CHARACTERIZATION OF ONCOLYTIC

BOVINE HERPESVIRUS TYPE 1

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BY

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Abstract

Oncolytic viruses (OV) are a promising alternative cancer therapy due to their specificity and lack of debilitating side effects, such as those which typically accompany conventional therapeutics such as chemotherapy and radiation. Bovine herpesvirus type 1 (BHV-1) is an *alphaherpesvirus* with the ability to infect and kill multiple human tumor cell types. In comparison to other species-specific viruses, for which deficiencies in type I interferon signalling pathways dictates cellular sensitivity to infection, mutations in KRAS were found to correlate with high levels of BHV-1 replication. Interestingly, BHV-1 is able to induce cellular cytotoxicity in the absence of a productive infection. In contrast to current breast cancer (BC) treatments, which are largely based on receptor expression status, BHV-1 is able to infect and kill BC cells and breast cancer initiating cells (BCICs) from luminal and basal subtypes. Furthermore, BHV-1-infected BC cells are significantly diminished in their capacity to form tumors *in vivo,* suggesting that BHV-1 reduces the tumor forming capacity of BCICs. Combination therapy involving OVs has been used to exploit differences in the mechanism of tumor cell death elicited by individual treatments. Treatment with epigenetic modifiers such as 5-Azacytidine (5-Aza), a DNA methyltransferase inhibitor, has been shown to increase the antitumor activity of OVs. Our data indicates that 5-Aza strongly synergises with BHV-1, increasing virus replication and cytotoxicity *in vitro*. *In vivo,* BHV-1 monotherapy did not significantly impact tumor growth or survival of CR bearing subcutaneous breast tumors; however, combination therapy with 5-Aza significantly decreased the number of

secondary lesions compared to BHV-1 monotherapy. Overall, the data presented in this dissertation indicate that BHV-1 is a promising broad spectrum OV with a unique mechanism of tumor cell targeting, and the ability to infect and kill tumor cells independent of a productive infection.

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List of Abbreviations

5-Aza	5-Azacytidine
ALDH1	aldehyde dehyrogenase 1
BC	breast cancer
BCIC	breast cancer initiating cell
BHV-1	bovine herpesvirus type 1
bICP0	bovine infected cell protein 0
CD	cluster of differentiation
CIC	cancer initiating cell
CR	cotton rat
CSC	cancer stem cell
DNA	deoxyribonucleic acid
DNMTi	DNA methyltransferase inhibitor
E	early
EGFR	epidermal growth factor receptor
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
gB	glycoprotein B
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GFP	green fluorescent protein
HCF	host cell factor
HDAC	histone deacetylase

HEL	human embryonic lung
HER2	human epidermal growth factor 2
HSPG	heparan sulphate proteoglycan
HSV-1	herpes simplex virus type 1
HVEM	herpesvirus entry mediator
ICP	infected cell protein
IE	immediate early
IFN	interferon
i.p	intraperitoneal
IRF	interferon regulatory factor
ISG	interferon stimulated gene
i.t	intratumoral
L	late
LUC	luciferase
MAPK	mitogen-activated protein kinase
MDBK	Madin Darby Bovine Kidney
MHC	major histocompatability complex
mRNA	messenger RNA
miRNA	micro RNA
NCI	National Cancer Institute
NOD	non-obese diabetic
oAd	oncolytic adenovirus
Oct-1	octamer transcription factor 1

OV	oncolytic virus
OVT	oncolytic virotherapy
PARP	poly ADP ribose polymerase
PBS	phosphate buffered saline
pfu	plaque-forming unit
pi	post infection
PI3K	phosphatidylinositol 3-kinase
PKR	dsRNA-dependent protein kinase R
PR	progesterone receptor
RB	retinoblastoma
RING	really interesting new gene
RNA Pol II	RNA polymerase II
sh	short hairpin
SP	side population
STAT	signal transducers and activators of transcription
Tk	thymidine kinase
TN	triple negative
tu	transcription unit
UV	ultraviolet
Vhs	virion-host shutoff protein
wt	wild type

Chapter 1: Introduction

1.1 Breast Cancer Biology

Breast cancer (BC) is a complex disease and its classification is ever evolving. The traditional classification system, which is based on histology, established four BC subtypes, including ductal, lobular, nipple and not otherwise specified. However, recent gene expression profiling using microarrays has redefined how we characterize BC (Sotiriou, Neo et al. 2003, Azim, Michiels et al. 2012, Network 2012). The expression of specific gene subsets has led to the identification of six different molecular subtypes: luminal A, luminal B, human epidermal growth factor receptor 2 (HER2), basal, normal-like and claudin-low (Eroles, Bosch et al. 2012, Network 2012). The classification of a breast tumor as benign or malignant depends on the cell growth pattern, originating cell type, stromal responses and cytological, nuclear and morphological characteristics (Mallon, Osin et al. 2000, Stingl and Caldas 2007). Benign lesions often occur from mutations in epithelial and myoepithelial cells, whereas malignant lesions usually originate from the mutation of luminal epithelial cells (Mallon, Osin et al. 2000). Carcinoma *in situ* is a form of low grade BC when the tumor cells have not invaded the surrounding tissue (Mallon, Osin et al. 2000). For example, ductal carcinoma *in situ* is characterized as the pre-invasive malignant proliferation of breast epithelial cells within the ductal tissue of the breast (Silverstein 1997). Metastatic BC primarily originates in regional lymph nodes with subsequent permeation of the capillaries and lymphatics by

breast tumor cells (Buerger, Otterbach et al. 1999, Weigelt, Peterse et al. 2005). Secondary or distal sites of metastasis include the lungs, liver, brain and to a lesser extent the bone and adrenal gland (Mallon, Osin et al. 2000, Weigelt, Peterse et al. 2005). Overall, patient prognosis is based on the size, type, stage, grade and receptor status (section 1.2) of the tumor (Mallon, Osin et al. 2000, Stingl and Caldas 2007). These factors also dictate the treatment that is prescribed.

1.1.2 Epigenetics in Breast Cancer

Epigenetic changes have been implicated in the development of BC, but it wasn't until recently that insights into the meaning behind these alterations and their implications in BC therapy have been elucidated. Epigenetic modifications are those which cause heritable changes to gene expression without altering the genome sequence. These can include deoxyribonucleic acid (DNA) methylation, post-translational modification of histones and gene expression regulation by non-coding micro RNAs (miRNAs). The aberrant methylation of DNA, commonly hyper- or hypomethylation of the 5' cytosine in CpG islands within gene promoter regions, can lead to the silencing of tumor suppressor genes and increased expression of oncogenes, respectively (Jones and Baylin 2002). Histone deacetylation is a common post-translational histone modification that is catalyzed by histone deacetylases (HDACs) and is associated with a transcriptionally closed, or inhibitory state (Jones and Baylin 2002). Lastly, miRNAs downregulate gene

expression by a variety of mechanisms, including messenger ribonucleic acid (mRNA) cleavage, translational repression and deadenylation (Shah and Chen 2014).

Changes in histone acetylation, methylation and miRNA expression patterns are early signs of BC and can be used to differentiate normal, benign and malignant breast tissue (Iorio, Ferracin et al. 2005, Ordway, Budiman et al. 2007, Elsheikh, Green et al. 2009). The Cancer Genome Atlas Network has identified distinct methylation patterns pertaining to specific BC molecular subtypes by performing methylation arrays on human breast tumor tissues (Network 2012). The degree of change in methylation correlates with the aggressiveness of the tumor, such that changes in certain genes affect carcinogenesis and metastatic potential (Rodenhiser, Andrews et al. 2008, Nickel and Stadler 2014). Furthermore, miRNA subtypes whose expression correlates with the clinical status (but not receptor expression itself) of estrogen receptor (ER), progesterone receptor (PR) and HER2 expressing tumors has been documented (Iorio, Ferracin et al. 2005). However, the miRNA expression profile in BC cells appear to be transient. The miRNAs which are expressed change in the transition from ductal carcinoma *in situ*, to invasive BC, and as metastasis progresses (Volinia, Galasso et al. 2012). These data can be used in the design of targeted therapeutic strategies implemented at various stages of disease progression.

The DNA methyltransferase inhibitor (DNMTi) 5-Azacytidine (5-Aza) has recently been used to study the role of gene methylation in the development and prognosis of BC (Yang, Ferguson et al. 2000, Byler, Goldgar et al. 2014). As DMNTi can have undesired effects on BC cells, including the upregulation of oncogenes, their combination with other cytotoxic drugs will likely be required to kill breast tumor cells (Mirza, Sharma et al.

2010, Radpour, Barekati et al. 2011). Alternatively, a recent study by Chik and colleagues showed that by balancing the effects of 5-Aza with its deoxy analog to block undesired methylating activity, they were able to induce augmented inhibition of BC cell growth and decrease pro-metastatic gene activation (Chik, Machnes et al. 2014). The combination of DMNTi with HDACs has been shown to act synergistically to elicit breast tumor cell cycle arrest and apoptosis (Mataga, Rosenthal et al. 2012). Furthermore, in an ongoing phase II clinical trial for the treatment of patients with triple negative (TN; section 1.2.3) metastatic BC, interim reports indicate that the combination of entinostat (an HDACi) and 5-Aza caused re-expression of the ER which sensitized tumor cells to ER antagonist therapy (NCT01349959).

1.2 Breast Cancer Therapy

Classical treatment modalities, such as radiation and chemotherapy are used in adjuvant therapy to treat BC. Adjuvant therapy is the use of chemotherapy and/or endocrine therapy following surgical removal of the primary tumor aimed at killing disseminated tumor cells in an effort to prevent metastases. The most common therapeutic regimens involve the combination of cyclophosphamide with doxorubicin and/or docetaxel, or methotrexate with fluorouracil (Nabholtz and Gligorov 2005, Rouzier, Perou et al. 2005). However, dose limiting toxicities have hindered the therapeutic success of chemotherapeutics (Montemurro, Redana et al. 2005, Nabholtz and Gligorov 2005). Radiation therapy is generally used after primary tumor resection surgery. It is applied to

the tumor bed and regional lymph nodes to kill any residual tumor cells which may cause relapse and the development of metastases (Ragaz, Olivotto et al. 2005, Wockel, Wolters et al. 2014).

Similar to other cancers, the main cause of BC patient mortality is the development of metastases, not the primary tumor itself. More accurate prognostic markers are urgently needed to identify patients who are at a high risk of developing breast metastases. Currently, adjuvant therapy is broadly prescribed to BC patients in an effort to prevent metastases. However, the wide spread toxicity of chemotherapy significantly impacts the patient's quality of life and approximately 40% of patients relapse and ultimately die from metastatic BC (EBCTCG 2005). Recent studies indicate that gene expression profiles of primary breast tumors can be used to more accurately predict their metastatic potential and clinical characteristics (van 't Veer, Dai et al. 2002, Ramaswamy, Ross et al. 2003, Sorlie, Tibshirani et al. 2003, Jonsson, Staaf et al. 2010). For instance, a 70 gene expression profile has been identified that predicts the likelihood of metastases in patients with luminal BC (van 't Veer, Dai et al. 2002).

Three receptors, namely the ER, PR and HER2, are the main therapeutic targets for BC treatments (Azim, Michiels et al. 2012). Receptor expression profiles generally correlate with BC subtype as follows: luminal A (ER/PR+, HER2-), luminal B (ER/PR+, HER2+), basal (ER/PR-, HER2-) and HER2 (ER/PR-, HER2+). However, not all tumors within a histological subtype are of the same molecular subtype and vice versa. The receptor status of breast tumors largely dictates the treatment prescribed and correlates with patient prognosis. Variation in receptor expression status within BC subtypes and the same

patient, compounded with multidrug resistance and high rates of patient relapse, warrant the development of broadly applicable treatment strategies whose efficacy is not confined to a particular tumor subtype.

1.2.1 ER Positive Breast Cancer Therapy

Breast tumors that are ER/PR+ depend on estrogen for their growth, and are therefore treated with endocrine therapy. The selective ER antagonist, Tamoxifen, is the most commonly prescribed endocrine therapy for ER/PR+ BC patients (Osborne 1998). Fulvestrant, a selective ER down regulator, is another widely used anti-estrogen therapeutic that is used to treat ER/PR+ BC (Ciruelos, Pascual et al. 2014). However, resistance to endocrine therapy (Tamoxifen in particular) is a well-established problem, but the mechanism(s) of resistance are unclear and numerous (as reviewed in (Zhao and Ramaswamy 2014)). For example, initial responses to endocrine therapy vary due to differences in ER expression levels in tumors, with lower ER expression correlating with resistance to endocrine therapy and increased aggressiveness (Britton, Hutcheson et al. 2006, Creighton 2012). Furthermore, PR co-expression impacts tumor sensitivity to endocrine therapy. Approximately 50% of ER+ tumors are also PR+, with receptor co-expression increasing responsiveness to endocrine therapy (Creighton 2012). Alternatively, ER/PR+ tumors can be treated with aromatase inhibitors such as anastrozole and letrozole, the front line therapy for post-menopausal patients (Gruber, Tschugguel et al. 2002, Carpenter and Miller 2005). Unfortunately, high rates of

resistance to ER antagonists and aromatase inhibitors limit therapeutic success, encouraging the development of novel treatment strategies. The interdependence of ER, HER2 and epidermal growth factor receptor (EGFR) signalling pathways has spawned interest in simultaneous targeting strategies to maximize therapeutic efficacy (Shou, Massarweh et al. 2004). Furthermore, the combination of Tamoxifen with Trastuzumab or Lapatinib (HER2 inhibitors), Gefitinib (EGFR inhibitor) and phosphatidylinositol 3-kinase (PI3K) pathway inhibitors are being used to overcome and/or prevent resistance (Gutteridge, Agrawal et al. 2010, Leary, Drury et al. 2010, Block, Grundker et al. 2012, Chen, Zhao et al. 2013, Massarweh, Romond et al. 2014, Morrison, Fu et al. 2014).

1.2.2 HER2 Positive Breast Cancer Therapy

Approximately 15-20% of BC is classified as HER2+ and is treated with Trastuzumab, a monoclonal antibody for HER2 (Slamon, Clark et al. 1987). Since the implementation of Trastuzumab as the frontline therapy for HER2+ BC, the 5 year survival of patients has increased by approximately 87% (Hortobagyi 2005). Trastuzumab has multiple modes of action, the primary mechanism being the uncoupling of HER2 with its primary binding partner HER3 (Saini, Azim et al. 2011). The broad treatment of HER2+ BC with Trastuzumab, although initially promising, has produced suboptimal long term results. New treatment strategies have been implemented, such as small molecule inhibitors of HER2 (Neratinib, Afatinib and Lapatinib) that impede receptor kinase activity (Blackwell, Burstein et al. 2012, Lin, Winer et al. 2012, Jankowitz, Abraham et al. 2013),

inhibitors of HER2 receptor dimerization (Pertuzumab) (Cortes, Fumoleau et al. 2012, Miller, Dieras et al. 2014) and heat shock protein inhibitors that interfere with proper HER2 receptor folding, maturation, conformation and stability (Modi, Saura et al. 2013). The combination of these treatment strategies with Trastuzumab (and typically chemotherapeutics) has improved patient response rates, especially in those whose tumors express the mutated form of HER2 (referred to as p95) which is resistant to Trastuzumab therapy (Blackwell, Burstein et al. 2012, Kumler, Tuxen et al. 2014). However, the lack of reliable biomarkers to identify HER2 subtypes and to determine the appropriate therapeutic regimen to prescribe to patients has limited treatment efficacy.

1.2.3 Triple Negative Breast Cancer Therapy

In cases where ER, PR and HER2 are not expressed, the tumor is classified as TN. Patients with TN BC have the worst prognosis as the tumors lack a clear molecular target (Sorlie, Tibshirani et al. 2003, Liedtke, Mazouni et al. 2008, Anders and Carey 2009). Moreover, the aggressiveness and rate of relapse is highest in patients with TN BC with an increased incidence of brain metastases (Sorlie, Tibshirani et al. 2003, Lin, Claus et al. 2008, Smid, Wang et al. 2008). The treatment of TN breast tumors with chemotherapy can initially lead to favorable responses; however, this is often followed by patient relapse and drug resistance, making these tumors difficult to treat and highlighting the need for novel treatment strategies (Carey, Dees et al. 2007, Liedtke, Mazouni et al. 2008). Alternative therapies for the treatment of TN BC include strategies to inhibit tumor

angiogenesis (Bevacizumab) (Sikov, Berry et al. 2014), the EGFR (Cetuximab) (Nechushtan, Vainer et al. 2014) and induces re-expression of the ER through epigenetic modification using HDACi (Vorinostat) (Tu, Hershman et al. 2014). However, majority of these methods still involve combination therapy with chemotherapeutics to improve antitumor efficacy.

