone cells, FOB cells, which are immortalized osteoblasts, were utilized as a normal cell type compared to the osteosarcoma cell line U2OS. Although CPE was induced at higher MOIs in FOB cells, KM100 and KM120 were much more cytotoxic to the tumour cell type U2OS (Table 1). In contrast, BHV-1 showed no preference for either the immortalized or transformed cells, as both FOB and U2OS were very permissive to BHV-1 (Table 1).

The immortalized prostate epithelial cell line RWPE-1 was assayed for sensitivity to ICP0-null HSV-1 oncolytic vectors and BHV-1, along with the tumour cell line PC-3P, and its tumourigenic variants PC-3M, PC-3M-M2, PC-3M-Pro4, as well as the highly metastatic variant PC-3M-LN4 (Pettaway et al., 1996). RWPE-1 cells, as well as all the prostate tumour cell lines, were very permissive to both KM100 and KM120, although KM100 induced slightly less CPE in PC-3M-LN4 cells (Table 1). BHV-1 was cytopathic in all prostate cell lines assayed, however, the virus was slightly more cytotoxic to the immortalized RWPE-1 cells compared to the PC-3 series of cell lines (Table 1). Similar to KM100, BHV-1 showed slightly less cytotoxicity in PC-3M-LN4 cells compared to the other prostate cells assayed (Table 1).

In addition to the human prostate cells discussed above, the patient matched immortalized normal and tumour prostate epithelial cells examined in section 3.1 were also assayed for sensitivity to BHV-1. As shown in Figure 1, KM100 and KM120 were very cytotoxic to both the normal and tumour cell lines from patient 1532 (1532-NPTX and 1532-CP2TX) and 1542 (1542-NPTX and 1542-CP3TX) (Figure 1, Table 1). However, 1535-NPTX was not very permissive to KM100 and KM120 compared to the tumour counterpart cell line 1535-CP1TX (Figure 1 and Table 1). In contrast, BHV-1 was more cytotoxic to the tumour cell line from patient 1532 (1532-CP2TX) compared to the normal cells (1532-NPTX). Furthermore, the normal and tumour cell lines from patients 1535 and 1542, were not very permissive to BHV-1, and no difference in cytotoxicity between the normal and tumour cell lines was observed (Table 1).

Primary human adult skin fibroblasts CCD-1140Sk were included in the panel in an effort to further examine the possible differences in permissiveness to BHV-1 between primary and immortalized cell lines. Similar to what was observed with the normal lung fibroblasts, CCD-1140Sk cells were not very

permissive to KM100 and KM120, as some CPE was observed only with high MOIs (Table 1). BHV-1 appeared to be restricted from this cell type, since no CPE was observed at any of the MOIs examined (Table 1).

In order to investigate the oncolytic capabilities of BHV-1 *in vivo* in future studies, a number of murine cell lines that could potentially be used as murine tumour models were assayed *in vitro* for permissiveness to BHV-1. The cell lines examined were the murine breast adenocarcinoma cells PyMT, the colon carcinoma CT26 cells, the neu/Ova breast tumour NOP32, as well as the melanoma cell line B16F10. Not all murine cell lines were assayed for sensitivity to KM100 and KM120 in this study, as the permissiveness has been described previously. Regardless of tumour cell type, BHV-1 was restricted from all murine cells (Table 1).

Overall, BHV-1 appeared to have some oncolytic properties in certain cell types with 7/11 (64%) normal or immortalized cell types relatively resistant (CPE scores of + or –), and 8/12 (67%) tumour cell types relatively permissive (CPE scores of +++ or ++). Furthermore, the process of immortalization appeared to affect permissiveness to BHV-1, since all of the phenotypically normal cell types that were permissive to BHV-1 were immortalized cell lines, whereas all of the normal, primary cell types were relatively resistant to BHV-1 infection.

Table 1. *In vitro* cytotoxicity of KOS, ICP0-null HSV-1 oncolytic vectors KM100 and KM120, and BHV-1 in normal and tumour cell types.

Tissue Type	Cell Line	State	CPE Assays			
			KOS	KM100	KM120	BHV-1
Human Lung	HEL	Normal	+++	+	++	+
	Ronald	Normal	+++	+	+	+
	Ventressca	Normal	+++	+	++	+
	A549	Transformed	+++	+++	+++	+++
Human Mammary	MCF-10A	Immortalized	+++	++	+++	++
	HME-1	Immortalized	+++	+++	+++	+++
	MDA-MB-468	Transformed	+++	+++	++	+++
	MDA-MB-231	Transformed	+++	++	++	+
Human Bone	FOB	Immortalized	+++	+	++	+++
	U2OS	Transformed	+++	+++	+++	+++
Human Prostate	RWPE-1	Immortalized	+++	+++	+++	+++
	PC3-P	Transformed	+++	+++	+++	++
	PC3-M	Transformed	+++	+++	+++	++
	PC3-M-M2	Transformed	+++	+++	+++	++
	PC3-M-Pro4	Transformed	+++	+++	+++	++
	PC3-M-LN4	Transformed	+++	++	+++	+
Human Patient Matched Prostate	1532-NPTX	Immortalized	+++	+++	++	+
	1532-CP2TX	Transformed	+++	+++	++	+++
	1535-NPTX	Immortalized	+++	+	+	+
	1535-CP1TX	Transformed	+++	++	++	+
	1542-NPTX	Immortalized	+++	+++	+++	+
	1542-CP3TX	Transformed	+++	+++	++	+
Human skin	CCD-1140sk	Normal	+++	+	+	–
Murine	PyMT	Transformed	+++	++	ND	–
	CT26	Transformed	+++	ND	ND	–
	NOP32	Transformed	+++	+++	+++	–
	B16F10	Transformed	–	ND	ND	–

ND, not determined

3.2.2 IFN Responsiveness of Human Normal and Tumour Cells

It has been shown for various oncolytic viruses, including VSV, NDV, MV and ICP0-null HSV-1 oncolytic vectors, that permissiveness of certain cell types correlates with impaired type I IFN signaling (Johnston et al., 2005, Krishnamurthy et al., 2006, Stojdl et al., 2000, Wang et al., 2004). The normal and tumour cell types screened for sensitivity to BHV-1 were assayed for IFN responsiveness in order to determine if IFN signaling may influence permissiveness to BHV-1 infection. Cells were treated with human IFNα for 24 hours prior to infection with VSV-GFP. VSV replication, as estimated by GFP fluorescence, was used to assess the IFN responsiveness of each cell line, as cells responsive to IFN will be unable to support VSV replication. Furthermore the EC_{50} of IFN required to protect each cell type was calculated to compare IFN responsiveness between cell lines.