1.3 Breast Cancer Initiating Cells

Controversy surrounding the origin of cancer stem cells (CSCs) and whether they arise from stem cells or cells with stem-like properties has led to the more accurate term cancer initiating cell (CIC). CICs possess the ability to self-renew and differentiate into all cell lineages contained within a tumor (Clarke, Dick et al. 2006, Kim and Dirks 2008, Korkaya and Wicha 2010). CICs in solid tumors were first described by Al-Hajj and colleagues, when they identified a tumorigenic subset of cluster of differentiation (CD)$44^{+}CD24^{-/low}$ breast CSCs, with as few as 10^{3} capable of initiating tumor growth in non-obese diabetic (NOD)/*scid* mice (Al-Hajj, Wicha et al. 2003). This cell population, now referred to as breast CICs (BCICs), are able to recapitulate the range of malignant progeny in the original tumor upon serial transplantation into NOD/*scid* mice (Al-Hajj, Wicha et al. 2003). While the frequency of BCICs within a tumor mass is variable they can be isolated based on the expression of cell surface markers. However, this isolation method is controversial as cell surface marker expression is largely context dependent

(Nguyen, Vanner et al. 2012). The study of BCICs can also be achieved by isolating the putative side population (SP) based on Hoechst 33342 dye exclusion. The SP is enriched for stem cell properties, including stem cell markers and *in vivo* tumor reconstitution ability. BCICs possess properties that confer resistance to conventional therapies, including resistance to apoptosis (Fulda and Pervaiz 2010), active DNA repair mechanisms (Diehn, Cho et al. 2009), and enhanced drug efflux (Dean, Fojo et al. 2005, Nguyen, Vanner et al. 2012). High expression of aldehyde dehydrogenase 1 (ALDH1) on BCICs allows for enhanced drug metabolism and correlates with poor prognosis (Ginestier, Hur et al. 2007, Kakarala, Brenner et al. 2010). Conventional BC therapies are often aimed at proliferating tumor cells. This strategy fails to target the highly tumorigenic BCIC population, which may result in relapse and progression to metastatic disease.

1.4 HSV-1 Biology

Herpes simplex virus type 1 (HSV-1) is a double stranded DNA virus which is a member of the *Herpesviridae* family, *Alphaherpesvirinae* subfamily. It has a linear genome of approximately 152 Kb contained in an icosahedral capsid. The capsid is connected to the outer envelope via the tegument, which contains viral-encoded proteins that aid in virus replication and abrogate the host antiviral response. HSV-1 is a neurotropic virus which establishes life-long latency within the trigeminal ganglia of the host. The virus

reactivates from latency during periods of stress, immunosuppression and increases in corticosteroids.

1.4.1 HSV-1 Cellular Entry and Replication

The initiation of HSV-1 entry occurs when glycoprotein B (gB) and/or gC binds to heparin sulphate proteoglycans (HSPG) on the cell surface triggering viral adsorption (Reske, Pollara et al. 2007, Connolly, Jackson et al. 2011). This allows for binding of gD to its target receptors, which include herpesvirus entry mediators (HVEM), nectin-1 and nectin-2, and 3-O sulfated heparin sulfate (Reske, Pollara et al. 2007, Connolly, Jackson et al. 2011). Binding of gD to its target receptor induces a conformational change in gD which elicits fusion via activation of gB and the gH/gL heterodimer (Reske, Pollara et al. 2007). Following fusion of the virion with the cell membrane, the viral capsid and components of the tegument enter the cytoplasm and are transported to the nucleus on microtubules. Entry of viral DNA into the nucleoplasm occurs at nuclear pore complexes (Chang and Roizman 1993).

HSV-1 replication occurs in a highly regulated manor involving sequential transcription of immediate early (IE), early (E) and finally, late (L) genes. Each gene functions as an independent transcriptional unit with its own promoter sequence. The viral genome consists of unique and repeated sequences within covalently linked long and short regions. Generally, genes are grouped based on function but the genome itself is not transcribed in order. Approximately 90 proteins are expressed, three of which are present

in more than one copy and include infected cell protein (ICP) 0, ICP4 and ICP34.5 (Shen and Nemunaitis 2006).

The initiation of HSV-1 gene transcription occurs when the viral tegument protein VP16 (an L gene product), together with the cellular binding proteins host cell factor (HCF) and octamer transcription factor 1 (Oct-1), bind IE gene promoters. This allows for subsequent binding of RNA polymerase II (RNA Pol II). There are five IE genes, ICP0, ICP4, ICP22, ICP27 and ICP47. ICP0 and ICP4 are transcriptional activators which play critical roles during virus replication (Shen and Nemunaitis 2006). In addition, ICP0 is extensively involved in counteracting the host interferon (IFN) response (Paladino and Mossman 2009). ICP22 enhances viral gene transcription by modulating RNA Pol II activity and ICP27 regulates post-translational processing. Functioning in multiple aspects of immune evasion, ICP47 inhibits antigen processing and CD8+ T cell activation. Once IE gene transcription is complete, ICP4 activates host RNA Pol II for transcription of E genes, including DNA polymerase, single-stranded DNA binding proteins, helicase-primase complex and ori-binding proteins. Finally, L genes which encode for structural proteins contained in the capsid and envelope are transcribed following DNA replication. The viral DNA circularizes and locates near replication centers containing promyelocytic leukemia protein nuclear bodies (Burkham, Coen et al. 2001). Viral DNA replication follows a rolling circle mechanism involving cleavage of sequential concatemers to form monomers, which are then packaged into a de novo nucleocapsid (Deiss, Chou et al. 1986). Envelopment occurs as the capsid buds from the

nuclear and cell membranes and then the mature virion is released into the extracellular space (Shen and Nemunaitis 2006).

1.4.2 HSV-1 and the Host Immune Response

HSV-1 infection, including lytic replication in epithelial cells and latent infection of trigeminal ganglion, is highly successful in part due to the production of viral proteins which combat the host antiviral immune response. For example, the effects of IFN signaling are pleiotropic (antiproliferative, antiviral and immunoregulatory functions), such that regulation of this signaling cascade has wide spread implications on virus replication and spread (Paladino and Mossman 2009).

Upon entry into the cell, the tegument virion host shutoff (vhs) protein degrades host and viral mRNA and disrupts polyribosomes to enhance mRNA turnover and expression of viral gene products (Pasieka, Lu et al. 2008). The vhs protein is also important in disrupting the type I and II IFN signaling cascades, specifically by regulating the production of IFN-stimulated genes (ISGs) (Liang and Roizman 2008, Pasieka, Lu et al. 2008). The IE gene product ICP0, in addition to acting as a transcriptional activator during productive infection, is the viral lynch pin in counteracting the IFN response. Specifically, ICP0-null HSV-1 mutants are hypersensitive to IFN such that virus replication and plaque forming ability is greatly diminished (Sacks and Schaffer 1987, Mossman, Saffran et al. 2000). Majority of the functions of ICP0 have been attributed to the really interesting new gene (RING) finger domain and its E3 ubiquitin ligase activity

(Paladino and Mossman 2009, Lanfranca, Mostafa et al. 2014). The multifactorial inhibition of the IFN response by ICP0 includes (but is not limited to) inhibition of interferon regulatory factor (IRF) 3 (Lin, Noyce et al. 2004, Paladino, Cummings et al. 2006), IRF7 (Lin, Noyce et al. 2004) and signal transducers and activators of transcription (STAT) 1 (Halford, Weisend et al. 2006). In addition to repressing host transcription, translation and decreasing mRNA stability and splicing, ICP27 blocks IFN induction during the early stages of infection by inhibiting IRF3 and STAT1 activation (Melchjorsen, Siren et al. 2006, Johnson, Song et al. 2008). Regulation of the IFN response extends to late phases of HSV-1 replication through the activity of the L gene products ICP34.5 and Us11. The production of IFN induces activation of the ISG dsRNA-dependent protein kinase R (PKR), which functions to inhibit viral protein synthesis. This effect is reversed by ICP34.5 and cellular protein phosphatase alpha (Chou, Chen et al. 1995, Leib, Machalek et al. 2000). Moreover, ICP34.5 has been shown to inhibit IRF3 phosphorylation, translocation to the nucleus and induction of ISGs (Verpooten, Ma et al. 2009). The L gene Us11 also inhibits PKR activation by direct protein-protein interaction and indirectly by mediating the association of activators of PKR, such as dsRNA (Poppers, Mulvey et al. 2000).

In addition to the inhibition of innate immune responses, several HSV-1 proteins inhibit the activity of cellular immune effectors and the adaptive immune response. ICP34.5 and vhs protein inhibit the maturation and activation of dendritic cells, thereby resulting in a decrease in HSV-1-specific antibody production and T cell activation (Kobelt, Lechmann et al. 2003, Cotter, Kim et al. 2011, Jin, Yan et al. 2011). Furthermore, ICP47 inhibits

transporter associated with antigen processing, decreasing antigen presentation by major histocompatibility complex (MHC) I (including expression of the MHC I receptor itself) as well as CD8+ T cell activation (Goldsmith, Chen et al. 1998, Jugovic, Hill et al. 1998).

1.5 Oncolytic Virotherapy

Since the early 1900s the lack of sustained patient responses to traditional cancer treatment modalities, such as chemotherapy and radiation, has resulted in a general skepticism towards patient prognosis. To date, a treatment which is tumor specific yet broadly applicable across multiple cancer types has yet to be developed, making the search for novel treatment strategies paramount.

Oncolytic viruses (OV) selectively replicate in and kill tumor cells while having minimal detrimental effects on normal cells. This characteristic is either natural (wild type (wt) OV) or gained through genetic engineering. Mutations inherent in tumor cells which alter cell survival, proliferation and immunoregulation also dictate cellular sensitivity to OVs (Russell, Peng et al. 2012). Furthermore, strategies for tumor targeting and methods used to improve antitumor efficacy appear to be limitless with new oncolytic vectors being described regularly. As a comprehensive discussion of targeting strategies is beyond the scope of this dissertation, we refer readers to Pol *et al.* (2014) for a current review of targeted OVs with specific focus on those in clinical phases of testing (Pol, Bloy et al. 2014). Briefly, OV tumor cell targeting can be achieved through 1) transductional targeting by modifying viral surface proteins to direct infection; 2) regulation of essential

viral gene transcription using tumor and/or tissue specific promoters; 3) translational regulation of viral mRNA expression using cloned miRNA binding elements and 4) the use of OVs with specific gene modifications which restrict virus replication to particular tumor types based on the mutations present.

OVs are appealing as an alternative cancer therapy due to the lack of off-target toxicities often seen with conventional therapies and their ability to target and kill drug-resistant cells, such as CICs. The safety and efficacy of OVs and their ability to induce antitumor activity has been demonstrated in clinical trials (as reviewed in (Russell, Peng et al. 2012)). In 2005, the first OV (H101) was approved by Chinese regulators (Garber 2006), highlighting the promise of oncolytic virotherapy (OVT).

1.5.1 Using Oncolytic Viruses to Target BCICs

The use of OVs to kill BCICs is attractive due to their unique targeting mechanisms compared to conventional therapeutics (Cripe, Wang et al. 2009, Patel, Ndabahaliye et al. 2010). The ability of oncolytic adenovirus (oAd) to target BCICs has been demonstrated in several studies. To limit off-target toxicity Bauerschmitz and colleagues used transcriptional targeting techniques to restrict replication of oAd to BCICs (Bauerschmitz, Ranki et al. 2008). Results indicated that oAd vectors infected and killed BCICs *in vitro.* Although reductions in tumor volume occurred, complete regression and eradication of BCICs cells was not observed *in vivo* (Bauerschmitz, Ranki et al. 2008). In another study, capsid-modified chimeric 5/3 oAd utilizing $\alpha_V\beta$-integrin or HSPG for cellular entry

effectively targeted BCICs *in vitro*. Pre-infection of BCICs with either construct prevented tumor formation in NOD/*scid* mice; however, treatment of established BCIC-derived tumors elicited a tumoristatic effect (Eriksson, Guse et al. 2007).

Other OVs have been shown to target BCICs. Reovirus, a wt OV, has been shown to induce regression of BC patient tumor xenografts in NOD/*scid* mice by killing both bulk tumor cells and BCICs (Marcato, Dean et al. 2009). Furthermore, the replication-competent oncolytic vaccinia virus GLV-1h68 exhibited higher levels of virus replication and cytotoxicity in BCICs (identified by high ALDH1 levels) compared to non-BCICs (Wang, Chen et al. 2012). The virus also elicited tumor regression in BCIC-derived tumor xenografts in nude mice (Wang, Chen et al. 2012).

Research on the ability of oncolytic HSV-1 to kill BCICs is only recent and confined to studies using G47Δ, a third generation oncolytic HSV-1 vector (section 1.4.7). Li and colleagues demonstrated the efficacy of G47Δ to infect and kill BCICs derived from the luminal epithelial human breast tumor cell line SK-BR-3 (Li, Zeng et al. 2012). *In vivo*, G47Δ treatment significantly inhibited the growth of subcutaneous SK-BR-3 BCIC-derived tumors in nude mice following intratumoral injection (Li, Zeng et al. 2012). In an additional study, G47Δ was shown to infect and kill BCICs derived from both luminal and basal-like human breast tumor cell lines and inhibit their ability to self-renew (Zeng, Hu et al. 2013). The virus equally targeted bulk BC cells and BCICs, as well as BCICs which showed resistance to the chemotherapeutic paclitaxel (Zeng, Hu et al. 2013). Moreover, the treatment of BCIC-derived tumor xenografts in nude mice with G47Δ

induced tumor regression, and in combination studies synergized with paclitaxel to kill bulk BC cells and BCICs *in vivo* (Zeng, Hu et al. 2013).

1.6 Ras Signaling in Cancer

The Rat sarcoma (Ras) superfamily, including HRAS, KRAS and NRAS, are plasma membrane associated proteins that have pleiotropic roles in cell growth, migration, adhesion, cytoskeletal integrity, survival, and differentiation (Downward 2003, Rodriguez-Viciana, Tetsu et al. 2005, Ferro and Trabalzini 2010). Ras is a guanosine triphosphate (GTP)ase which hydrolyzes GTP (active) to guanosine diphosphate (GDP) (inactive) to initiate signalling from downstream effectors, including the activation of antiviral pathways (Everts and van der Poel 2005, Battcock, Collier et al. 2006, Noser, Mael et al. 2007, Shmulevitz, Pan et al. 2010, Christian, Zu et al. 2012).

Mutations in Ras are found in approximately 20% of all tumors, with the most common occurring in the GTP binding cleft causing constitutive activation of the protein (Bos 1989, Downward 2003, Wee, Jagani et al. 2009). KRAS mutations have been shown to play a role in the progression of mammary tumors, lung cancer and gastrointestinal cancer (Ayllon and Rebollo 2000, Leslie, Gao et al. 2010, Zhao, Wang et al. 2010).

The evasion of apoptosis, a common hallmark of malignant cell types, is often the result of gain-of-function mutations in components of the Ras signalling cascade (Hanahan and Weinberg 2011). Ras inhibits dsRNA activated PKR, an ISG, which shuts off viral

protein synthesis (Everts and van der Poel 2005). OVs that are sensitive to the inhibitory effects of PKR, such as reovirus, can replicate in tumor cells with activating Ras mutations (Farassati, Yang et al. 2001, Marcato, Shmulevitz et al. 2007, Shmulevitz, Pan et al. 2010). Alternatively, a virus can be made oncotropic by modifying or deleting proteins which counteract the effects of PKR. For example, ICP34.5-null HSV-1 vectors preferentially replicate in cancer cells with mutation in Ras and its downstream signalling effectors (Cassady, Gross et al. 1998, Farassati, Yang et al. 2001, Smith, Mezhir et al. 2006).

1.7 Oncolytic HSV-1 Vectors

HSV-1 was the first virus used to demonstrate that a genetic mutation can render a virus oncolytic (Martuza, Malick et al. 1991). Multiple characteristics make HSV-1 an ideal candidate for use in OVT. Its large dsDNA genome (~125-240 kb) allows for the insertion of transgenes, such as suicide and immunostimulatory genes which augment antitumor efficacy (Everts and van der Poel 2005, Shen and Nemunaitis 2006). HSV-1 has been shown to infect a broad number of cell types due to the expression of its cognate receptors in multiple human tissues. The virus kills infected cells as result of lytic replication and does not insert its DNA into the host genome, reducing the risk of insertional mutagenesis (Varghese and Rabkin 2002, Shen and Nemunaitis 2006). Moreover, HSV-1 rarely causes severe illness in immunocompetent adults (Varghese and

Rabkin 2002, Shen and Nemunaitis 2006) and antiherpetic drugs, such as acyclovir and ganciclovir, can be used to control viral replication.

1.7.1 First Generation Oncolytic HSV-1 Vectors

First generation oncolytic HSV-1 vectors, so named as they are engineered to express one mutation in order to achieve tumor selectivity. The first oncolytic HSV-1 vector (dlsptk) contained a mutation in the gene that encodes for thymidine kinase (tk) (Martuza, Malick et al. 1991). Another common mutation in first generation vectors such as the HSV-1 recombinant hrR3, is in the gene encoding for ribonucleotide reductase (ICP6) (Mineta, Rabkin et al. 1994). ICP6 functions with tk to synthesize deoxynucleotide triphosphates for virus replication. In theory, the mutation of tk and ICP6 should restrict replication to rapidly dividing (G1/S phase) tumor cells; however, off target toxicities have been reported, resulting from viral spread and replication in normal tissues (Valyi-Nagy, Gesser et al. 1994). Adding to their safety profile, the expression of tk confers sensitivity to antiherpetic drugs which can be used to control unwanted replication. Furthermore, deletion of one or both copies of the neurovirulence factor ICP34.5 has been shown to significantly reduce pathogenicity of the virus by inhibiting virus replication in neurons and slowly dividing cell types. ICP34.5-null HSV-1 vectors, such as HSV1716 (deleted for both copies of ICP34.5), selectively replicate in tumor cells with Ras gain-of-function mutations (Randazzo, Kesari et al. 1995). HSV1716 has shown antitumor efficacy and conferred a survival advantage in multiple animal tumor models and is now in clinical

trials for the treatment of patients with malignant glioma, metastatic melanoma and oral squamous cell carcinoma (MacKie, Stewart et al. 2001, Harrow, Papanastassiou et al. 2004, Mace, Ganly et al. 2008). The risk of reversion to wt and concerns over off target replication and toxicity in normal cells have led to the development of second generation HSV-1 oncolytic viruses, containing mutations in multiple genes to increase their safety profile and enhance antitumor efficacy.