Human lung cells, despite KM100, KM120 and BHV-1 showing preferential cytotoxicity in A549 cells compared to the normal fibroblasts, were all responsive to IFN (Figure 5A). For Ronald and Ventressca fibroblasts, cells were protected with low concentrations of IFN (Figure 5A). Although VSV-GFP was able to replicate in HEL and A549 cells with pre-treatment of 10 U/mL of IFN, the majority of cells remained protected (Figure 5A). Similarly, all lung cells showed low EC_{50} values ranging from 0.54 to 3.55 U/mL of IFN (Table 2).

Human mammary cells were all permissive to ICP0-null HSV-1 oncolytic vectors, regardless of normal or tumour origin. Furthermore, all of these cell types were also permissive to BHV-1, with the exception of the mammary tumour cell line MDA-MB-231. This sensitivity did not correlate with IFN signaling, as all four cell lines were responsive to IFN, showing no VSV-GFP replication with 10 U/mL of IFN (Figure 5B). In addition, these cell lines had low EC_{50} values, which ranged from 0.5 to 3.5 U/mL of IFN (Table 2).

The IFN responsiveness of FOB and U2OS cells correlated with the susceptibility to KM100 and KM120. FOB cells were resistant to KM100 and KM120 and were very responsive to IFN (Figure 5C) with a low EC_{50} value (Table 2). U2OS cells, however, showed significant defects in type I IFN signaling, as VSV-GFP was able to replicate with IFN pre-treatments as high as 1000 U/mL (Figure 5C). This is reflected in the high EC_{50} value for U2OS cells (Table 2). In contrast, these differences in IFN responsiveness did not correlate with permissiveness to BHV-1, as both cell lines were sensitive to BHV-1 (Table 1).

The prostate cell lines examined showed a range of responsiveness to IFN treatment. The normal prostate epithelial cell line RWPE-1, as well as the prostate adenocarcinoma cell line PC-3M, were responsive to IFN (Figure 5D, Table 2). The remaining prostate adenocarcinoma cell lines PC-3P, PC-3M-M2, PC-3M-Pro4 and PC-3M-LN4 were only responsive to higher doses of IFN, upwards of 100 U/mL (Figure 5D, Table 2). This pattern of IFN responsiveness did not correlate with susceptibility to KM100, KM120, or BHV-1. RWPE-1 cells were

the most IFN responsive prostate cell line examined, and yet were very sensitive to KM100, KM120 and BHV-1 (Table 1). Although most of the prostate adenocarcinoma cell lines were not responsive to IFN and were also highly sensitive to KM100 and KM120, PC-3M was just as sensitive as the other prostate tumour cells but was responsive to IFN. BHV-1, however, was slightly less cytotoxic in the prostate adenocarcinoma cells compared to RWPE-1, despite the fact that all of the prostate tumour cells, except PC-3M, were not responsive to IFN treatment.

Three of the six cell lines generated from the patient matched normal prostate epithelial and tumour cells were not permissive to VSV-GFP and therefore could not be assayed for IFN responsiveness. However, the remaining cell lines, including 1532-CP2TX, 1542-NPTX and 1542-CP3TX were all very responsive to low concentrations of IFN (Figure 5E) and had EC_{50} values less than 1 U/mL (Table 2). KM100 and KM120 were cytotoxic to all three cell lines, therefore there was no correlation with IFN responsiveness (Table 1). Despite the IFN responsiveness of 1532-CP2TX, 1542-NPTX and 1542-CP3TX, 1532-CP2TX was sensitive to BHV-1, whereas 1542-NPTX and 1542-CP3TX were resistant (Table 1), making it difficult to correlate IFN responsiveness with permissiveness to BHV-1.

Finally, the IFN responsiveness of the normal adult skin fibroblast cell line CCD-1140Sk was also characterized. This cell line was highly responsive to IFN with small concentrations of IFN able to block VSV-GFP (Figure 5F). Furthermore, CCD-1140Sk cells had the lowest EC_{50} value of all the cell lines screened (Table 2). These results correlated with permissiveness to KM100, KM120 and BHV-1, since all three viruses were not very cytotoxic to this cell type.

Overall, IFN responsiveness did not appear to play a role in permissiveness to BHV-1. Out of the 12 cell lines that showed some permissiveness to BHV-1, only 4 (33%) were unresponsive to IFN treatment. Similarly, permissiveness to KM100 and KM120 did not appear to correlate with type I IFN signaling in the cell lines screened. Only 5 out of 15 (33%) cell lines that were permissive to KM100 and 18 (28%) cell lines that were permissive to KM120, as well as assayed for IFN responsiveness, showed defects in IFN signaling.