1.7.2 Second and Third Generation Oncolytic HSV-1 Vectors

There exists a multitude of second generation HSV-1 oncolytic vectors, some of which are currently being tested in clinical trials. The premier second generation HSV-1 mutant G207, which contains deletions in both copies of ICP34.5 and an inactivating lac Z gene insertion in the ICP6 coding region, has been shown to have antitumor efficacy in a phase I (Markert, Medlock et al. 2000) and phase Ib trial for the treatment of malignant glioblastoma (Markert, Liechty et al. 2009). NV1020, which retains one copy of ICP34.5 and an intact ICP6 gene was generated because the deletion of both copies of ICP34.5 has been shown to result in attenuated virus replication and antitumor efficacy (McAuliffe, Jarnagin et al. 2000, Bennett, Delman et al. 2002). A phase I/II study evaluated the efficacy of NV1020 in patients with treatment refractory metastatic colorectal cancer in the liver. Results indicate that NV1020 treatment was well tolerated and stabilized liver metastases but did not result in any complete responses (Geevarghese, Geller et al. 2010). Recent studies highlighting the role of the immune system in OVT emphasizes the need

for immunostimulatory HSV-1 vectors which augment the antiviral and antitumor immune response to aid in tumor regression. G47Δ, a derivative of G207 with an added deletion in the gene encoding for ICP47, showed enhanced MHC I expression in infected cells resulting in improved antigen presentation and an increase in antitumor T cells responses relative to G207 (Todo, Martuza et al. 2001). As an alternative to the deletion of immunoregulatory genes, several groups have engineered HSV-1 vectors that express immunostimulatory genes as a method by which to increase immunostimulation. The oncolytic HSV-1 vector Talimogene laherparepvec (T_{vec}), formerly known as OncoVEX$^{GM\text{-}CSF}$, expresses granulocyte macrophage colony-stimulating factor under the control of a cytomegalovirus promoter and is deleted for ICP47 and both copies of ICP34.5 (Liu, Robinson et al. 2003). Furthermore, the clinical isolate JS1 was used as the viral backbone as it was found to increase tumor cell cytotoxicity in comparison to the laboratory strain 17^{+} (Liu, Robinson et al. 2003). In syngeneic murine tumor models both injected and non-injected tumors showed significant decreases in size or complete regression and were refractory to tumor cell re-challenge, all indicating the establishment of a sustained antitumor immune response (Liu, Robinson et al. 2003). T_{vec} is currently in phase III clinical trials for the treatment of patients with unresected Stage III B/C or Stage IV melanoma (NCT00769704).

1.8 BHV-1 Biology

Like HSV-1, Bovine herpesvirus type 1 (BHV-1) is a member of the *Herpesviridae* family and *Alphaherpesviridae* subfamily. BHV-1 initiates bovine respiratory disease complex in cattle through transient immunosuppression (Nataraj, Eidmann et al. 1997, Zhang, Wood et al. 1997). This manifests in symptoms such as ocular and nasal secretions, lesions on mucosal surfaces, anorexia, dyspnoea, conjunctivitis, and abortions (Turin, Russo et al. 1999, Hushur, Takashima et al. 2004). In approximately 10% of affected animals bacterial superinfection occurs, resulting in bronchopneumonia (Turin, Russo et al. 1999). However, in the absence of bronchopneumonia the infection is self-limiting due to the immune response, and recovery occurs within 1 to 2 weeks (Turin, Russo et al. 1999). BHV-1 is a neurotropic virus which establishes life-long latency in neurons, with reactivation of the virus resulting from parturition, pregnancy, transport, entrance into a new herd, concomitant bacterial or viral infections, poor living conditions, deficient diet and increases in corticosteroids (Turin, Russo et al. 1999, Jones and Chowdhury 2007).

BHV-1 binds the same attachment and entry receptors used by HSV-1, such as HSPGs and nectin-1 (Campadelli-Fiume, Cocchi et al. 2000). However, it is unable to bind nectin-2 and thus binds CD155 instead (Campadelli-Fiume, Cocchi et al. 2000). CD155 is a poliovirus receptor associated with tumor cell migration and invasion, and has been shown to be upregulated in human cancers (Merrill, Bernhardt et al. 2004). Like HSV-1,

the replication of BHV-1 occurs in three distinct phases of gene expression, IE, E and L (Jones and Chowdhury 2007). There are two transcription units (tu), IEtu1 and IEtu2, which encode functional homologues of HSV-1 ICP0, ICP4 and ICP22, respectively (Wirth, Gunkel et al. 1989, Jones and Chowdhury 2007). Expression of BHV-1 ICP0 (bICP0) is also regulated by an E promoter (Wirth, Vogt et al. 1991, Jones and Chowdhury 2007). During productive infection bICP0 acts as the major regulatory protein due to its ability to activate all other viral promoters (Wirth, Gunkel et al. 1989, Wirth, Vogt et al. 1991, Fraefel, Zeng et al. 1994). Overall, genes expressed by BHV-1 are generally named after the coinciding HSV-1 gene, which often have similar functions although there are some functional differences (Henderson, Zhang et al. 2005, Saira, Zhou et al. 2007, Everett, Boutell et al. 2010).

1.8.1 BHV-1 as an Oncolytic Virus

Of particular interest is the narrow host range of BHV-1, as it is unable to productively infect murine and normal human cells (Hushur, Takashima et al. 2004, Rodrigues, Cuddington et al. 2010). Furthermore, Rodrigues and colleagues showed that both human immortalized and transformed cells are sensitive to BHV-1 infection to varying degrees (Rodrigues, Cuddington et al. 2010). In contrast to other species-specific wt OVs, cellular sensitivity to BHV-1 does not correlate with a defect in type I IFN signalling (Stojdl, Lichty et al. 2000, Wang, Ma et al. 2004, Krishnamurthy, Takimoto et al. 2006, Rodrigues, Cuddington et al. 2010). BHV-1 holds promise as an effective oncolytic virus

as it possesses advantages over HSV-1 vectors. The lack of pre-existing immunity and non-pathogenic nature of BHV-1 in humans may allow for successful systemic treatment with increased safety. Finally, BHV-1 can be used as a non-attenuated virus, and as result it may possess more powerful tumor killing activity.

1.9 Hypotheses and Objectives

The central hypothesis of this project is that:

"BHV-1 is a novel oncolytic virus with the ability to infect and kill a wide variety of human tumor cell types. Specifically, cellular sensitivity to BHV-1 is dictated by a ubiquitous mechanism allowing for the effective targeting of bulk cancer cells and cancer initiating cells."

From this, three testable objectives were determined and tested. The results of which are presented in the publications within this dissertation.

The major objectives used are:

1) Evaluate the oncolytic breadth of BHV-1 *in vitro*
2) Determine the factor(s) which dictate cellular sensitivity to BHV-1 infection
3) Assess the *in vivo* antitumor ability of BHV-1 in an immunocompetent model

Chapter 2: Permissiveness of human cancer cells to oncolytic Bovine herpes virus type 1 is mediated in part by KRAS activity

This chapter consists of an author-generated version of the manuscript entitled "Permissiveness of human cancer cells to oncolytic Bovine herpes virus type 1 is mediated in part by KRAS activity", published in Journal of Virology in June 2014. The paper is reproduced with permission from the **Journal of Virology**, as stated in the ASM Journals Statement of Authors' Rights.

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For this paper, I performed all assays with two exceptions. The National Cancer Institute (NCI) panel screen for virus replication and cytotoxicity was assisted by Michael Herman and Nicole Vidinu, two undergraduate thesis students whom I supervised. Graphical analysis of the NCI panel data was assisted by Dr. Dora Ilieva. All figures were created by myself and I wrote and did revisions for the manuscript. Dr. Karen Mossman provided intellectual direction and aided in revising the manuscript.

Context and background information

The foundation of my project was based on work done by Rebecca Rodrigues, a former Masters student in Dr. Karen Mossman's laboratory. Her project was on the characterization of oncolytic herpesviruses, one of which was BHV-1. Specifically, preliminary studies conducted by Rebecca investigated the ability of BHV-1 to initiate replication and induce cytotoxicity in a panel of normal (6), immortalized (7) and transformed (12) cell types. Overall, normal primary cells were resistant to BHV-1 infection, however; both immortalized and transformed cells were sensitive to BHV-1 at varying degrees (Rodrigues, Cuddington et al. 2010).

Other species-specific OVs are sensitive to the effects of type I IFN signalling. However, this is not the case for BHV-1 as IFN-responsiveness does not correlate with sensitivity to BHV-1 and IFN-production was absent in BHV-1 infected cells (Rodrigues, Cuddington et al. 2010). This suggests that a broader factor, or group of factors, may limit BHV-1 replication in cells.

To further evaluate the oncolytic breadth of BHV-1, I performed a comprehensive screen for virus replication and cellular viability in the NCI panel of 59 established human tumor cell lines. BHV-1 infectivity was compared to that of KM100, our prototypic HSV-1 vector (Hummel, Safroneeva et al. 2005, Sobol, Boudreau et al. 2011). Cell lines were examined for the initiation of virus replication, measured as a function of green fluorescent protein (GFP) fluorescence, and reductions in cellular viability two days post-infection (pi). Heat maps were generated representing virus replication and changes in cellular viability. To ensure that GFP fluorescence is an accurate measure of initiation of

virus replication, I isolated protein from a subpanel of tumor cell lines to study the expression of bICP0, an IE/E gene that acts as a transcriptional activator throughout productive infection (Fraefel, Zeng et al. 1994, Saira, Chowdhury et al. 2008). Box and whisker plots were generated with the assistance of Dr. Dora Ilieva and used to compare the distribution, variability and median values of BHV-1 and KM100 data within each tissue type for both virus replication and cytotoxicity. Overall, the ability of BHV-1 to infect and kill a wide variety of human tumor cell types suggested that a pleiotropic factor(s) dictates cellular sensitivity to the virus.

The mutation status of common oncogenes and tumor suppressors was examined using the Sanger Institute online database, highlighting that mutations in KRAS correlate with high levels of BHV-1 replication. Next, I overexpressed mutated KRAS in a panel of human tumor cell lines which express wt KRAS using a retroviral transduction system. However, I failed to detect any changes in virus replication and cytotoxicity between overexpression and control cell lines. In contrast, when I knocked down mutated KRAS in human tumor cells using a lentiviral shRNA system a decrease in viral titers was observed between knockdown and control (short hairpin (sh) luciferase (LUC)) cell lines. To determine the effects of a KRAS mutation on BHV-1 infectivity, in the absence of underlying mutations as is the case in human tumor cells, I overexpressed mutated KRAS using a retroviral transduction system in normal human embryonic lung (HEL) cells. The overexpression of mutated KRAS in HEL cells conferred permissivity to BHV-1 infection.

A member of the E2F family of transcription factors, E2F1, has been shown to play a role in increasing the efficiency of BHV-1 infection (Workman and Jones 2010, Workman and Jones 2011). To determine whether overexpression of mutated KRAS increases E2F1 expression, thereby enhancing BHV-1 infection, I examined whole cell lysates for basal E2F1 levels in control and HEL cells overexpressing mutated KRAS. In fact, increases in E2F1 expression which correlate with KRAS expression were detected by western blotting.

These studies indicate that BHV-1 is able to infect and kill a wide variety of human tumor cell types and that KRAS is a major contributing factor which dictates permissivity to the virus. We have also suggested a direct link between KRAS activity and E2F1. The proposed mechanism is that additional pathways and/or factors, activated directly or indirectly by KRAS, work in concert with E2F1 to confer permissivity to BHV-1 infection. Understanding the factor(s) that govern permissivity of human cells to BHV-1 will allow for more efficient tumor cell targeting and enhanced cytotoxicity.

Paper: Permissiveness of human cancer cells to oncolytic Bovine herpes virus type 1 is mediated in part by KRAS activity

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Running Head: KRAS activity determines permissiveness to BHV-1

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Abstract

Oncolytic viruses (OV) are attractive avenues of cancer therapy due to the absence of toxic side effects often seen in current treatment modalities. Bovine herpesvirus type 1 (BHV-1) is a species-specific virus that does not induce cytotoxicity in normal primary human cells, but can infect and kill various human immortalized and transformed cell lines. To gain a better understanding of the oncolytic breadth of BHV-1, the NCI panel of established human tumor cell lines was screened for sensitivity to the virus. Overall, 72% of the panel is permissive to BHV-1 infection with corresponding decreases in cellular viability. This sensitivity is in comparison to only 32% for an HSV-1 based oncolytic vector. Strikingly, while 35% of the panel supports minimal or no BHV-1 replication, significant decreases in cellular viability still occur. These data suggest that BHV-1 is an OV with tropism for multiple tumor types and is able to induce cytotoxicity independent of significant virus replication. In contrast to other species-specific OVs, cellular sensitivity to BHV-1 does not correlate with type I IFN signaling; however, mutations in KRAS were found to correlate with high levels of virus replication. The knockdown or overexpression of KRAS in human tumor cell lines yields modest changes in viral titres; however, overexpression of KRAS in normal primary cells elicits permissivity to BHV-1 infection. Together these data suggest that BHV-1 is a broad spectrum OV with a distinct mechanism of tumor targeting.

Importance

Cancer remains a significant health issue and novel treatments are required, particularly against tumors that are refractory to conventional therapies. Oncolytic viruses are a novel platform given their ability to specifically target tumor cells, while leaving healthy cells intact. For this strategy to be successful, a fundamental understanding of virus-host interactions is required. We previously identified Bovine herpesvirus type 1 as a novel oncolytic virus with many unique and clinically relevant features. Here, we show that BHV-1 can target a wide range of human cancer types, most potently lung cancer. In addition, we show that enhanced KRAS activity, a hallmark of many cancers, is one of the factors that increases BHV-1 oncolytic capacity. These findings hold potential for future treatments, particularly in the context of lung cancer, where KRAS mutations are a negative predictor of treatment efficacy.

Introduction

Oncolytic virotherapy (OVT) is based on the observation that viruses, either through genetic engineering or by an inherent mechanism, preferentially replicate in and kill cancer cells while having minimal detrimental effects on normal cells (1). Oncolytic viruses (OVs) elicit the destruction of cancer cells as a direct result of viral replication and the induction of tumor-specific immune responses (2). The safety of OVs and their ability to induce antitumor activity in patients has been demonstrated in phase I and II clinical trials (as reviewed in (3)). Wild-type OVs, such as Reovirus, Newcastle disease virus, Vesicular stomatitis virus (VSV) and Bovine herpesvirus type 1 (BHV-1), do not require mutations to render them oncotropic. Alternatively, OVs that require genetic modification for selective oncolysis include herpes simplex virus type 1 (HSV-1) and adenovirus (1). The collection of gain or loss-of-function mutations within a tumor type dictates permissivity to OVs. A common aberration in cancer cells involves loss-of-function mutations within the interferon (IFN) signaling pathway (4).

HSV-1 was the first virus used to show that gene deletion can render a virus oncolytic (5). The HSV-1 OV KM100 ($ICP0^{n212}VP16^{in1814}$ (6)) possesses lesions in infected cell protein 0 (ICP0), an immediate early (IE) protein that acts as a transcriptional activator during infection and counteracts host cellular responses to viral infection mediated by IFN (6-8). Accordingly, KM100 is unable to infect and replicate in non-transformed, non-immortalized fibroblasts because it cannot effectively block the IFN-induced antiviral state (9, 10), but effectively replicates in human and murine

transformed cells with deficiencies in IFN responsiveness (10). Moreover, unlike small RNA viruses such as VSV, the ability of HSV-1 OVs to elicit a viral burst and induce cytotoxicity in vitro does not correlate with *in vivo* efficacy (11-13).

Bovine herpesvirus type 1 (BHV-1) is a member of the *Herpesviridae* family, *Alphaherpesviridae* subfamily. BHV-1 is a species-specific, neurotropic virus that initiates bovine respiratory disease complex in cattle through transient immunosuppression (14). It establishes life-long latency in neurons, with reactivation occurring due to stress (14-16). The structure of BHV-1 is similar to that of HSV-1. BHV-1 binds attachment and entry receptors used by HSV-1, such as heparan-sulfate and nectin-1 (17). However, it is unable to bind nectin-2, but binds CD155 instead (17-19). Genes expressed by BHV-1 are generally named after the coinciding HSV-1 gene, which often have similar functions (8, 20, 21). While BHV-1 is unable to productively infect normal human cells (14, 22), human immortalized, transformed and breast cancer initiating cells (BCICs) are permissive to infection (22, 23). Interestingly, the ability of BHV-1 to kill human breast tumor cells and BCICs is not contingent upon virus replication or the production of a viral burst (23). Furthermore, in contrast to other species-specific viruses, sensitivity to BHV-1 does not correlate with type I IFN signaling (22). Thus, the determinants of permissivity for BHV-1 in human cells are unknown.