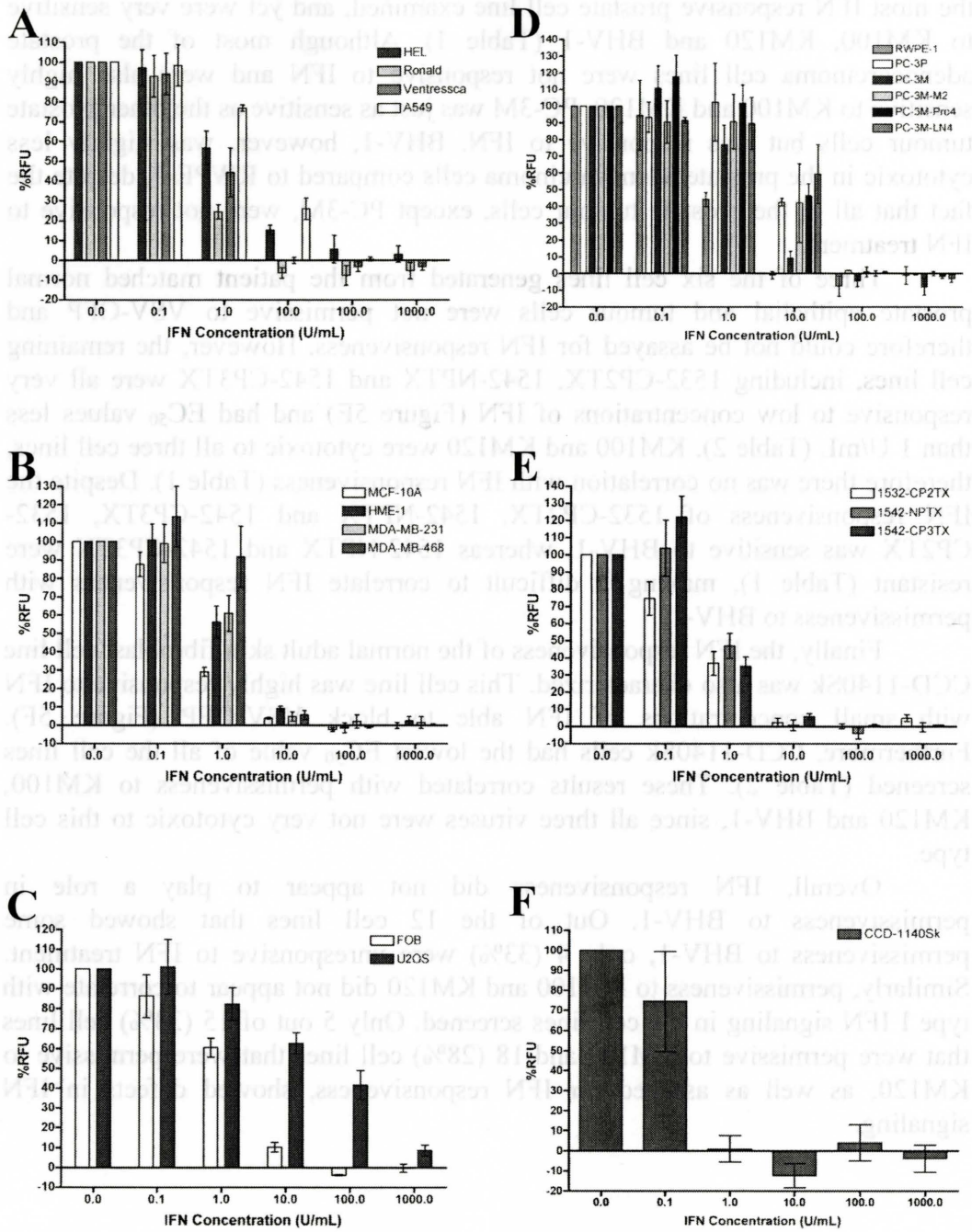


Figure 5. IFN responsiveness of various normal and tumour cell lines, including human lung (**A**), mammary (**B**), bone (**C**), prostate (**D**), as well as patient matched normal and tumour prostate (**E**), and skin (**F**) cells. Cells lines were pre-treated with varying concentrations human IFNα for 24 h before infection with VSV-

GFP. GFP fluorescence was quantified 24 h post-infection and fluorescence relative to an untreated control was calculated. Data are expressed as means ± SEM.

Table 2. The EC_{50} (U/mL) values of IFNα for protection from VSV-GFP challenge of human normal and tumour cell types from multiple histological origins.

Tissue Type	Cell Line	State	EC_{50} (U/mL)*
Human Lung	HEL	Normal	1.499
	Ronald	Normal	0.538
	Ventressca	Normal	0.705
	A549	Transformed	3.554
Human Mammary	MCF-10A	Immortalized	0.500
	HME-1	Immortalized	1.198
	MDA-MB-468	Transformed	3.548
	MDA-MB-231	Transformed	1.319
Human Bone	FOB	Immortalized	1.215
	U2OS	Transformed	41.249
Human Prostate	RWPE-1	Immortalized	0.680
	PC3-P	Transformed	7.226
	PC3-M	Transformed	2.867
	PC3-M-M2	Transformed	10.327
	PC3-M-Pro4	Transformed	8.303
	PC3-M-LN4	Transformed	10.047
Human Patient Matched Prostate	1532-NPTX	Immortalized	np
	1532-CP2TX	Transformed	0.414
	1535-NPTX	Immortalized	np
	1535-CP1TX	Transformed	np
	1542-NPTX	Immortalized	0.769
	1542-CP3TX	Transformed	0.862
Human skin	CCD-1140sk	Normal	0.362

*Expressed as means from at least 3 independent experiments.
np, non-permissive to VSV-GFP

3.2.3 Cross-Reactivity of Antibodies Against HSV-1 with BHV-1

One of the potential advantages of using BHV-1 as an oncolytic vector over HSV-1 is the possibility of circumventing pre-existing immunity, which may allow for more effective systemic delivery in HSV-1 seropositive cancer patients. Therefore, we investigated if neutralizing antibodies against HSV-1 could cross-react with BHV-1. Previously, it has been reported that a rabbit polyclonal antibody against HSV-1 gB cross-reacted with BHV-1 gB and vice versa (Misra et al., 1988). We investigated the ability of a rabbit polyclonal pan-HSV-1 antibody to bind to BHV-1 proteins. U2OS cells were infected with either BHV-1 or KOS and whole cell extracts harvested at 24 and 36 hours post-infection for Western blot analysis. A spectrum of HSV-1 proteins were detected at 24 and 36 hours post-infection, however no BHV-1 proteins were recognized by this antibody at the time points examined (Figure 6A). Although no positive control for BHV-1 infection was included, CPE from BHV-1 infection was observed in U2OS cells at the time that whole cell extracts were collected.