Ras is a superfamily of plasma membrane associated proteins whose members, particularly HRAS, KRAS and NRAS, can be found in almost every human cell type (24, 25). Ras proteins and their downstream signaling effectors are pleiotropic with roles in cell growth, migration, adhesion, cytoskeletal integrity, survival, and differentiation (25-

27). Ras is a GTPase, active in its GTP-bound form and inactive in its GDP-bound form. In addition, Ras is able to mediate cellular responses to virus infection by mediating the activity of antiviral pathways (28-32).

Mutations in Ras, the most common involving constitutive activation through mutation of the GTP binding cleft (33), are associated with particular tumor types with approximately 20% of all tumors have an activating Ras mutation (25, 34). KRAS mutations have been associated with the progression of mammary tumors, acute myelogenous leukemia, lung cancer and gastrointestinal cancer (24, 35, 36).

Many cancer cells are resistant to apoptosis due to mutations in the Ras pathway, which functions to inhibit dsRNA activated protein kinase R (PKR), an IFN-stimulated gene (ISG) (28). Viruses that are sensitive to the effects of PKR, such as reovirus, can infect and replicate in cells with a Ras activating mutation, such as cancer cells, while remaining inhibited in normal cells (37). Additionally, HSV-1 ICP34.5-null mutants preferentially replicate in cancer cells with Ras gain-of-function mutations (38, 39).

Here we report that BHV-1 is able to infect and kill human tumor cell lines from a variety of histological origins and that the oncolytic activity of BHV-1 does not correlate with the extent of virus replication. Of interest, high levels of virus replication were observed in lung, colon and prostate tumor cell lines, which have been associated with mutations in KRAS. While the knockdown or overexpression of oncogenic KRAS in human tumor cell lines yielded a modest effect, overexpression in normal primary cells conferred permissivity to BHV-1 infection. Together, these data indicate that BHV-1 holds promise as a broad spectrum oncolytic with the ability to infect and kill a wide

variety of human tumor cell types, particularly those expressing oncogenic KRAS. These studies also shed light on aspects of the cellular environment within human cells that determine permissivity to this species-specific virus.

Materials and Methods

Cell Lines. All cell types were maintained at 37°C, 5% CO2, in media supplemented with 2 mM L-glutamine. Cell lines with KRAS knockdown or overexpression were maintained in the appropriate medium supplemented with 2 mM penicillin/streptomycin. Madin-Darby bovine kidney (MDBK) cells were obtained from Vikram Misra (University of Saskatchewan) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% horse serum. Human osteosarcoma U2OS cells and human embryonic lung (HEL) cells were maintained in DMEM with 10% fetal bovine serum (FBS). 293 T cells were grown in DMEM with 10% FBS. Human lung carcinoma cells (A549; ATCC) were grown in α-minimal essential medium (α-MEM) with 10% FBS. Human immortalized fibroblast cell lines MSU 1 and MSU 1.1 were maintained in Eagle's MEM with 10% FBS supplemented with sodium pyruvate (1 mM) L-serine (0.25 mM, Sigma) and L-aspartic acid (0.15 mM, Sigma). Cells within the National Cancer Institute (NCI) panel of 60 established human cancer cells lines were maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS.

Viruses. BHV-1 constructs expressing green fluorescent protein (GFP) under control of an endogenous immediate early (IE) promoter was a kind gift from Dr. Guenther Keil (Friedrich-Loeffler-Institut, Germany). Viral stocks were propagated and titrated on MDBK cells followed by sucrose cushion purification (23). The oncolytic HSV-1 vector KM100 ($ICP0^{n212}VP16^{in1814}$), expressing GFP under the control of an IE cytomegalovirus promoter, was propagated and titrated on U2OS. Stocks of this virus were also prepared by sucrose cushion purification (23).

Measurement of Virus Replication and Cytotoxicity. Cells were seeded into 96-well plates and one day later 90-95% confluent cell monolayers were infected with BHV-1 or KM100 at MOIs of 10, 5, 2.5, 1, and 0.5 on each half of the plate respectively. Infection was carried out for 1 hour at 37ºC, after which a maintenance overlay of RPMI+1% FBS was applied. One and two days post infection (pi) plates were scanned on a Typhoon BioAnalyzer (GE Healthcare, Piscataway, NJ) and virus replication was quantified as a function of GFP fluorescence and expressed as a fold change over background. Minimum (1.00) and maximum (22.06) fold change values were set at dark blue and red, respectively. White represents median values. Virus replication fold change values less than one were set to one for heat map analysis. Cytotoxicity, in terms of decreases in cellular metabolism, was assessed two days pi using alamarBlue (5% v/v, Biosource, Carlsbad, CA). Cells were incubated for 30 min at 37ºC, after which fluorescence was read using a Safire fluorescence plate reader (Tecan, Mannedorf, Switzerland). Data was analyzed relative to uninfected controls, corrected for background fluorescence and used to generate a heat map. Minimum and maximum levels of toxicity are represented by dark blue and red, respectively. White represents median values. At least three independent experiments were performed for each cell line.

Graphical Analysis of NCI panel data. Comparison of virus replication and cellular viability data was used to assess whether cell death of a particular cancer type correlates with high or low levels of viral replication. Partek Genomics Suite software (Partek Inc., St. Louis, MO) was used to generate box and whisker plots and perform Principle Component Analysis (PCA). Box and whisker plots were used to indicate the amount and

distribution of variability in panel data. Data was grouped according to tissue origin and analyzed at MOI 0.5. PCA was used to visualize the correlation between virus replication, cellular metabolism and membrane integrity. Each point represents the xyz coordinates of each cell line within the multivariate data space. Data was analyzed at MOI 0.5.

Western Blot Analysis. Cells were mock or infected with BHV-1 and whole cell lysates were collected at various times post infection in 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, St. Louis, MO]) and lysed on ice for 30 min. Lysates were centrifuged at 1,000 rpm for 10 min at 4^{o}C and the supernatants were collected. Protein was quantified using a Bradford assay kit (Bio-Rad Laboratories, Mississauga, ON). Whole cell extracts were boiled in sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol, run on a 10% polyacrylamide gel for bICP0, 12% for E2F-1 and 15% for KRAS knockdown/overexpression confirmation. Gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) with a wet transfer apparatus at 100 V for 1 hour. All blots were blocked in 5% non-fat milk in Tris-buffered saline (TBS) at room temperature for 2 hours. Blots were probed with primary antibodies specific for bICP0 (1:500, courtesy of Dr. Clinton Jones, University of Nebraska), E2F-1 (1:500, Sigma, St. Louis, MO) or KRAS (1:1000, Sigma) diluted in TBS-Tween (0.1%), overnight at 4^{o}C. Blots were probed with an anti-rabbit (bICP0 and KRAS) or anti-mouse (E2F-1) secondary antibody conjugated to horseradish peroxidise (HRP; Sigma) diluted

1:2000 in 5% non-fat milk in 0.1% TBS-Tween. Blots were visualized by chemiluminescence.

Factors dictating cellular sensitivity to BHV-1. NCI panel cell lines were divided into groups based on low (0-5,000 RFU), medium (5,000-20,000 RFU) and high (>20,000 RFU) levels of BHV-1 replication. The Sanger Institute COSMIC database was used to determine the mutations present in each cell line. The number of cell lines possessing each mutation in low, medium and high replication groups was graphed.

Knockdown of KRAS in NCI panel cell lines. To achieve knockdown of KRAS, a short hairpin RNA (shRNA) was made with the sequence 5'-CGATACAGCTAATTCAGAATC-3' (sense) (33). The sense sequence was used to design forward and reverse oligos that have a sense loop and antisense, respectively:

5' CCGGCGATACAGCTAATTCAGAATCCTCGAGGATTCTGAATTAGCTGTA TCGTTTTTG-3' (forward)

5' AATTCAAAAACGATACAGCTAATTCAGAATCCCGAGGATTCTGAATTAG CTGTATCG-3' (reverse)

Forward and reverse oligos were annealed using 1X annealing buffer (100 mM potassium acetate, 30 mM HEPES pH7.4, 2 mM magnesium acetate) and heated at 95^{o}C for 5 min followed by 30 min incubation at 70^{o}C. Oligos were phosphorylated using T4 polynucleotide kinase, and then purified via phenol chloroform extraction. The lentiviral vector pLKO-puro was digested with EcoRI and AgeI restriction enzymes and ligated with the insert using T4 ligase. Plasmid was amplified on 2x low salt Lysogeny broth (LB) agar plates containing 100 μg/ ml ampicillin. The following day select colonies were

inoculated to 2x low salt LB broth and incubated overnight at 37^{o}C with shaking. Plasmid DNA was extracted using the QiaPrep Miniprep Spin kit (Qiagen, Hilden, Germany). Clones were verified by sequencing.

293 T cells were co-transfected with 16 µg of pLKO-shLUC, 8 µg of pCMV.DR.8.91, 4 µg of pVSV-G, and 48 µl of lipofectamine 2000 (Life Technologies, Carlsbad, CA) in OPTI-MEM for 4 hours, after which medium was changed to DMEM + 10% FBS. The following day medium was changed to DMEM + 30% FBS and the supernatant virus harvested 24 hours later and medium replaced with DMEM + 30% FBS for a second round of supernatant harvest 24 hours later. Cell debris present within the supernatant fraction was pelleted by centrifugation at 1500 rpm for 5 min and subsequently filtered through a 0.45 µM filter and a sterile syringe. Virus was pelleted by centrifugation at 25000 rpm for 1.5 hours, after which pellets were resuspended in serum free DMEM.

Cell lines in the KRAS knockdown panel include HCT116, which has a G13D amino acid mutation, and A549, which has a G12S amino acid mutation (Sanger Institute, 2010). KRAS was also knocked down in HELKRAS as a control. Cells were seeded into 6 well plates and a day later 60% confluent monolayers were infected with lentivirus expressing shKRAS or control shLUC for 1 hour at 37^{o}C, after which a maintenance overlay of DMEM + 5% FBS was applied. Medium was changed 24 hours pi followed by puromycin (Sigma) selection at 48 hours pi using 2 µg/µL (A549), 1.5 µg/µL (HCT-116) or 1.0 µg/µL (HELKRAS). Cells were maintained and split two days later to expand the

population of survivor cells carrying the shKRAS or shLUC constructs. Western blot analysis was used to confirm KRAS knockdown.

Overexpression of Oncogenic KRAS in Human Tumor and Normal Primary Cells. The human tumor cell lines COLO-205, NCI-H226 and BT-549, as well as the normal primary cell line HEL were included in KRAS overexpression studies. Activated KRAS effector mutant constructs were a generous gift from Patrick Lee (Dalhousie University). All over-expression plasmids are based on the pBABE-puro retrovirus backbone with activated KRAS generated through the introduction of an activating G12V mutation (40). Cells were transduced with retroviral constructs to make stable cell lines for use in subsequent experiments. Specifically, 293 T cells were transfected with 2 μg pVSV-G, 12 μg pHITgag/pol, and 10.5 μg pBABE vectors in OPTI-MEM for 16 hours. Retrovirus-containing supernatant was collected at 24 and 48 hours post transfection. Cell debris was pelleted by centrifugation at 1000 rpm for 10 min and supernatant was collected and subsequently filtered through a 0.45 μM filter. Virus was flash-frozen and stored at -80^{o}C until use.

Cell lines were infected with the retrovirus for three hours at 37^{o}C, after which a maintenance overlay of RPMI+5% FBS was applied. Two days post pi, cells were subjected to puromycin selection at 1.0 μg/mL (COLO-205), 1.0 μg/mL (NCI-H226), 1.5 μg/mL BT-549, and 1.0 μg/mL (HEL). Western blot analysis was performed to verify KRAS overexpression.

Viral Burst. Cells were infected with BHV-1 at MOI 1, 3, 5 and 10. Viral supernatants and infected cells were collected 1, and 2 days pi. Samples were freeze/thawed three

times prior to centrifugation at 1,000 rpm for 10 min at 4^{o}C. Supernatant was collected and titrated by serial dilution in serum-free DMEM. Dilutions were applied to 90-95% confluent monolayers of MDBK for 1 hour at 37^{o}C. MDBK monolayers were maintained in DMEM supplemented with 0.5% horse serum. At 2 days pi cells were scanned on a Typhoon BioAnalyzer (GE Healthcare) and pfu were counted.

Cytopathic Effect Assays. HEL, HELempty, HELKRAS and HELKRASshKRAS were mock or BHV-1-infected at the indicated MOIs for 1 hour at 37^{o}C. Following viral adsorption cells were maintained in DMEM+5% FBS. Two days pi cells were fixed with methanol and stained using Giemsa to visualize cytopathic effect (CPE).

Results

BHV-1 replicates and reduces cytotoxicity in a wide range of human tumor cell lines. To fully appreciate the oncolytic capacity of BHV-1, particularly in light of its unique properties (22, 23), we screened the NCI panel of 59 established human tumor cell lines comparing BHV-1 to our prototypic HSV-1 vector KM100. The capacity of BHV-1 and KM100 to initiate replication in and decrease viability of the NCI panel was assessed.

Initiation of virus replication, as a function of GFP fluorescence, was analyzed as a fold change over background and used to construct a heat map (Figure 1). GFP expression is not a measure of productive virus infection, but was used to assess viral entry and the initiation of replication. For simplicity, we refer to the initiation of virus replication, as indicated by GFP fluorescence, as virus replication. Cellular toxicity, in terms of reduction in cellular viability, was evaluated 2 days pi and used to generate a heat map (Figure 1).

In general, NCI panel cell lines fall into five different categories: 1) high levels of virus replication with significant decrease in cellular viability; 2) low levels of virus replication with significant decrease in cellular viability, 3) minimal to no virus replication with significant decrease in cellular viability, 4) virus replication with no significant decrease in cellular viability and 5) no significant virus replication or decrease in cellular viability (Tables 1 and 2).

Overall, 95% of panel cell lines support some extent of BHV-1 replication (Figure 1A) with 72% having a corresponding increase in cytotoxicity, defined as a decrease in cellular viability assays of at least 20% at MOI 10 (Figure 1A). In reference, a decrease of

80% in cellular viability (MOI 10) was observed in U2OS, a human osteosarcoma cell line which acts as a prototypic cell line in our screen as it is highly permissive to BHV-1 infection (Data not shown). Taken together, 22% (13/60) of the cell lines showed high BHV-1 replication and a corresponding decrease in cellular viability, 15% (9/60) showed low BHV-1 replication and a decrease in cellular viability, 35% (21/60) showed minimal or no replication and a decrease in cellular viability, 23% (14/60) showed replication but no effect on cellular viability, and finally 5% (3/60) showed no replication and no effect on cellular viability (Table 1).

In contrast, the ability of KM100 to replicate and induce cytotoxicity in panel cell lines was markedly decreased in comparison to BHV-1 (Figure 1B). Only 3% (2/60) of cell lines showed high KM100 replication and a corresponding decrease in cellular viability, 15% (9/60) showed low KM100 replication and a decrease in cellular viability, 13% (8/60) showed minimal or no replication and a decrease in cellular viability, 63% (38/60) showed replication but no effect on cellular viability, and finally 5% (3/60) showed no replication and no effect on cellular viability (Figure 1B and Table 2).

To ensure that GFP fluorescence is an accurate measure of initiation of virus replication, SF-268 (category 1), T47D (category 3) and UACC-257 (category 5) were selected to study the expression of bICP0, an IE/early (E) gene that is required for efficient BHV-1 infection of bovine cells by acting as a transcriptional activator throughout productive infection (41, 42). Expression of bICP0 was not detected in T47D and UACC-257 at any of the time points examined, but was apparent in SF-268 cells, similar to control permissive cell lines MDBK and U2OS (Figure 2). For category 5 cells,

such as UACC-257, it is possible that BHV-1 fails to bind and enter, or this process is inefficient. However, wild type HSV-1, which uses similar receptors for viral entry as BHV-1, is able to initiate replication in this cell line (Data not shown), suggesting that the blockade to replication may be downstream of entry.

Box and whisker plots were used to compare the distribution, variability and median values of BHV-1 and KM100 data sets within each tissue type relative to virus replication and cytotoxicity. Box and whisker plots show a large range of variability within leukemic cell lines with regards to BHV-1 and KM100 replication while lung and colon cell lines possesses a large range in variability in cytotoxicity for both viruses (Figure 3). The median values for BHV-1 replication and cytotoxicity were greater compared to that of KM100. This indicates that BHV-1 is able to initiate replication and induce cytotoxicity at lower MOIs relative to KM100. PCA validated box and whisker plot analysis (Data not shown).

Knockdown of mutant KRAS Decreases BHV-1 Titres. Knowledge of pathways that dictate the permissivity of human tumor cells to OVs allows for more efficient targeting and aids in increased antitumor efficacy due to an improved understanding of host-virus interactions. Data from our group indicates that BHV-1 oncolysis does not correlate with defects in type I IFN signaling (22). Furthermore, BHV-1 is able to distinguish between normal and immortalized cell types (22), suggesting that immortalization and cellular changes occurring during this process may confer sensitivity to BHV-1. However, mutations in TP53 and retinoblastoma (RB1) protein, which are commonly deregulated during immortalization, do not correlate with permissivity to BHV-1 (Figure 4).

NCI panel cell lines were divided into groups based on low, medium, and high levels of BHV-1 replication. The mutations present within each cell line were determined using the Sanger Institute COSMIC database (www.cancer.sanger.ac.uk/cancergenome/projects/cosmic/). The incidence of each mutation in low, medium and high replication groups was quantified to determine which genes are differentially expressed between low and high cohorts. Only genes for which a large difference was observed between low and high replication groups were explored. Results indicate that mutations in KRAS correlate with high levels of virus replication (Figure 4). Moreover, cancer types most commonly associated with KRAS mutations (e.g. lung and colon cancers) were highly permissive to BHV-1 (Figure 1). Ras proteins are the principle activators of multi-component signaling cascades with implications in cellular differentiation, proliferation, and apoptosis. Given the cellular processes controlled by Ras signaling cascades, it is not surprising that they are commonly mutated during immortalization and transformation (34).

The impact of KRAS knockdown on BHV-1 replication in A549 (lung; G12S) and HCT-116 (colon; G13D) cells, which express a constitutively active form of the protein (http://cancer.sanger.ac.uk), was evaluated using a lentiviral shRNA system. These cell types were selected as representative of lung and colon cancer cells, tissue types which are commonly associated with KRAS mutations and are also highly permissive to BHV-1. Stable cell lines were generated by puromycin selection and maintained for subsequent experiments. KRAS knockdown was confirmed by Western blot (Figure 5A). Assessment of the effects of KRAS knockdown on BHV-1 replication (Figure 5B) and cellular

viability (Data not shown) did not delineate differences between control and KRAS knockdown cell lines. However, when viral titres were examined, a decrease of one order of magnitude was observed 1 day pi at MOIs 1 and 3 between $A549_{shLUC}$ and $A549_{shKRAS}$ (Figure 5C). This effect was not present at MOI 5 and 10. Conversely, a decrease of approximately half an order of magnitude was observed 2 days pi at each MOI (Figure 5C). Similar results were observed in HCT-116 cells (Data not shown).

The overexpression of oncogenic KRAS in COLO205, NCI-H226 and BT-549, which express wild-type (wt) KRAS (http://cancer.sanger.ac.uk), was achieved by retroviral transduction with stable cell lines created through puromycin selection. However, differences in BHV-1 replication and cellular viability between control and KRAS overexpression cell lines were not detected (Data not shown). This observation is not surprising as these cell lines contain a plethora of mutations (http://cancer.sanger.ac.uk) that likely make the addition of active KRAS redundant and its individual effect difficult to ascertain.

KRAS Overexpression Sensitizes Normal Primary Cells to BHV-1 Infection. Increased susceptibility of KRAS overexpression lines to BHV-1 infection was not detected. Therefore, studying the effects of oncogenic KRAS within the context of a normal primary cell type will allow for determination of the effects of this single gene mutation on BHV-1 infection. To better address whether active KRAS confers enhanced sensitivity to BHV-1 infection, the non-immortalized, non-transformed cell line HEL was transduced with control retrovirus (empty) or retrovirus expressing constitutively activated KRAS (G12V). Western blot analysis revealed that retrovirus transduction

alone increases cellular levels of KRAS (Figure 6A). HEL, HEL_{empty} and HEL_{KRAS} cells were subsequently infected with BHV-1 (Figure 6B). In comparison to wt HEL, for which CPE was observed at MOI 10 three days pi, almost complete destruction of HEL_{KRAS} monolayers was present at MOI 2.5 at the same time point, with CPE noticeable at MOI 0.5. Intermediate levels of CPE were apparent in the control line HEL_{empty}, suggesting that the process of transduction itself or the resultant increase in endogenous KRAS expression predisposes these cells to BHV-1 infection. To confirm that the increase in sensitivity of HEL cells to BHV-1 infection is due to the overexpression of functionally active KRAS, KRAS was knocked down in HEL_{KRAS} cells. Results show that CPE is apparent at MOI 10 in $HEL_{KRASshKRAS}$ at levels similar to that seen in wt HEL. To evaluate whether KRAS overexpression increases permissivity of HEL cells to BHV-1, viral titres were determined 1 and 2 days pi and compared between wt HEL, HEL_{empty}, HEL_{KRAS} and $HEL_{KRASshKRAS}$. One day pi, BHV-1 titres were 1 to 2 logs higher in HEL_{empty} and HEL_{KRAS} cells relative to wt HEL cells, with little difference observed between HEL_{empty} and HEL_{KRAS} cells (Figure 6C). However, two days pi, BHV-1 titres were significantly higher in HEL_{KRAS} cells, compared to both wt HEL and HEL_{empty}. Knock-down of KRAS in $HEL_{KRASshKRAS}$ cells significantly lowered BHV-1 titres. Together these data suggest that overexpression of endogenous or mutant KRAS in HEL cells increases their permissivity to BHV-1 infection.

MSU-1.0 and MSU-1.1 are cell lines derived by sequential clonal selection of human foreskin fibroblast cells following introduction of the v-myc oncogene, which is sufficient for immortalization but not transformation (43). MSU-1.1 additionally

possesses mutations in HRAS and KRAS (43). However, neither cell line is able to form tumors in athymic mice (43). Therefore, these cell lines allow us to examine BHV-1 infection at early stages in the transformation process. An increase in permissivity to BHV-1 was observed in MSU-1.1 in comparison to MSU-1.0, suggesting that cells that have incurred additional mutations at late stages of the immortalization process have increased sensitivity to BHV-1 infection (Data not shown).

KRAS Overexpression Increases Expression of E2F1 in Normal Primary Cells. The BHV-1 IE/E protein bICP0 stimulates productive infection by inducing viral gene synthesis (42, 44). Infection of MDBK cells with a bICP0-null virus results in a reduction in titres of approximately 100 fold (44). The E2F family of transcriptional regulators has roles in cell proliferation, cell cycle progression, activity of tumor suppressor proteins and p53-dependent/independent apoptosis. A member of the E2F family, E2F1, binds and activates the bICP0 E promoter 100-fold in transient transfection assays (45). BHV-1 has also been shown to increase E2F1 protein levels during productive infection (46). Conversely, knockdown of E2F1 has been shown to significantly reduce the efficacy of infection (46). Mutations in KRAS have been shown to impact many cellular processes, including cell cycle progression (25-27, 47, 48). To determine whether overexpression of activated KRAS elicits an increase in E2F1 expression thereby enhancing BHV-1 infection, we examined basal E2F1 levels in the HEL series of cell lines. Western blot analysis shows a correlation between levels of KRAS (refer to Figure 6A) and E2F1 (Figure 7). Furthermore, siRNA-mediated knockdown of E2F1 in MCF7, a breast cancer cell line that is highly permissive to BHV-1 infection, reduces viral titres by

approximately three orders of magnitude at all MOIs examined (Data not shown). These data suggest a potential mechanism by which oncogenic KRAS enhances BHV-1 infection in human transformed and HELKRAS cells.

Discussion

The use of OVs to target and lyse cancer cells is a novel approach to cancer therapy that lacks the toxic side effects of many current cancer treatments. HSV-1 was the first virus used to demonstrate that a genetic mutation can render a virus oncolytic (5, 49, 50). In fact, HSV-1 has been studied extensively as an OV. The safety of oncolytic HSV-1 at current maximum feasible doses has been demonstrated in phase I and II clinical trials (as reviewed in (3)). Although cross-priming and amplification of antitumor immunity has been demonstrated following i.t administration of oncolytic HSV-1 (51, 52), systemic delivery will be required for the treatment of metastatic and minimal residual disease. However, the high incidence of pre-existing immunity to HSV-1 may limit systemic delivery of the virus. To date, clinical trials have failed to demonstrate whether direct tumor lysis is required for patient responses. In fact, evidence suggests that direct tumor lysis may be linked to dose (53), which could restrict the use of certain OVs due to manufacturing difficulties. These obstacles warrant the development of non-human viruses for OVT. Furthermore, the use of wt non-human viruses circumvents safety concerns over the risk of unexpected toxicities due to the use of genetically manipulated viruses (54).

Results presented herein indicate that BHV-1 is an OV with the ability to infect a large range of human tumor cell types and induce cytotoxicity at low MOIs in comparison to KM100, which has been well characterised for its *in vivo* and *in vitro* oncolytic capacity (10, 12). While the majority of panel lines support both BHV-1 and KM100 replication, BHV-1 displays a more significant increase in virus replication over

background at lower MOIs than KM100, with a corresponding decrease in cellular viability. In the majority of cases where low/no BHV-1 replication was observed, decreases in cellular viability still occurred. Overwhelmingly, while 72% of panel cell lines screened with BHV-1 showed a decrease in cellular viability, only 32% show a decrease with KM100 infection. Furthermore, 35% of the panel supported minimal/no BHV-1 replication; however, a decrease in cellular viability occurred. Together these data suggest that BHV-1 holds potential as an OV possessing tropism for multiple cancer types and is able to induce cytotoxicity independent of significant virus replication.

We have previously reported that in the majority of human breast tumor cells studied, cellular death occurs in the absence of a viral burst (23). Thus, although we did not measure viral bust on NCI panel cell lines, previous studies indicate that virus replication data are not predictive of cytotoxicity and cellular death from BHV-1 infection (23). While these data suggest that a soluble cytotoxic factor may be responsible for cellular death, we have shown that supernatants from breast cancer cells infected with BHV-1 are unable to decrease the cellular viability of MDBK cells (23). The mechanism by which BHV-1 elicits cellular death remains unknown; however, possible methods include epigenetic alterations and microRNA (miRNA) production ((55-59) and as discussed in (23)). Future studies will investigate these possibilities.

The ability of BHV-1 to infect and kill a large range of human tumor cell types suggests that a ubiquitous factor(s), pathway or process is responsible for dictating cellular sensitivity to BHV-1. Panel screen data implicates a broader mechanism for restriction of BHV-1 replication other than sensitivity to type I IFN signaling, unlike

other species-specific viruses such as MV, NDV and VSV. Furthermore, the ability of BHV-1 to infect and kill human immortalized cells is exciting, as it suggests that the virus may be able to infect pre-neoplastic cells and therefore target developing lesions (22).

The Ras family of proteins has pleotropic roles in the cell including mediation of PKR activity. Activated Ras inhibits auto-phosphorylation of PKR blocking its downstream effects, including inhibition of viral protein synthesis by phosphorylating eIF2α (28). Some viruses, such as HSV-1, have developed mechanisms to counteract the effects of PKR and thus productively infect cells without the aid of activated Ras. Other viruses, such as reovirus, rely on activated Ras in order to counteract the effects of PKR. BHV-1 does not encode a homologue of ICP34.5 and, like reovirus, may rely on oncogenic Ras to establish productive infection. Our data suggests that KRAS plays a role in dictating sensitivity to BHV-1 infection; however, it is not the sole factor in this process. While the effects of KRAS knockdown and overexpression on the sensitivity of human tumor cell lines to BHV-1 are variable, this observation is not surprising as these cell lines contain a plethora of mutations which may make the addition or knockdown of oncogenic KRAS insignificant. Most striking are the changes in permissivity of normal primary HEL cells to BHV-1 infection from KRAS overexpression. Unexpectedly, control retroviral transduction increased sensitivity of HEL cells to BHV-1, likely due to a corresponding increase in endogenous KRAS. In fact, HEL_{empty} was intermediate in terms of permissivity to BHV-1 relative to parental HEL and HEL_{KRAS}, in all assays performed. However, HEL_{KRAS} was found to have an altered phenotype in comparison to wt HEL and HEL_{empty}, adopting a spindle-like and elongated appearance (Data not

shown). Although activated KRAS is insufficient for transformation (43), its ability to enhance BHV-1 replication is consistent with our previous observations that immortalized, but non-transformed, cells are permissive for infection (22).

The intricacies of Ras signaling, including its role in multiple cell processes and multitude of downstream effectors, makes comprehensive interrogation of this pathway difficult. Although, to our knowledge, a direct link between KRAS activity and E2F1 has not been established, it is not surprising that we observed increased E2F1 levels in HEL_{empty} and HEL_{KRAS}, as E2F1 levels correlate with the mitotic index of many cancer cells (60, 61). Although E2F1 directly stimulates BHV-1 replication due to E2F1-binding sites within the bICP0 E promoter, it is likely that additional pathways and factors, activated directly or indirectly by KRAS, work in concert with E2F1 to render HEL_{empty} and HEL_{KRAS} cells permissive to infection by BHV-1. Further studies are required to unravel the relationship between KRAS, E2F1, immortalization and cellular permissivity to BHV-1. Although KRAS is not the only factor that dictates permissivity to BHV-1, understanding the relationship between KRAS and BHV-1 activity has clinical relevance, particularly in lung cancer, given that KRAS mutations are predictive of poor response rates to lung cancer therapy (62-65). By understanding the factors that govern permissivity (or lack thereof) of human cells to non-human viruses, important insights are gained into the evolution of host anti-viral mechanisms, which can ultimately be exploited for the development of novel therapeutics.

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Table 2.1: Summary of NCI panel categories based on the effects of BHV-1 infection

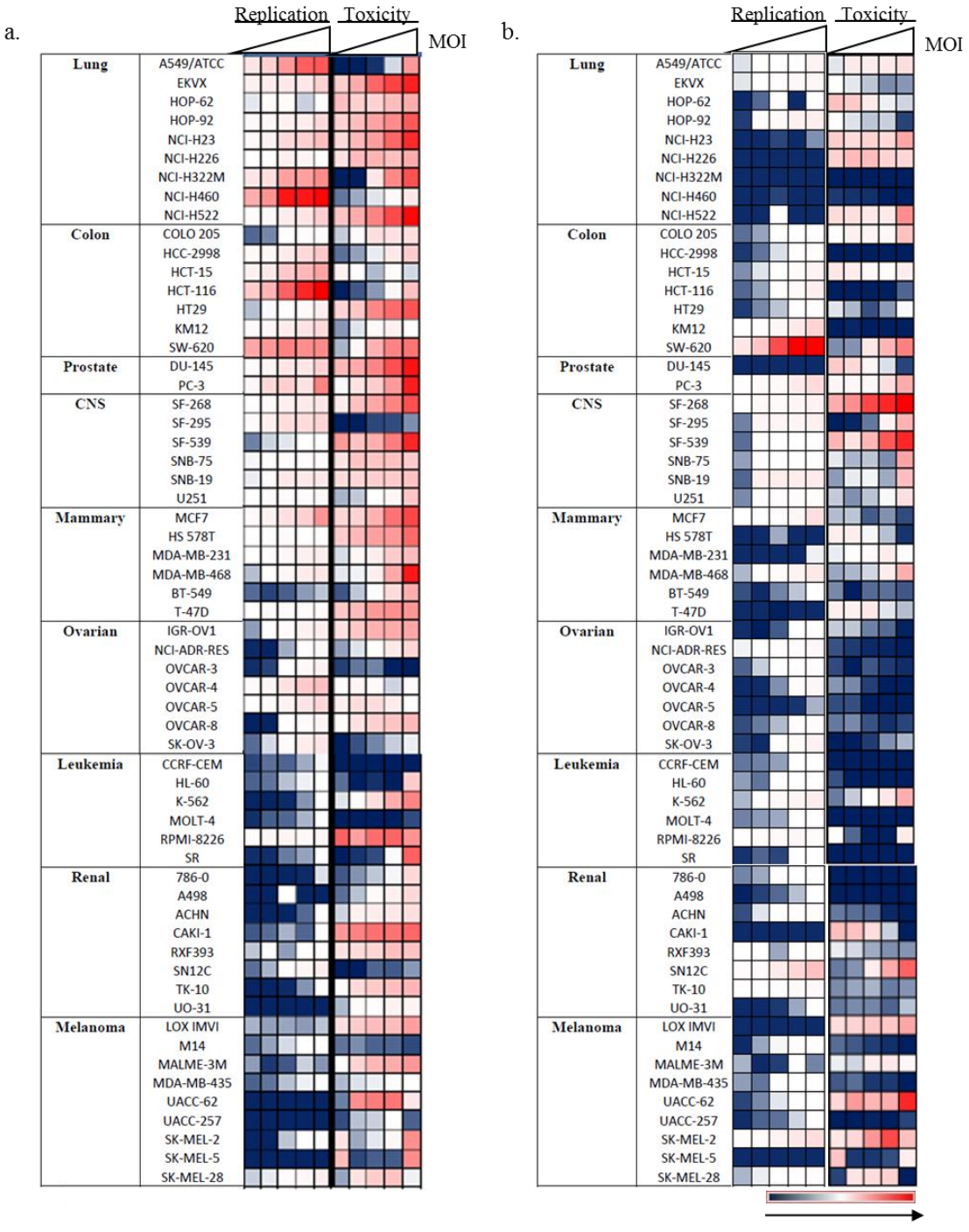
Category 1	A549, DU-145, EKVX, HCT-15, HCT-116, HOP-92, MCF-7, NCI-H23, NCI-H322M, NCI-H522, OVCAR-4, PC-3, SW-620
Category 2	HL-60, HT-29, IGR-OV1, MDA-MB-231, MDA-MB-468, OVCAR-8, RPMI-8226, SK-MEL-28, SNB19
Category 3	786-O, A498, ACHN, BT-549, CAKI-1, COLO 205, HS-578T, HOP-62, K562, LOX-IMV1, MALME-3M, NCI-H226, RXF-393, SF-539, SK-MEL-2, SNB75, SR, T47D, TK-10, U251, UACC-62
Category 4	CCRF-CEM, HCC-2998, KM12, M14, MDA-MB-435, MOLT4, NCI-ADR-RES, NCI-H460, OVCAR-3, OVCAR-5, SF-268, SF-295, SK-OV-3, SN12C
Category 5	SK-MEL-5, UACC-257, UO-31

NCI panel cell lines were divided into five different categories: 1) high virus replication (20,000+ RFU), significant decrease in cellular viability (minimum decrease of 20% at MOI 10); 2) low levels virus replication (5-20,000 RFU), significant decrease in cellular viability, 3) min/no virus replication (0-5,000 RFU), significant decrease in cellular viability, 4) virus replication, no significant decrease in cellular viability and 5) no significant virus replication or decrease in cellular viability.