Next we investigated the ability of components of human serum to neutralize BHV-1. Both KOS and BHV-1 were incubated with 2-fold dilutions of human serum prior to infection of U2OS cells. KOS plaque formation was inhibited with higher concentrations of pooled human serum, and was completely abrogated at a serum dilution of 1:8 (Figure 6B). In contrast, BHV-1 plaque formation was not affected by human serum, as the number of plaques formed did not change over the human serum dilutions (Figure 6B), showing that components of human serum specific to HSV-1 were unable to neutralize BHV-1.

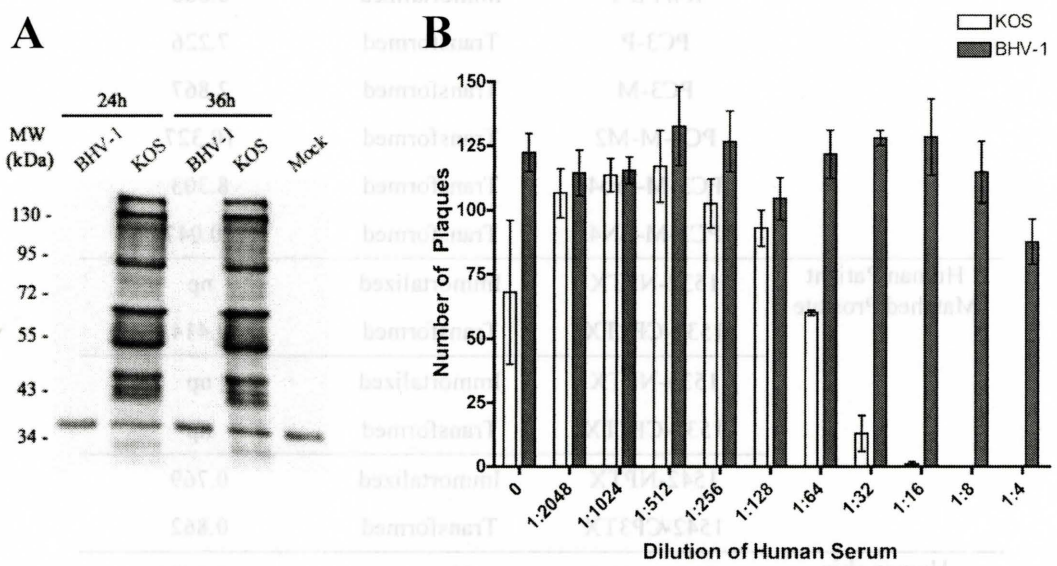


Figure 6. Antibodies against HSV-1 did not cross-react with BHV-1. A rabbit polyclonal pan-HSV-1 antibody did not recognize BHV-1 proteins in a Western blot (**A**). U2OS cells were infected with 10 pfu/cell of BHV-1 or HSV-1 strain KOS. Whole cell extracts were harvested at 24 and 36 h post-infection for Western blot analysis. Human serum did not inhibit BHV-1 plaque formation (**B**). 2-fold dilutions of pooled human serum were incubated with 100 pfu of virus for 1 h prior to infection of U2OS cells. Cells were stained at 3 days post-infection and plaques were counted. Data are expressed as means from 3 independent experiments ± SEM.

3.2.4 ACV Does Not Inhibit BHV-1 Replication

One of the advantages of HSV-1 oncolytic vectors is the availability of clinically utilized drugs, such as ACV, as a safeguard in the event of unwanted viral replication. These anti-herpetic drugs are nucleoside analogs that target the activity of the viral encoded TK. During viral replication, TK will phosphorylate the nucleoside analogue drugs, and therefore they will be incorporated into the newly synthesized DNA and subsequently cause chain termination. Since BHV-1 also possesses a TK gene with the same function as TK from HSV-1, we sought to determine if ACV could inhibit BHV-1 replication, potentially adding another safety advantage of BHV-1 as an oncolytic vector.

A plaque reduction assay with 2-fold serial dilutions of ACV was used to determine if the presence of ACV would inhibit either KOS or BHV-1 plaque formation in U2OS cells. The number of KOS plaques decreased with the addition of ACV and the virus could be inhibited completely with an ACV concentration of 10 ug/mL (Figure 7). However, the presence of ACV did not appear to affect the number of plaques formed by BHV-1 infection, up to an ACV concentration of 20 ug/mL (Figure 7), suggesting that BHV-1 replication was not inhibited by ACV.

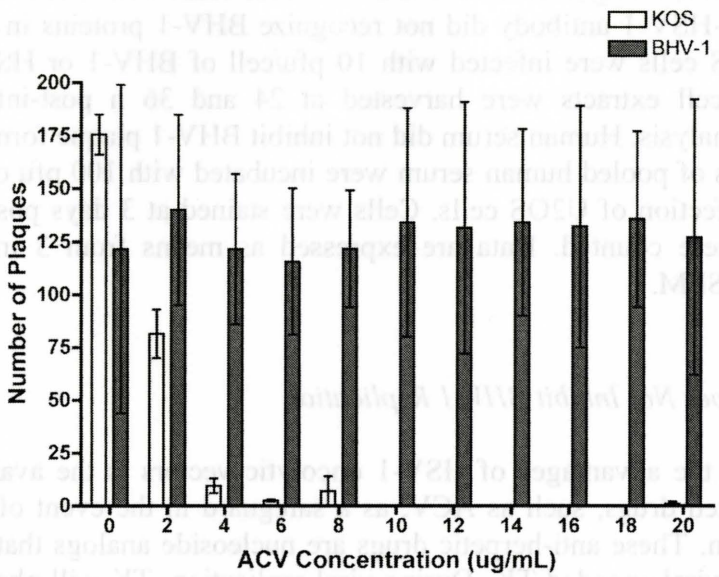


Figure 7. The anti-herpetic drug ACV did not inhibit BHV-1 replication. U2OS cells were infected with 100 pfu of BHV-1 or HSV-1 strain KOS. Following viral adsorption 2% methylcellulose was added to cell monolayers containing 2-fold dilutions of ACV. At 3 days post-infection cells were stained and plaques counted. Data are expressed as means of 2 independent experiments ± SEM.

CHAPTER 4: DISCUSSION

4.1 The Role of PML in Permissiveness to ICP0-null HSV-1 Oncolytic Vectors

Given the lack of efficacy of oncolytic viruses observed in clinical trials for cancer therapy, it is beneficial to elucidate factors that contribute to viral oncolysis, since this knowledge may be useful in designing more effective oncolytic vectors. Furthermore, understanding mechanisms of oncolysis may be valuable in screening tumour types to choose an oncolytic vector that would be most suitable to target that particular tumour.

A role for PML has been suggested in repressing HSV-1 infection, as well as tumour suppression, with down-regulation of PML shown in various cancer types, but particularly in prostate cancer. This investigation sought to determine

whether the down-regulation of PML in prostate tumour cells correlated with sensitivity to ICP0-null HSV-1 mediated oncolysis, by screening prostate cell lines for viral induced CPE and by evaluating the expression of PML. Furthermore, patient matched normal prostate epithelial and tumour cells were utilized to provide biologically relevant comparisons of PML status between normal and tumour cells. Of the three patient matched normal and tumour pairs, only the pair from patient 1535 showed a difference in permissiveness to KM100 and KM120. The normal cell type 1535-NPTX was more resistance to infection with ICP0-null HSV-1 vectors than the tumour counterpart 1535-CP1TX, which appeared to correlate with PML expression.

Unexpectedly, the normal prostate epithelial cell lines generated from patients 1532 and 1542 were just as sensitive to ICP0-null HSV-1 mediated oncolysis as the tumour counterpart cell lines from these patients. In the case of patient 1542, this permissiveness appeared to correlate with relatively lower levels PML, as estimated by the PML to pan-Acetyl ratio, similar to the ratio calculated for the sensitive cell line 1535-CP1TX. Although it appeared that PML was reduced in patient 1542 cell lines, this was not verified by Western blot analysis. While PML expression and sensitivity to ICP0-null HSV-1 oncolysis appeared to correlate in the cell lines from patients 1535 and 1542, a correlation was not observed in the cell lines from patient 1532. Both 1532-NPTX and –CP2TX were permissive to KM100 and KM120, but both had levels of PML similar to the levels observed in 1535-NPTX, which was resistant to ICP0-null HSV-1 oncolysis.

From these data, it is difficult to establish whether or not PML influenced permissiveness of these cell types to ICP0-null HSV-1 oncolysis. It is possible that there were more subtle differences in PML expression among these cell lines that were not observed through these assays. Since the method for quantitating PML fluorescence utilized in this study does not quantify fluorescence throughout the z-plane of the nucleus, the levels of PML estimated may not be accurate. A quantitative Western blot may have been a more sensitive method of detecting differences in PML expression since every cell is collected in a whole cell extract analyzed by a Western blot. Furthermore, this study, as well as others in the field, only evaluated expression levels of PML, however, it is not clear how expression correlates with PML function. Therefore, if there are mutations in PML that may affect the function in some of the tumour cell lines studied, it may not be reflected in the expression observed. Ultimately, however, the disruption of PML through PML-RARα expression, followed by the over-expression of PML, as was performed by Sobol et al. (unpublished data), or short hairpin RNA (shRNA)-mediated knockdown of PML expression, would have been the more effective in establishing a link between PML and permissiveness to ICP0-null HSV-1 oncolysis (Everett et al., 2006).

Three of the six cell lines generated from patient samples were also assayed for IFN responsiveness in section 3.2.2. It has been documented that PML-NBs increase in size and number upon IFN treatment and that PML is

responsible for mediating some of the downstream effects of IFN in the sensitivity of an ICP0-null HSV-1 to IFN (Chee et al., 2003). Given that 1532-CP2TX, 1542-NPTX and 1542-CP3TX were IFN responsive (Figure 5E, Table 2) and that presumably, production of IFN upon infection with ICP0-null HSV-1 oncolytic vectors in these cell lines would result in an increase in PML expression, it was surprising that these cells were not resistant to ICP0-null HSV-1 oncolysis. It may be that PML was not upregulated in these cell lines following the stimulation of IFN signaling upon ICP0-null HSV-1 infection, or that PML does not contribute to the repression of ICP0-null HSV-1 oncolysis in these cell lines. However, it has recently been demonstrated that the depletion of PML in human fibroblasts did not rescue the IFN sensitivity of an ICP0-null HSV-1, however the plaque forming efficiency of an ICP0-null HSV-1 was greater in IFN-treated PML-depleted cells than in IFN-treated PML-positive cells, suggesting that the sensitivity of ICP0-null HSV-1 viruses to the effects of IFN and PML in human cells may be separable (Everett et al., 2008). Furthermore, it has been observed that the effects of IFN and PML on ICP0-null HSV-1 mutants can vary depending on the assay employed and that assaying for virus yield reveals greater differences between ICP0-null and wild-type HSV-1 (Everett et al., 2006, Everett et al., 2008). It is possible that an assay for virus yield in the normal and tumour pairs may be revealed more differences in permissiveness of these cell lines to KM100 and KM120 that may have correlated with PML status. Whether or not PML plays a significant role in the permissiveness of these cell lines to ICP0-null HSV-1 vectors remains to be determined through further investigation.