Table 2.2: Summary of NCI panel categories based on the effects of KM100 infection

Category 1	PC-3, SW-620
Category 2	K562, NCI-H522, RPMI-8226, SF-268, SF-295, SK-MEL-2, SN12C, SNB 19, SNB75
Category 3	COLO 205, HCT-15, MALME-3M, NCI-H23, NCI-H226, SF-539, U251, UACC-62
Category 4	786-O, A498, A549, ACHN, CCRF-CEM, DU-145, EKVX, HCC-2998, HCT-116, HL-60, HOP-62, HOP-92, HS-578T, HT-29, IGR-OV1, KM12, LOX-IMV1, M14, MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB- 435, MOLT4, NCI-ADR-RES, NCI-H322M, NCI-H460, OVCAR-3, OVCAR-4, OVCAR-5, OVCAR-8, RXF-393, SK-OV-3, SK-MEL-28, SR, T-47D, TK-10, UACC-257, UO-31
Category 5	BT-549, CAKI-1, SK-MEL-5

NCI panel cell lines were divided into five different categories: 1) high virus replication (20,000+ relative fluorescence units (RFU)), significant decrease in cellular viability (minimum decrease of 20% at MOI 10); 2) low levels virus replication (5-20,000 RFU), significant decrease in cellular viability, 3) min/no virus replication (0-5,000 RFU), significant decrease in cellular viability, 4) virus replication, no significant decrease in cellular viability and 5) no significant virus replication or decrease in cellular viability.


a.
Replication
Toxicity
MOI
Lung
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H23
NCI-H226
NCI-H322M
NCI-H460
NCI-H522
Colon
COLO 205
HCC-2998
HCT-15
HCT-116
HT29
KM12
SW-620
Prostate
DU-145
PC-3
CNS
SF-268
SF-295
SF-539
SNB-75
SNB-19
U251
Mammary
MCF7
HS 578T
MDA-MB-231
MDA-MB-468
BT-549
T-47D
Ovarian
IGR-OV1
NCI-ADR-RES
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3
Leukemia
CCRF-CEM
HL-60
K-562
MOLT-4
RPMI-8226
SR
Renal
786-0
A498
ACHN
CAKI-1
RXF393
SN12C
TK-10
UO-31
Melanoma
LOX IMVI
M14
MALME-3M
MDA-MB-435
UACC-62
UACC-257
SK-MEL-2
SK-MEL-5
SK-MEL-28
b.
Replication
Toxicity
MOI
Lung
A549/ATCC
EKVX
HOP-62
HOP-92
NCI-H23
NCI-H226
NCI-H322M
NCI-H460
NCI-H522
Colon
COLO 205
HCC-2998
HCT-15
HCT-116
HT29
KM12
SW-620
Prostate
DU-145
PC-3
CNS
SF-268
SF-295
SF-539
SNB-75
SNB-19
U251
Mammary
MCF7
HS 578T
MDA-MB-231
MDA-MB-468
BT-549
T-47D
Ovarian
IGR-OV1
NCI-ADR-RES
OVCAR-3
OVCAR-4
OVCAR-5
OVCAR-8
SK-OV-3
Leukemia
CCRF-CEM
HL-60
K-562
MOLT-4
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LOX IMVI
M14
MALME-3M
MDA-MB-435
UACC-62
UACC-257
SK-MEL-2
SK-MEL-5
SK-MEL-28

Figure 2.1. Heat map showing BHV-1 (A) and KM100 (B) replication and effects on cellular viability on NCI panel cell lines. Cells were infected at MOI of 0.5, 1.0, 2.5, 5.0 and 10.0 for 1 hour at 37ºC. Virus replication as a function of GFP fluorescence was quantified two days pi using a Typhoon Bioanalyzer and is represented as a fold change over background. Minimum (1.00) and maximum (22.06) fold change values were set at dark blue and red, respectively. White represents median values. Cellular toxicity, in terms of decreases in cellular metabolism, was quantified two days pi as measured by alamarBlue. Data were analyzed as a fold change over background and minimum and maximum fold change values were set at dark blue and red, respectively. White represents median values. Replication and cellular toxicity are arranged in order of increasing MOI. At least three independent trials were completed for each cell line.

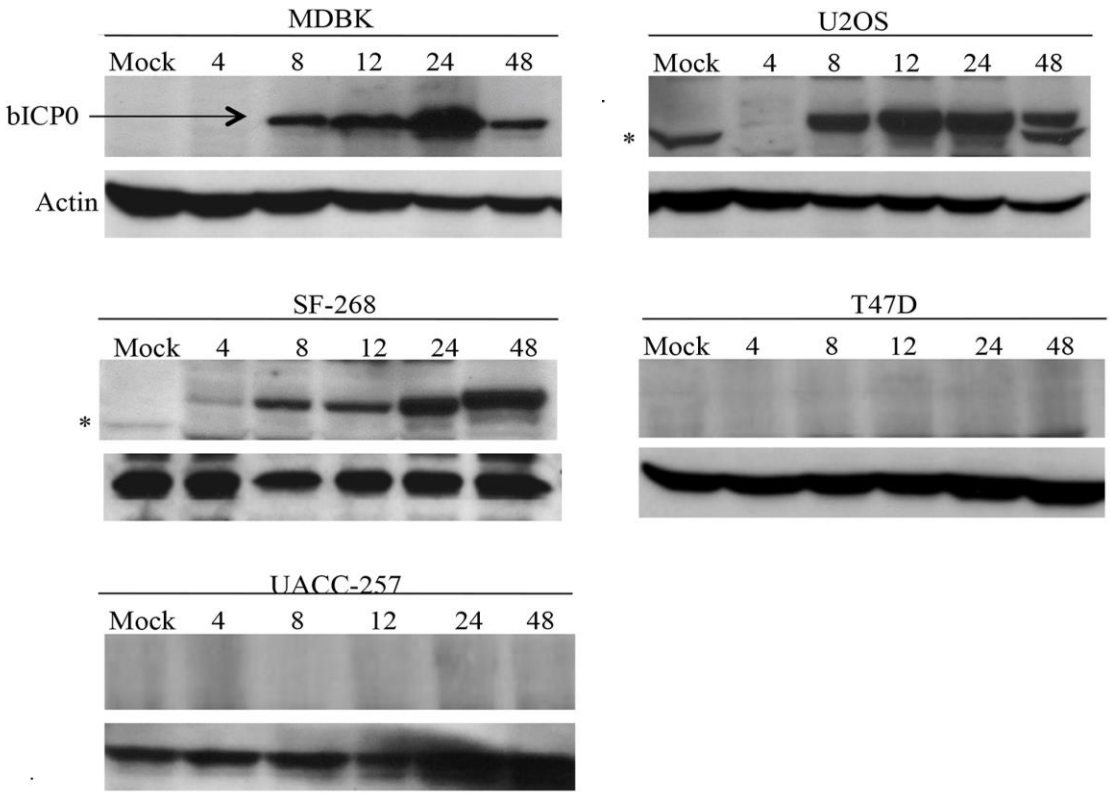

Figure 2.2. bICP0 expression in NCI panel cell lines. Cells were infected with BHV-1 at MOI 3 for 1 hour at 37ºC. Whole cell extracts were harvested at 4, 8, 12, 24 and 48 hours pi for Western Blot analysis. MDBK cells serve as a positive control as they are highly permissive to the virus. The human osteosarcoma cell line U2OS is also used as a positive control as it is a human tumor cell line which has been shown to be highly permissive to BHV-1. * indicates a non-specific band

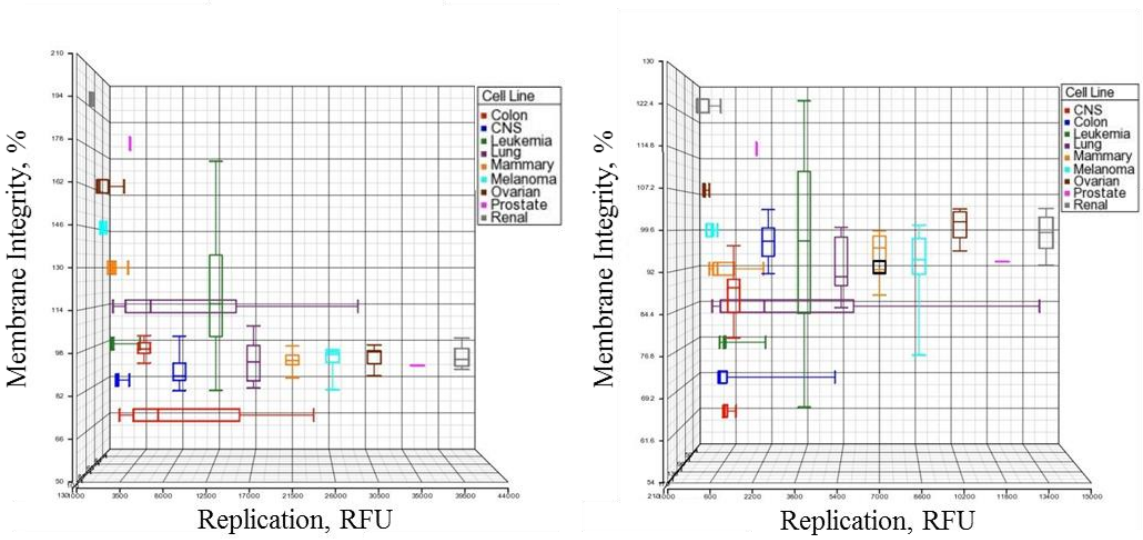

Figure 2.3. Box and whisker plots of NCI panel data with BHV-1 and KM100. Box and whisker plot showing BHV-1 (left) and KM100 (right) replication and cellular toxicity at MOI 0.5 on the NCI panel. Virus replication, as a function of GFP fluorescence, was quantified 2 days pi using a Typhoon BioAnalyzer and is represented in relative fluorescence units. Cellular toxicity, in terms of decreases in cellular metabolism, was quantified 2 days pi using alamarBlue. Box and whisker plots were generated using Partek Genomics Suite software.

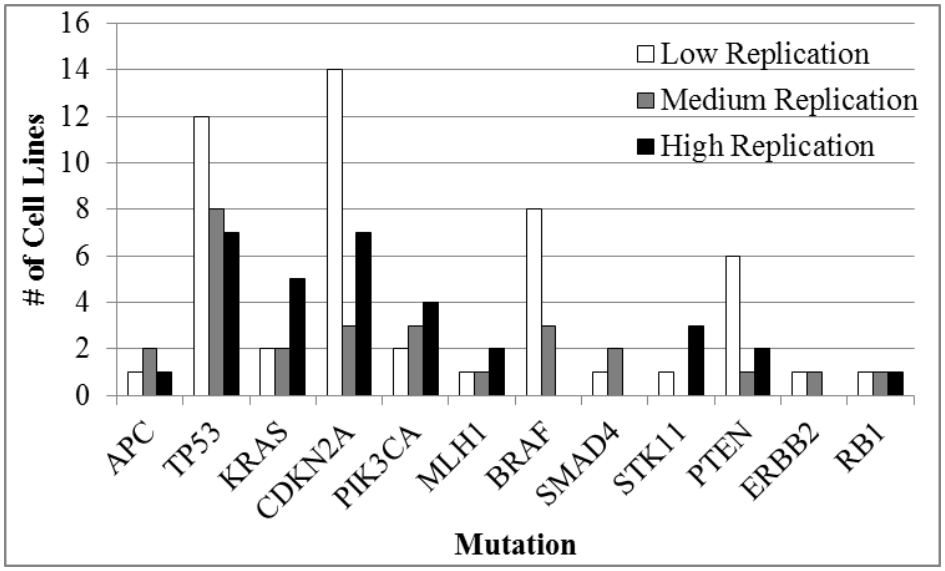

Figure 2.4. Correlation of gene mutations with BHV-1 replication on NCI panel cell lines. NCI panel cell lines were divided into groups based on low (0-5,000 RFU), medium (5,000-20,000 RFU) and high (>20,000 RFU) levels of replication. Mutation status of major oncogenes and tumor suppressors was determined using the Sanger Institute NCI panel database.

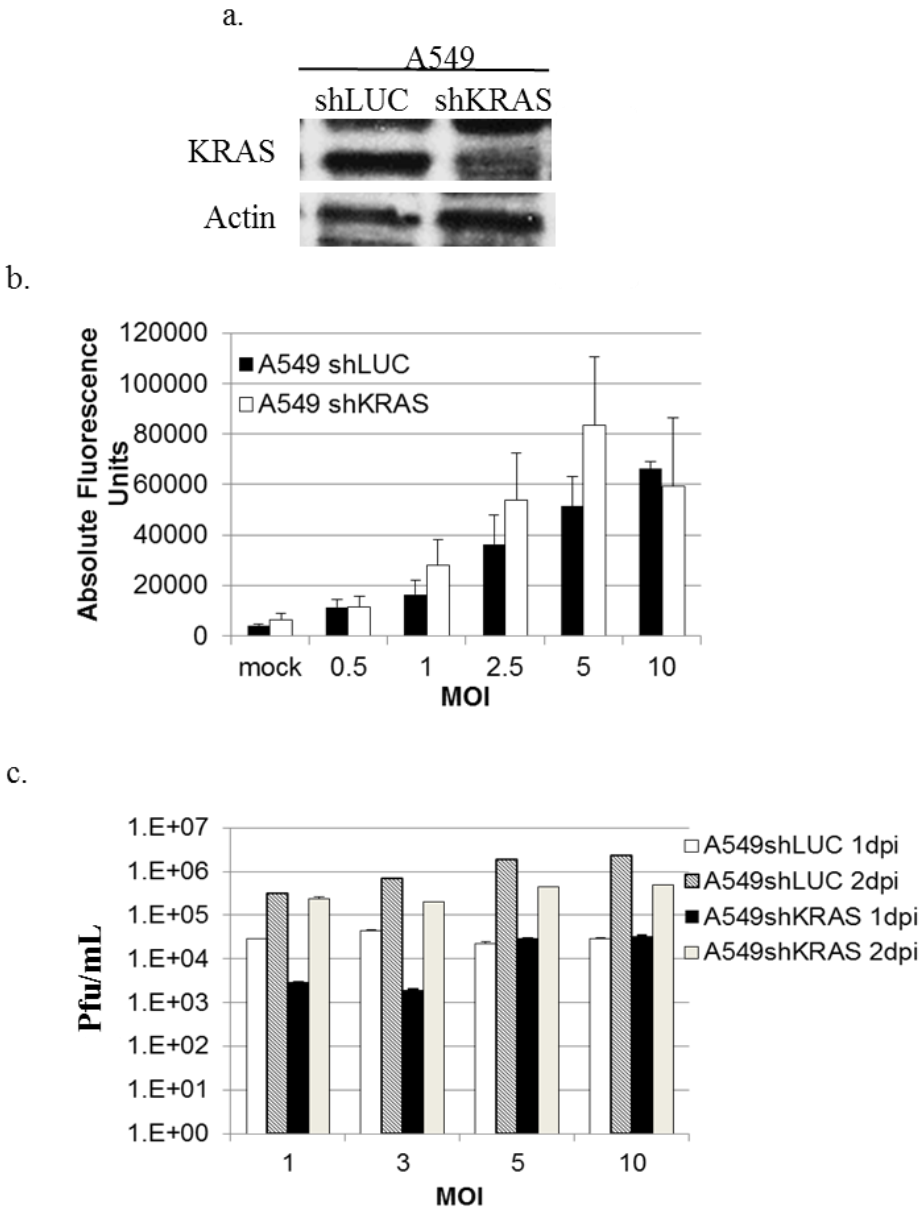

Figure 2.5. KRAS knockdown decreases sensitivity of A549 to BHV-1 infection. (A) Confirmation of KRAS knockdown in $A549_{shKRAS}$. Whole cell extracts were harvested for Western Blot analysis to confirm knockdown of KRAS. (B) BHV-1 replication in $A549_{shLUC}$ and $A549_{shKRAS}$ cell lines. Virus replication, as a function of GFP fluorescence,

was quantified 2 days pi using a Typhoon BioAnalyzer and is represented in absolute fluorescence units. (C) BHV-1 titres on $A549_{shLUC}$ and $A549_{shKRAS}$ cell lines. Cells were infected with BHV-1 at the indicated MOIs for 1 hour at 37^{o}C. Triplicate samples of viral supernatants and cell-associated virus particles were collected one and two days pi and titrated on naïve MDBK monolayers. Error bars represent mean + SEM, n=3.