4.2 Characterization of BHV-1 as an Oncolytic Virus

One of the advantages of using oncolytic vectors as a cancer therapeutic is the potential of successful systemic delivery, without toxic side effects. This may allow for the treatment of multiple tumour foci. Furthermore, this treatment modality would be advantageous for tumours that are difficult to reach and therefore problematic to remove surgically. Although HSV-1 oncolytic vectors possess many advantages as a therapeutic, the ability to treat tumours by intravascular injection may not be as effective as other oncolytic viruses, since it has been estimated that a substantial proportion of the population is seropositive for HSV-1 (Baringer & Swoveland, 1973, Corey & Spear, 1986). Furthermore, the use of genetically altered viruses as anti-cancer agents may be less advantageous in that over-attenuation may hinder efficacy, and genetic defects may make virus manufacturing more costly. As a result, we were interested in characterizing BHV-1 as an oncolytic vector, as an alternative to HSV-1, which may allow for circumvention of pre-existing immunity. Furthermore, as a virus that is not naturally pathogenic in humans, BHV-1 may not have to be genetically

altered to target tumour cells, offering another advantage of mutated HSV-1 vectors.

The *in vitro* cytotoxicity of BHV-1 to various human and murine cell types was evaluated and compared to the cytotoxicity of characterized ICP0-null HSV-1 oncolytic vectors to determine if BHV-1 has oncolytic properties. In general, KM100 and KM120 appeared to be more cytotoxic to tumour cells than BHV-1, since every tumour cell type assayed was very sensitive to ICP0-null HSV-1 oncolysis, whereas BHV-1 was cytotoxic in 8/12 (67%) human tumour cell lines. However, BHV-1 appeared to be less cytopathic to normal or immortalized cells, since 7/11 (64%) normal or immortalized cells were relatively resistant to BHV-1 induced cytopathic effects. In contrast, KM100 and KM120 were less cytopathic in 6/11 (54%) and 3/11 (27%) normal or immortalized cells respectively. The *in vitro* cytotoxicity of KM100 and KM120 evaluated in this study shows that these ICP0-null viruses were generally more cytotoxic to normal cells than was previously described by Hummel *et al.* (Hummel et al., 2005). In particular, slightly more CPE from ICP0-null HSV-1 infection was observed in HEL, MCF-10A and RWPE-1 cells in this study. This may be a result of differences in the assays, since CPE assays were carried out for three days in this study, whereas Hummel *et al.* allowed only two days of infection (Hummel et al., 2005). This is supported by previous observations showing that KM100 was capable of inducing more CPE in HEL cells over a three-day infection than the CPE reported by Hummel *et al.* (Mossman & Smiley, 1999).

Interestingly, the effects of immortalization on phenotypically normal cell lines may have influenced permissiveness to BHV-1. Of the four primary, normal cell types examined, including HEL, Ventressca, Ronald and CCD-1140Sk cells, all were consistently resistant to BHV-1 infection. In particular, CCD-1140Sk cells were non-permissive at all MOIs examined. Therefore, BHV-1 may be more cytotoxic to both immortalized and transformed human cells *in vitro*, compared to normal cell types, suggesting that BHV-1 may indeed have oncolytic properties, however *in vivo* experiments would be valuable in further elucidating the specificity of BHV-1 for tumour cells. Previous data suggests that BHV-1 had anti-tumour effects in a heterotransplantable human tumour in hamster cheek pouches (Yohn et al., 1968). Inoculation of HeLa tumours with BHV-1 caused regression in 5/17 tumours, three of which showed complete regression (Yohn et al., 1968). Furthermore, BHV-1 was shown to replicate in this tumour type given that 6/13 tumours yielded virus (Yohn et al., 1968). Although this suggests that BHV-1 may have oncolytic effects, this study failed to detect definitive evidence of viral cytopathology in tumours, suggesting further *in vivo* investigation is required to understand the mechanism of BHV-1 inhibition of tumour growth (Yohn et al., 1968).

When assayed for cytotoxicity, all murine cell types were consistently resistant to BHV-1. Furthermore B16F10 cells were also resistant to KOS, which has previously been documented and is attributed to a lack of receptors, since the transfection of HVEM and nectin-1 renders B16F10 cells permissive to HSV-1

(Miller, 2001, Randazzo et al., 1995). The resistance of murine cells to BHV-1 was not surprising, since it has been documented that both attachment and penetration of BHV-1 in non-permissive murine embryo fibroblasts is impaired compared to permissive MDBK cells (Murata et al., 1999). This impairment could be greatly improved by the expression of pseudorabies virus (PrV) glycoproteins C (PgC) and PgB, suggesting some differences in binding of heparan sulfate on murine cells compared to permissive bovine cells (Nakamichi et al., 2001, Nakamichi & Otsuka, 2000). This link between the roles of BHV-1 gB and gC in the binding and entry to murine cells is supported by data showing that the murine homologue of nectin-1 is able to mediate the gD-dependent entry of BHV-1 in Chinese hamster ovary (CHO) cells without impairments compared to PrV and HSV-1, suggesting that interactions between gD and murine nectin-1 may not be impaired (Shukla et al., 2000). Furthermore, beyond binding and entry of BHV-1 to murine cells, there are additional blocks to BHV-1 replication. It was demonstrated with the BHV-1 recombinants expressing PgC and PgB that IE gene expression was still restricted in murine cells, despite successful binding, entry, transport of the nucleocapsid to the nucleus and uncoating (Nakamichi et al., 2001). Moreover, it has been shown that the bICP4 promoter was not functional in murine cells, indicating that there are multiple blocks to BHV-1 replication in non-permissive cells (Hushur et al., 2004).

Receptor binding and entry of BHV-1 may have contributed the varying permissiveness of human cells as well, since it was observed that in general, BHV-1 was less cytotoxic to all human cells examined than KM100 and KM120. This may reflect an overall impairment of BHV-1 to infect human cells at the level of binding and entry. Previous studies have shown that one of the blocks to BHV-1 infection in a semi-permissive hamster lung (HmLu-1) cell line was binding and penetration, and that viral titres could be increased following infection with a recombinant BHV-1 expressing PgC and PgB (Nakamichi & Otsuka, 2000). An impaired ability of BHV-1 to bind and penetrate into human cells may have contributed to the relatively slower rate of CPE induced by BHV-1 compared to KOS in U2OS cells (data not shown). Furthermore, although BHV-1 induced significant CPE in U2OS cells, titres of BHV-1 were on average 15-fold lower in U2OS cells (data not shown), suggesting that U2OS are not fully permissive to BHV-1 infection. Although expression of PgC and PgB resulted in improved BHV-1 viral titres on HmLu-1 cells by approximately 1 log unit, they were still approximately 2 log units lower than the viral titres observed on fully permissive MDBK cells, suggesting that in a semi-permissive cell type, binding and penetration are not the only obstacles in BHV-1 infection (Nakamichi & Otsuka, 2000). Given the varying degrees of cytotoxicity observed in human cells, it is possible that there are multiple factors that contribute to permissiveness to BHV-1.

A factor that may influence permissiveness to BHV-1 that was explored in this study was type I IFN signaling. Since IFN signaling is important in determining the host range of certain viruses, as well as contributing to the

permissiveness of certain tumour types to IFN-sensitive viruses, we characterized the IFN responsiveness of each cell line screened for BHV-1 sensitivity, in order to determine if there was a correlation between type I IFN signaling and sensitivity to BHV-1. Out of the 23 cell lines assayed for IFN responsiveness, 12 were sensitive to BHV-1 infection and only 3/12 sensitive cell lines showed an impaired ability to respond to IFN treatment, suggesting that permissiveness to BHV-1 does not correlate with IFN signaling. The factors involved in permissiveness of human cells to BHV-1 remain unclear, however, the observation that the removal of both the innate and adaptive immune responses in mice through type I and type II IFN receptors and *RAG-2* gene deletions was required for mice to succumb to BHV-1 infection, suggests that an interplay of different factors may be involved in permissiveness to BHV-1. However, it is also possible that the factors restricting BHV-1 from murine cells may be different than in human cells. This was shown for MV, in that type I IFNs were responsible for the species barrier in mice, but TNF signaling was a contributor to the restriction in human cells (Wang et al., 2008, Wang et al., 2004). Either way, these data suggest that IFN responsiveness is not closely linked with restricting BHV-1 infection in human cells.

In this study, the sensitivity of human normal and tumour cells to ICP0-null HSV-1 viruses did not correlate with responsiveness to IFN. KM100 was relatively cytotoxic in 15/23 cell lines assayed for IFN responsiveness and 18/23 cell lines were sensitive to KM120 infection, however only 5 of these cell lines showed a decreased responsiveness to IFN. Previous data showed that of the 8 tumour cell lines that were sensitive to ICP0-null HSV-1 oncolytic vectors, 7 possessed defects in their ability to produce and/or respond to IFN (Hummel et al., 2005). However, the assays employed to characterize IFN responsiveness were different between this study and the work of Hummel *et al.* In this study, GFP fluorescence was quantified as a measure of VSV-GFP replication, however, Hummel *et al.* counted the number of plaques formed by VSV in the presence and absence of IFN (Hummel et al., 2005). Since different outcomes are measured in these assays, different results may be observed. Measuring GFP fluorescence may be an overestimation of permissiveness to VSV since it is more likely that more cells will express viral genes than will yield infectious virus, eventually forming plaques, as this requires multiple rounds of viral replication and spread. Differences such as these were observed in RWPE-1 cells, which when infected with VSV-GFP, no CPE or plaquing was observed visually, however, GFP fluorescence could be detected. Furthermore, this study required the use of much more virus compared to Hummel *et al.*, in which cells were challenged with only 100 pfu of VSV in a 12-well. In addition, Hummel *et al.* also evaluated the ability of cells to make IFN, while this study did not. Regardless of previous data, this study did not show a correlation between sensitivity to ICP0-null HSV-1 oncolytic vectors and responsiveness to IFN, as determined by measuring GFP fluorescence from VSV-GFP in these particular cell types. Although IFN signaling has been linked to sensitivity to ICP0-null HSV-1 oncolysis, it is not the

only factor that determines permissiveness to these vectors, as demonstrated by CT26 cells. This murine colon carcinoma cell line has been shown to be very defective in type I IFN signaling, nevertheless, these cells were not sensitive to KM100 and KM120, suggesting that additional factors may be involved in influencing permissiveness to ICP0-null HSV-1 vectors (Hummel et al., 2005).

One of the concerns with using HSV-1 oncolytic vectors in cancer therapy is the possibility that pre-existing immunity to HSV-1 may hinder the systemic efficacy of the virus. As a result, we were interested in determining if antibodies against HSV-1 could cross react with BHV-1, which would suggest that BHV-1 therapy may not be as effective in patients with pre-existing immunity to HSV-1. Although it has been demonstrated that a rabbit polyclonal antibody against HSV-1 gB was able to immunoprecipitate BHV-1 gB and vice versa, this study showed that a polyclonal pan-HSV-1 antibody was unable to recognize any BHV-1 proteins on a Western blot (Misra et al., 1988). Harvesting Vero cells infected with HSV-1 cells and injecting rabbits generated the polyclonal pan-HSV-1 antibody used in this study. Therefore, with all of the possible epitopes from HSV-1 as well as Vero cells in this scenario, it is possible that antibodies against the particular epitopes from gB that a capable of cross-reacting with BHV-1 were not generated.