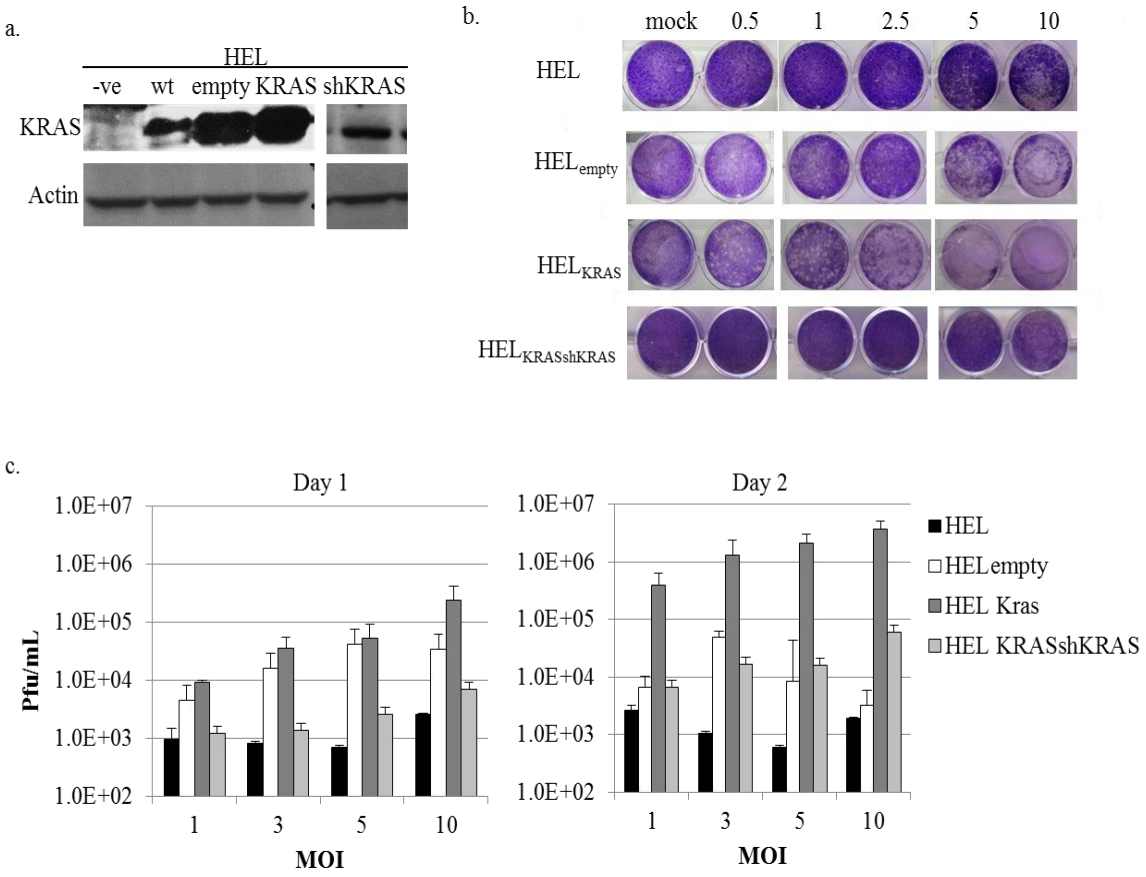

Figure 2.6. KRAS overexpression increases susceptibility of HEL cells to BHV-1. (A) Confirmation of KRAS overexpression and knockdown in HEL$_{KRAS}$ and HEL$_{KRASshKRAS}$, respectively. Whole cell extracts were harvested for Western Blot analysis to confirm overexpression or knockdown of KRAS. Negative (-ve) control 293 T whole cell extract. (B) Permissiveness of wt HEL, HEL$_{empty}$, HEL$_{KRAS}$ and HEL$_{KRASshKRAS}$ to BHV-1. Cells were infected at the indicated MOIs for 1 hour at 37^{o}C. Two days pi cells were Giemsa stained to visualize CPE. (C) BHV-1 titres on wt HEL, HEL$_{empty}$ HEL$_{KRAS}$ and HEL$_{KRASshKRAS}$ cell lines. Cells were infected with BHV-1 at the indicated MOIs for 1

hour at 37ºC. Triplicate samples of viral supernatants and cell-associated virus particles were collected one and two days pi and titrated on naïve MDBK monolayers. Error bars represent mean + SEM, n=3.

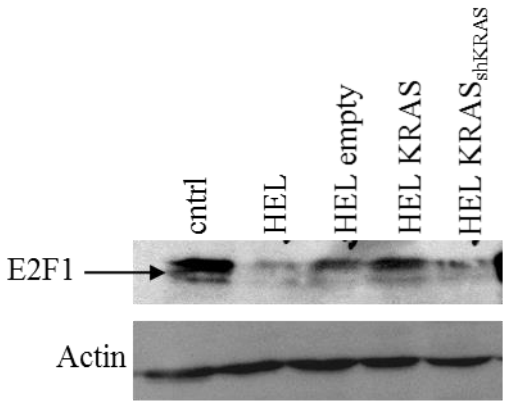

Figure 2.7. Overexpression of KRAS increases expression of basal E2F1 in normal primary cells. Whole cell extracts were harvested for Western Blot analysis to examine basal E2F1 expression in wt HEL, HEL_{empty}, HEL_{KRAS} and $HEL_{KRASshKRAS}$ cell lines. 293 T cell lysate was used as a positive control.

Chapter 3: Oncolytic bovine herpesvirus type 1 infects and kills breast tumor cells and breast cancer initiating cells irrespective of tumor subtype

This chapter consists of an author-generated version of the manuscript entitled "Oncolytic bovine herpesvirus type 1 infects and kills breast tumor cells and breast cancer initiating cells irrespective of tumor subtype", published in Cancer Gene Therapy in May 2013. The paper is reproduced with permission from **Cancer Gene Therapy**.

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For this paper, I performed all cell culture, virus propagation and purification for *in vitro* and *in vivo* studies, viral burst assays and measurements of virus replication and cellular viability. Sphere forming assay and colony forming assays were performed with advisement from Adele Girgis-Gabardo, a laboratory technician employed by Dr. John Hassell (McMaster University). Sorting and *in vivo* protocols were established by Amanda Dyer, a former Masters student in Dr. Karen Mossman's laboratory. Sorting experiments were carried out with the assistance of Dr. Liang Hong (McMaster Flow Cytometry Facility Technician). Dr. Samuel Workenhe provided assistance for *in vivo* studies, specifically pertaining to anesthesia and quality control for tumor measurements.

All figures were created by myself and I wrote and did revisions for the manuscript. Dr. Karen Mossman provided intellectual direction and aided in revising the manuscript.

Context and background information

BHV-1 is able to infect and kill a wide variety of human tumor cell types, including BC cells (Chapter 2). Due to the broad tropism of the virus, I became interested in whether BHV-1 was able to overcome the drawback of current targeted BC therapies, which are efficacious against only particular subtypes of the disease, depending on which cellular receptor they target. I also chose to focus on BC as Dr. Karen Mossman's laboratory has multiple established murine models of breast adenocarcinoma (Hummel, Safroneeva et al. 2005, Sobol, Boudreau et al. 2011, Workenhe 2013).

Current BC treatments are designed to target specific attributes such as tumor stage, grade and molecular profile, including tumor subtype and receptor status. Although these treatments have improved patient outcomes, multidrug resistance and patient relapse has emphasized the need for novel treatment strategies with unique tumor targeting mechanisms. Recent studies have implicated BCICs as major contributors in BC relapse and metastasis. Their ability to self-renew, differentiate to give rise to multiple cell lineages and resist conventional therapeutics, such as radiation and chemotherapy, make them an important anticancer target (Cripe, Wang et al. 2009, Patel, Ndabahaliye et al. 2010).

The ability of BHV-1 to infect and kill BC cells from a variety of subtypes and with varied receptor expression profiles was tested. I examined cell lines for the initiation of virus replication, measured as a function of GFP fluorescence, and reductions in cellular viability two days pi. A heat map was generated representing virus replication and changes in cellular viability, showing that BHV-1 is able to infect and kill human BC cells from a variety of subtypes. Interestingly, decreases in cellular viability did not always correlate with virus replication and in the majority of cases (with the exception of two cell lines) an appreciable viral burst was not detected. Due to the absence of a productive infection I determined whether a soluble factor excreted during infection was responsible for BC cell death. However, this was not the case and the mechanism by which BHV-1 elicits BC cell death is yet unknown.

In addition to bulk BC cells, I tested the ability of BHV-1 to infect and kill BCICs from both luminal and basal subtypes. To do this, I sorted BCICs (with the assistance of Dr. Liang Hong) based on their Hoechst 33342 dye exclusion ability and immediately infected them with BHV-1. Measurements for virus replication and decreases in cellular viability were taken two days pi. I determined that BHV-1 was able to kill human BCICs from luminal and basal subtypes, even in the absence of the initiation of virus replication. With the assistance of Adele Girgis-Gabardo, the effect of BHV-1 on BCIC self-renewal and differentiation was assessed using sphere and colony-forming assays, respectively. I found that BHV-1 infection decreased the self-renewal and differentiation abilities of BCICs from luminal and basal BC cell lines.

BCICs have been described as having enhanced tumor forming ability *in vivo* (Al-Hajj, Wicha et al. 2003, Han and Crowe 2009). Given that I had determined that BHV-1 is able to infect and kill human BCICs, I wanted to ascertain whether it was also able to reduce their tumor forming ability. Bulk MCF7 cells (luminal BC cell line) were mock or BHV-1-infected and injected subcutaneously into NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NOD *scid* gamma or NSG) mice. I monitored tumor formation for 66 days post injection and found that the tumor volumes of mice injected with BHV-1-infected MCF7 cells was significantly less in comparison to mock.

My data shows that BHV-1 is able to infect and kill bulk BC cells and BCICs from a variety of BC subtypes with varied receptor expression profiles. Interestingly, tumor cell death does not require the initiation of virus replication or the presence of a productive infection. BHV-1 is also able to reduce the self-renewal and differentiation of BCICs, as well as their tumor forming ability. Together these data suggest that BHV-1 holds promise as a broadly applicable, yet targeted BC therapy. I believe that with further study BHV-1 can be developed into a BC treatment strategy which circumvents the problems associated with molecular subtypes and receptor expression profiles. Most importantly, the ability of BHV-1 to infect and kill BCICs may result in sustained and increased patient survival.

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Paper: Oncolytic bovine herpesvirus type 1 infects and kills breast tumor cells and breast cancer initiating cells irrespective of tumor subtype

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Running Title: Bovine herpesvirus type 1 targets breast cancer cells

Key words: oncolytic virus, breast cancer therapy, cancer initiating cells

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Abstract

Oncolytic viruses are attractive breast cancer therapeutics due to their unique mechanisms of tumor cell targeting and the absence of toxic side effects associated with current treatments. Bovine herpesvirus type 1 (BHV-1) is a species-specific herpesvirus which fails to induce cytopathic effects in normal human cells, but is capable of infecting and killing a variety of immortalized and transformed human cell types, including human breast tumor cell lines from luminal, basal A and basal B subtypes representing a variety of receptor expression profiles. BHV-1 is able to initiate replication in and kill both bulk and side population cells, the latter of which have enhanced tumor initiating capacity. Despite the lack of a productive infection or secretion of cytotoxic factors, BHV-1 infection elicited decreases in cellular viability even from long term culture following low multiplicity infection. Moreover, BHV-1-infected MCF7 cells are significantly diminished in their capacity to form tumors *in vivo*. Overall, these studies suggest that oncolytic BHV-1 targets bulk breast cancer cells and cancer initiating cells from luminal and basal subtypes by a novel mechanism that is not contingent upon receptor expression status.

Introduction

Recombinant and naturally occurring oncolytic viruses (OVs) preferentially replicate in and kill cancer cells while having minimal detrimental effects on normal cells by exploiting biochemical differences between normal and tumor cells.[1] Oncolytic virotherapy (OVT) holds potential as a novel therapy given its two-pronged approach, the lytic destruction of cancer cells and induction of tumor-specific immune responses.[2] Although human viruses such as Herpes simplex virus (HSV) are safe and show signs of efficacy in clinical trials[3-6], pre-existing immunity and thus the treatment of metastatic disease warrants the development of wild type, non-human viruses.

Bovine herpesvirus type 1 (BHV-1), a member of the *Alphaherpesviridae* subfamily, shares many similarities with HSV. Although BHV-1 uses HSV attachment and entry receptors heparan-sulfate and nectin-1, it does not bind nectin-2 but instead binds CD155, a poliovirus receptor associated with tumor cell migration and invasion that is upregulated in human cancers.[7-10] Of clinical relevance is the narrow host range of BHV-1, as it is unable to productively infect normal murine and human cells.[11,12] However, BHV-1 efficiently targets immortalized and transformed human cells.[12] Wild-type mice are not susceptible to infection with BHV-1; however, RAG-2 gene deleted mice lacking type I and II interferon (IFN) receptors die within several days of infection.[13] Unlike most naturally occurring OVs, however, permissivity of human cells to BHV-1 does not correlate with type I IFN signaling.[12]

Cancer stem cells, also called cancer-initiating cells (CICs), are characterized by two properties, self-renewal and the ability to generate progeny consisting of all cell lineages

contained within a tumor.[14,15] Although CICs are typically thought to represent only 1 to 2% of total tumor cells within a tumor mass, recent studies indicate that the frequency of CICs is variable.[16] Experimentally, CICs are often referred to as tumor initiating cells due to their ability to support continual tumor growth *in vivo*. CICs possess properties that confer resistance to conventional therapies, including self-renewal, differentiation, high proliferative capacity, resistance to apoptosis, active DNA repair mechanisms, relative quiescence, and enhanced drug efflux.[17]

Goodell et al. first described a 'side population' (SP) of cells enriched for stem cell properties such as efflux of the DNA-binding dye Hoechst 33342, which express stem cell markers and have *in vivo* reconstitution abilities.[18] SPs have been identified in a variety of human tissues and cancers.[19,20] An SP, ranging from 0.2-7.5%, has been described in the MCF7 human breast cancer cell (BCC) line. This population is enriched for colony forming ability, quiescence, CD44, efflux pump components, tumorsphere formation capacity and enhanced *in vivo* tumorigenesis.[21-26] Thus, BCC line SPs are prime targets for the investigation of new therapeutics capable of selectively eliminating tumorigenic cancer cells. The use of OVs to target CICs, and in particular breast CICs (BCICs), is attractive as OVs kill cancer cells through mechanisms distinct from conventional therapeutics.[27,28] Recent studies have validated the ability of OVs to target CICs.[27,29, 30, 31]

We previously showed that BHV-1gfp targets immortalized and transformed human cells, but not normal cells.[12] Here, we report that BHV-1gfp targets bulk and BCICs regardless of subtype or receptor status and reduces the sphere and colony forming ability of BCICs.

In majority of cases, cell death occurs in the absence of a productive virus infection. Moreover, BHV-1gfp-infected MCF7 cells are unable to initiate tumor formation *in vivo*. Collectively, these data suggest that BHV-1gfp possesses multiple strategies to target human BCICs to limit or prevent de novo tumor formation.

Material and Methods

Cell Lines

All cell lines were maintained at 37ºC + 5% CO2 in medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human osteosarcoma cells (U2OS; American Type Culture Collection [ATCC], Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Madin Darby bovine kidney (MDBK) cells were obtained from Dr. Vikram Misra (University of Saskatchewan) and were cultured in DMEM supplemented with 5% horse serum. Human breast adenocarcinoma cells (MCF7; ATCC) were cultured in high glucose DMEM (Gibco) supplemented with 10% FBS. Additional human breast adenocarcinoma cell lines (HS-578T, MDA-MB-468, SkBr3) were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS. MCF7 and HCC1954 spheres were maintained in stem cell medium (SCM) comprised of DMEM-F12 (Gibco) supplemented with 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor (EGF), 20 ng/mL B27, and 1 mg/mL heparin (Sigma).

Viruses

BHV-1 expressing GFP (BHV-1gfp) was a kind gift from Dr. Günther Keil (Friedrich-Loeffler-Institut, Germany) and was propagated and titrated on MDBK cells. Virus preparations were sucrose cushion purified. Specifically, 80% confluent 150cm^2 dishes were infected with BHV-1gfp for 1 hour at 37ºC at a multiplicity of infection (MOI) of 0.01 plaque forming units (pfu)/cell in serum-free DMEM. Following infection, cells were maintained in the appropriate medium and harvested three days post infection (pi).

Samples were centrifuged, supernatant collected, and cell pellets resuspended in serum-free DMEM and stored at -80ºC. Virus was isolated from supernatant fractions by ultracentrifugation at 25,000 rpm for 2 hours at 4ºC. Virus was isolated from cell pellets following three freeze-thaw and vortex cycles by Dounce homogenization and centrifugation at 1,000 rpm for 10 minutes at 4ºC. The virus-containing pellet was resuspended using the supernatant-associated fraction. The combined fractions were sonicated for 1 minutes and then purified by ultracentrifugation through a 36% sucrose gradient at 30,000 rpm for 2 hours at 4ºC. Purified virus was resuspended in serum-free DMEM, or PBS for animal experiments, and stored at -80ºC.

Measurement of Virus Replication and Cellular Viability

Cells were seeded into 96-well plates and one day later 90-95% confluent cell monolayers were infected with BHV-1gfp at the indicated MOI. One and two days pi plates were scanned on a Typhoon BioAnalyzer (GE Healthcare, Piscataway, NJ) and virus replication was quantified as a function of GFP fluorescence, analyzed as a fold change over background and used to generate a heat map. Two days pi cellular viability was assessed using a Safire fluorescence spectrophotometer (Tecan, Männedorf, Switzerland). Measures of cellular metabolism and membrane integrity were assessed using alamarBlue and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), respectively (Invitrogen, Grand Island, NY). Cells were incubated with alamarBlue (5% v/v) and CFDA-AM (4 µM) for 30 minutes at 37ºC, after which fluorescence was read. Data were analyzed relative to uninfected controls and corrected for background fluorescence.