This study demonstrated that a component of human serum specific to HSV-1 was unable to neutralize BHV-1 *in vitro*, suggesting that pre-existing immunity to HSV-1 may not impede the efficacy of BHV-1. However, the effects of pre-existing immunity against HSV-1 oncolysis are variable. Delman *et al.* demonstrated that immunization against KOS, generating both cellular and humoral immunity to HSV-1, prior to intravenous administration of a high dose of G207 or NV1020 (10^7 pfu), did not affect the number of CT26 liver metastases compared to control non-immunized animals, however the low dose of 10^6 pfu was inhibited (Delman et al., 2000). The inhibition observed could be circumvented by administration of the virus in close proximity to the tumour through intraportal injection, suggesting that pre-existing immunity may not be affect HSV-1 oncolytic vectors, depending on the dose and route of injection. Ikeda *et al.* have also implicated an inhibitory effect of pre-existing immunity (Ikeda et al., 1999). A considerable increase in the transduction of rat intracerebral tumours following intra-arterial administration of an HSV-1 oncolytic vector along with the B-cell immunosuppressive agent cyclophosphamide (CPA) was observed (Ikeda et al., 1999). This effect was achieved rapidly (2 days following viral and CPA administration) and therefore was attributed to innate immune responses (Ikeda et al., 1999). However, it was observed that IgM serum levels decreased, suggesting that pre-existing IgM played a role in interacting with the complement pathway to inhibit HSV-1 infection (Ikeda et al., 1999). Furthermore, it was subsequently demonstrated that depletion of complement through administration of cobra venom factor (CVF), as well as the inhibition of innate and elicited anti-HSV-1 neutralizing antibody response with CPA, increased oncolytic HSV-1 propagation in multiple brain

tumours, further demonstrating an inhibitory effect of interactions between innate and adaptive anti-responses (Ikeda et al., 2000).

Finally, another factor considered in employing BHV-1 as an oncolytic vector was the ability of anti-herpetic nucleoside analogue drugs to inhibit BHV-1, as this would add a safety advantage to BHV-1 as an oncolytic vector. This study demonstrated ACV could not inhibit that BHV-1 replication, with concentrations as high as 20 ug/mL. The resistance of BHV-1 to ACV has previously been observed by Weinmaster *et al.* (Weinmaster et al., 1982). These previous data also show that BHV-1 could be partially inhibited by high concentrations of ACV, such as 100 ug/mL in MDBK cells (Weinmaster et al., 1982). Weinmaster *et al.* also found that BHV-1 could not be inhibited by ganciclovir - another anti-herpetic drug used in the clinical setting (Weinmaster et al., 1982). However, BHV-1 could be inhibited by bromovinyldeoxyuridine (BVDU), also known as brivudine, at low concentrations of less than 1 ug/mL (Weinmaster et al., 1982). Although BVDU is clinically utilized to treat herpes zoster, it is only available in several European countries (De Clercq, 2004, De Clercq, 2005).

CHAPTER 5: CONCLUSIONS

The elucidation of PML as a factor contributing to ICP0-null HSV-1 oncolysis of tumour cells may contribute to understanding the mechanism of ICP0-null HSV-1 mediated oncolysis. This information may be useful in the predicting the tumor types that may be sensitive to ICP0-null HSV-1 oncolytic therapy for more effective cancer treatment. Although a role for PML in sensitivity of various transformed cells to ICP0-null HSV-1 oncolysis has been demonstrated (Sobol et al., unpublished data), this study failed to observe a link between PML expression and permissiveness to ICP0-null HSV-1 vectors in three sets of patient matched normal prostate epithelial and tumour cell lines. Since the roles of PML in permissiveness to ICP0-null HSV-1 mutant viruses can depend on cell types as well as assays employed, further investigation would be useful in establishing a link between PML and ICP0-null HSV-1 oncolysis in the cell types examined in this study.

Furthermore, this study sought to determine whether BHV-1 has oncolytic properties that correlate with type I IFN signaling. The *in vitro* cytotoxicity of BHV-1 in human cells observed in this study suggests that BHV-1 may have oncolytic effects, since BHV-1 was less cytotoxic in normal human cells than in immortalized and transformed cell lines. However, the sensitivity of the human cells assayed, could not be linked to type I IFN signaling. The evaluation of BHV-

1 oncolysis *in vivo* would be valuable in delineating if BHV-1 preferentially replicates in tumour cells over normal cells, and if BHV-1 has potential as a cancer therapeutic.

It is becoming appreciated that different oncolytic viruses will be more effective against different tumour types, and that multiple treatments with different vectors may be more effective for successful cancer therapy. It is hoped that the results of this study will contribute to the understanding of how herpesvirus-mediated oncolysis occurs. Furthermore, it is likely that a single viral agent cannot be administered multiple times in an individual due to host immune responses. To this end, it is hoped that this initial characterization of BHV-1 as a potential oncolytic vector may be useful in ultimately elucidating whether BHV-1 can be an effective oncolytic virus to provide an alternative herpesvirus vector in cancer therapy.

CHAPTER 6: REFERENCES

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CHAPTER 7: APPENDIX

Appendix 1

Western blot of PML in normal and tumour prostate epithelial cell lines from patients 1532 and 1535. Whole cell extracts of mock treated cells were prepared and 60 ug of total protein was loaded onto 10% SDS-PAGE gels. Actin blots were used as a loading control. Results are representative of three independent experiments. Data obtained from P. Sobol.