Viral Burst

Cells were infected with BHV-1gfp at MOI 3. Viral supernatants and infected cells were collected 1, 2 and 3 days pi. Samples were either freeze/thawed three times or frozen once and sonicated for 1 minute prior to centrifugation at 1,000 rpm for 10 minutes at 4^{o}C. Supernatant was collected and titrated by serial dilution in serum-free DMEM. Dilutions were applied to 90-95% confluent monolayers of MDBK for 1 hour at 37^{o}C. MDBK monolayers were maintained in DMEM supplemented with 0.5% horse serum in 1% methyl cellulose. At 2 days pi cells were scanned on a Typhoon BioAnalyzer (GE Healthcare, Piscataway, NJ) and pfu were counted and viral burst determined.

Soluble Factor Cytotoxicity

HS-578T was seeded into 6 well plates and a day later 90-95% confluent monolayers were infected with BHV-1gfp at MOI 3. At 4, 24 and 48 hours pi supernatants were collected and transferred to naïve HS-578T and MDBK monolayers. Cellular viability was assessed 2 days pi using alamarBlue and CFDA-AM.

Side Population Cell Sorting

Cells were trypsinized, filtered through a 40 μm mesh, and resuspended in DMEM supplemented with 2% FBS and 10mM HEPES buffer at 10^6 cells/ml. Control samples were incubated with 50 μM reserpine (Sigma) for 30 minutes at 37^{o}C. All samples were then incubated with 5 μg/ml Hoechst 33342 dye for 90 minutes in a 37^{o}C water bath with intermittent shaking. Following incubation, cells were washed and resuspended in HBSS buffer supplemented with 2% FBS, 10mM HEPES buffer, 2 μg/ml propidium iodide (Sigma) and kept on ice. Prior to flow analysis cells were filtered through a 40μm mesh to

obtain a single cell suspension. Sorting was performed using a FACSVantage SE (Becton Dickinson, Franklin Lakes, NJ) with FACSDiva Option. A wavelength of 350 nm was used to excite the Hoechst 33342, while Hoechst blue was detected at 424/44 nm and Hoechst red at 675LP. Channels were separated by a 640LP dichromic beam-splitter, and sorting was carried out at 16 ψ sheath pressure using a 100 μm nozzle. The SP population was defined as cells diminished in the presence of reserpine. Data were analyzed using Flow Jo 8.8.6 software. Immediately following sorting, SP and non-SP cells were plated in 96-well pates and infected with BHV-1gfp at MOIs of 3 and 10 for 1 hour at 37ºC. Virus replication and cellular viability were examined as previously indicated.

Sphere forming assay

Spheres were dissociated by trypsinization at 37ºC for 2 minutes with pipetting and resuspended in SCM. Cells were plated at $3.0x10^4$ cells/mL and mock or BHV-1gfp-infected at MOI 1, 3, 5 or 10 for 30 minutes at 37ºC. Following infection, cells were resuspended in SCM and transferred to 24 well plates. The number of spheres for each well was counted after 4 days of culture and compared between mock and BHV-1gfp-infected groups.

Colony forming assay

Spheres were dissociated by trypsinization at 37ºC for 2 minutes with pipetting and resuspended in complete medium. Cells were plated into 96 well plates at a density of $3.0x10^4$ cells/mL and mock or BHV-1gfp-infected at MOI 1, 3, 5 and 10 for 45 minutes at 37ºC. Following infection cells were collected and resuspended in complete medium and seeded into 10 mm^2 plates and cultured for 10 days. Cells were then washed with PBS,

fixed in methanol for 2 minutes, and Giemsa stained. Colonies were counted and compared between mock and BHV-1gfp-infected groups.

***In vivo* tumor analysis**

MCF7 cells were either mock-infected or infected with BHV-1gfp at MOI 3. Cells were incubated for 3 hours at 37^{o}C, trypsinized, centrifuged at 1000 rpm for 10 minutes at 4^{o}C and resuspended in a 1:1 PBS/Matrigel mixture. Anaesthetized 5-week-old female NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NOD scid gamma or NSG) mice (Jackson Laboratory, Bar Harbor, ME) were injected subcutaneously (SC) with 10^6 cells resuspended in 200 μL of PBS/Matrigel one day following SC implantation of slow-release 17β-estradiol pellets (1.5 mg/pellet, 60 day release; Innovative Research of America, Sarasota, FL) via trocar. Tumor growth was monitored every 2 to 3 days. Tumor volume was determined by digital caliper measurement and calculated using: $4/3\pi(\text{length}/2)(\text{width}/2)^2$. All animal work was performed in full compliance with the Canadian Council on Animal Care with approval from the McMaster University Animal Research Ethics Board.

Results

BHV-1gfp infection of human breast cancer cells.

The in vitro oncolytic capacity of BHV-1gfp has been demonstrated in several human BCC lines.[12] Breast cancer (BC) treatments are designed based on tumor stage, grade and molecular profile, including tumor subtype and receptor status. Multidrug resistance and subsequent patient relapse warrants the development of novel and broadly applicable treatment strategies. BCC lines representing basal A, basal B and luminal subtypes, as well as varying estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) expression were included in our screen (Table 1). The capacity of BHV-1gfp to initiate replication in and decrease cellular viability of BCCs was determined. The initiation of virus replication, as a function of GFP fluorescence, was quantified two days pi and represented as a fold change over background. These data were used to generate a heat map. Cellular viability, in terms of cellular metabolism and membrane integrity, was quantified and is represented as a fold change relative to uninfected cells. Initiation of virus replication increased with increasing MOI, which resulted in a coinciding decrease in both measures of cellular viability, with the exception of MDA-MB-436, which did not show a decrease in either measure of cellular viability (Figure 1). Interestingly, increases in virus replication did not always correlate with a decrease in cellular viability and vice versa. Although low levels of virus replication were observed on BT-549, Hs-578T and HCC1954 decreases in both measures of cellular viability occurred (Figure 1).

The ability of BHV-1gfp to productively replicate in BCCs was assessed by determining the number of infectious virus particles produced per cell. The bovine cell line MDBK was used as a positive control. Interestingly, the initiation of virus replication did not yield a burst of infectious virus in BCC lines, with the exception of MCF7 and SK-BR-3 (Fig. 2 and data not shown). Although BHV-1gfp initiated low levels of replication in Hs-578T and HCC1954, it was unable to produce a burst of infectious virus. However, decreases in cellular viability detected were similar to that of MCF7, for which an appreciable burst was detected (Figure 2). These data suggest that the presence of an appreciable virus burst is not required to elicit BCC death.

Hs-578T cellular death is not the result of a soluble cytotoxic factor.

Due to the absence of a productive infection in Hs-578T cells, we were surprised to observe nearly complete cellular death 10 days pi with BHV-1gfp at a MOI as low as 0.1 and at 20 days pi at MOI 0.01 (Figure 3a). Thus, we investigated whether a soluble cytotoxic by-product of infection contributes to Hs-578T cellular death. Supernatant was collected from BHV-1gfp-infected Hs-578T cells at 24 and 48 hours following infection at MOI 3, applied to naïve Hs-578T cells, and cellular viability was assessed 2 days post treatment. We failed to detect a significant decrease in cellular metabolism and membrane integrity (Figure 3b). These data suggest Hs-578T cellular death does not occur due to the presence of a soluble cytotoxic by-product of infection. Transfer of supernatants to naïve permissive cells also failed to alter cellular viability and no hallmarks of infection were observed (data not shown), confirming that Hs-578T cells secrete neither a soluble

cytotoxic factor nor infectious virus particles. Instead, these data suggest that exposure of Hs-578T cells to BHV-1gfp is sufficient to induce cell death.

BHV-1gfp infection of breast cancer initiating cells.

BC relapse and metastasis has been, in part, attributed to the presence of BCICs. BCICs are highly tumorigenic and possess the ability to self-renew, form differentiated progeny and resist conventional therapeutics, such as radiation and chemotherapy.[27,28] Targeting bulk BCCs and BCICs may result in sustained tumor remission and increased patient survival. To address whether BCICs from luminal and basal subtypes are permissive to BHV-1gfp infection, SP cells were isolated from MCF7 (luminal), Hs-578T (basal B) and MDA-MB-468 (basal A) cultures, immediately plated following sorting, and infected with BHV-1gfp at an MOI of 3 and 10. As accumulation of Hoechst 33342 dye is toxic, sorted non-SP cells were not assessed. Initiation of virus replication and a decrease in both measures of cellular viability were observed in the SP populations isolated from MCF7 (Figure 4a) and Hs-578T (data not shown). Although virus replication was not detected in MDA-MB-468 SP cells, a decrease in cellular viability occurred (Figure 4b). Cytopathic effects were observed at all MOIs tested (data not shown). The ability of BHV-1gfp to productively replicate in MCF7 BCICs was assessed by determining the number of infectious virus particles produced per cell. The viral burst of BHV-1gfp was not significant in MCF7 SP cells indicating that the presence of an appreciable viral burst is not required for MCF7 BCIC death (Figure 4c).

BHV-1gfp reduces the self-renewal and differentiation of human breast cancer initiating cells.

The sphere forming and colony forming assays can be used to assess the self-renewal and differentiation of BCICs, respectively. Therefore, these methods were employed to analyze the effect of BHV-1gfp infection on the self-renewal and differentiation of MCF-7 and HCC1954 BCICs. MCF7 and HCC1954 tumorspheres were dissociated and mock or BHV-1gfp-infected at MOI 1, 3, 5 and 10. Spheres were counted at 4 days pi and colonies at 10 days pi and compared between mock and BHV-1gfp-infected groups. The sphere-forming ability of MCF7 and HCC1954 decreased by ~48% and ~69% respectively, and colony formation decreased by ~39% and ~93% as the result of BHV-1gfp infection using an MOI as low as 3 (Figure 5). Together these data indicate that BHV-1gfp reduces both the self-renewal and differentiation of BCICs from luminal and basal BCC lines.

BHV-1gfp treated MCF7 have reduced tumor-forming ability *in vivo*.

Our data suggest that BHV-1gfp initiates replication in, and reduces cellular viability of MCF7 SP cells. MCF7 SP cells have been described as having increased tumor-forming ability *in vivo*.[21-23] To confirm that exposure to BHV-1gfp reduces the *in vivo* tumor-forming ability of MCF7 SP cells, bulk MCF7 cells were mock or BHV-1gfp-infected and injected subcutaneously into NSG mice. Tumor volumes in mice injected with BHV-1gfp-infected cells were significantly less than mock for the duration of the experiment ($p < 0.01$), which was performed for 66 days to ensure the effect was sustained (Figure 6). Overall, these data indicate that exposure of bulk MCF7 cells to BHV-1gfp leads to a

reduction in tumor formation *in vivo*, consistent with the ability of BHV-1gfp to reduce cellular viability *in vitro*.

Discussion

Although somewhat controversial, it has been generally accepted that a population of BCCs, referred to as BCICs, possess increased tumorigenicity and enhanced resistance to conventional cancer therapy. BCICs are thought to be largely responsible for patient relapse and development of metastatic disease; therefore, development of treatment strategies which kill these cells would lead to sustained and increased patient survival. The depletion of BCICs using OVs is an attractive avenue of cancer therapy due to the ability of OVs to evade host resistance mechanisms, their utility as delivery vehicles for therapeutic transgenes that target the tumor-propagating phenotype of BCICs and their resistance to commonly used chemotherapeutics, permitting combination therapy approaches.[27] Oncolytic reovirus infects BCICs and non-CICs within tumors, and replicates to similar levels in both cell types eliciting cellular death via apoptosis.[29,32] Similarly, oncolytic adenovirus vectors target $CD44^{+}CD24^{-/low}$ BCCs and show efficacy in animal models of both advanced local and metastatic BC.[30,31] Due to their neurotropic nature, HSV oncolytic vectors effectively target glioma CICs.[27,33] While these vectors show promise as OVs in pre-clinical and clinical trials, an inherent disadvantage of using human viruses is the potential for pre-existing immunity within the population.

The results of this study indicate the ability of BHV-1gfp to infect and kill BCCs and BCICs from both luminal and basal subtypes, regardless of receptor status. Interestingly, cell death occurred both in the presence and absence of productive infection in BCCs and BCICs. The lack of availability of antibodies for E and L BHV-1 proteins, toxic effects of Hoechst 33342 dye accumulation and low SP cell numbers confound comprehensive

analysis of the progression of infection in SP versus non-SP cells. Several studies have examined factors dictating sensitivity of CICs to OV infection. Specific to BCICs, defects in the induction of the type I IFN response were found to mediate susceptibility to oncolytic adenovirus.[34] Although many naturally occurring oncolytic viruses exploit defects in IFN signalling to replicate, BHV-1gfp replication does not depend on type I IFN status.[12] Moreover, cells that are immortalized, but not transformed, are also susceptible to BHV-1gfp[12], suggesting that BHV-gfp may target pre-neoplastic cells. Overall, CIC sensitivity to OV infection depends on the virus used and the mutations present. Understanding stem cell biology in the context of OV infection will allow for development of more effective immunotherapies to target CICs.

Despite the lack of productive infection, BHV-1gfp infected cultures showed significant cell loss in long term culture, even following low multiplicity infection (MOI 0.1). The observed cell death could not be explained by the production of a soluble cytotoxic factor, suggesting that BHV-1gfp elicits cell death overtime through a different mechanism. These data suggest that the presence of a permissive cellular environment is not required for BHV-1gfp to elicit oncolytic activity. It is unknown at this time how BHV-1gfp kills cells following low multiplicity infection; however, studies are ongoing to elucidate these mechanisms.

Although lytic infection of cells with herpesviruses such as BHV-1 typically lead to the induction of apoptosis and other forms of cell death, viral infection can also influence cell status through epigenetic alterations and microRNA (miRNA) production. Epigenetic modifications can influence imprinting, germ line silencing, DNA rearrangements, overall

chromosome stability and stem cell division.[35] It is well appreciated that disruption of epigenetic processes can lead to altered gene function and tumorigenesis and that epigenetic changes of both viral and host genomes in virally infected cells can affect malignant cellular transformation.[36,37] Herpesvirus in particular have been shown to elicit host epigenetic changes, in part by encoding genes that alter host methylation and histone deacetylation.[38-40] Herpesviruses are also known for their expression of high levels of multiple miRNA species that regulate viral replication and pathogenesis and for their ability to reshape the pattern of host miRNA expression.[41] Indeed, the human herpesvirus Kaposi's sarcoma-associated herpesvirus encodes a miRNA that regulates global cellular epigenetic reprogramming.[42] As both epigenetic modifications and miRNAs regulate CICs[43-45], it is entirely plausible that infection of BCICs with BHV-1gfp induces cellular alterations that rendered them less efficient in tumor initiation, despite maintaining a population of cells with efflux properties. Moreover, under conditions where we observed cell death in long-term culture following a low multiplicity infection, it is also possible that BHV-1gfp-induced alterations of the cellular genome led to cell death. Although an MOI of 0.1 suggests that only one in ten cells received an infectious, or plaque-forming, virus particle, given that a typical particle to plaque-forming unit ratio for wild type *Alphaherpesviruses* is in the order of 100, it is likely that each cell in the culture was infected with at least one virus particle. It will be intriguing to elucidate the mechanism of cell death under low multiplicity infection and to understand how cells that survive BHV-1gfp infection are altered.

Overall, these data have implications for BC therapy. Given the strict species specificity of BHV-1, the lack of neutralizing antibodies within the human population and the inability of BHV-1 to induce toxicity in healthy cells[12], BHV-1 is an excellent candidate for oncolytic virotherapy requiring intravenous administration to reach difficult to access and/or metastasized tumors. Of particular interest is the ability of BHV-1gfp to initiate replication in and kill bulk BCCs and BCICs irrespective of tumor subtype or receptor status. In contrast, conventional BC treatments are designed based on tumor stage, grade and molecular profile, including tumor subtype and receptor status. Tumor heterogeneity both within the tumor and between patients represents a significant road block to the efficacy of current treatment strategies. Furthermore, multidrug resistance and subsequent patient relapse prevent lasting patient cures. The ability of BHV-1gfp to infect and kill BCICs has important implications for the prevention of metastases and relapses. Moreover, BHV-1gfp infected BCICs are restricted in their capacity to initiate tumor formation *in vivo*. These data do not evaluate the efficacy of BHV-1gfp against established human BC tumors, as the presence of an intact immune response has been shown to play a pivotal role in OVT.[46-48] Despite advances in the isolation and molecular characterization of murine BCICs, their roles as putative tumor initiating cells within the context of spontaneous and genetically engineered murine models of BC remain unclear.[49,50] Therefore, data obtained using these models may not be indicative of results in humans. Future studies aimed at understanding the mechanism of BHV-1gfp-induced cell death in BCCs will provide valuable information on basic tumor biology and enable

the development of novel BC therapies with the ability to overcome the specificity of currently used treatments.

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Cell Line	Subtype	ER	PR	HER2
MCF7	luminal	+	+	-
T47D	luminal	+	+	-
SK-BR-3	luminal	-	-	+
MDA-MB-468	basal A	-	-	-
HCC1954	basal A	-	-	+
Hs-578T	basal B	-	-	-
MDA-MB-231	basal B	-	-	-
BT-549	basal B	-	-	-
MDA-MB-436	basal B	-	-	-

Table 3.1: Breast cancer cell line subtype and receptor status.

Abbreviations: Estrogen receptor (ER); progesterone receptor (PR); human epidermal growth factor receptor 2 (HER2).

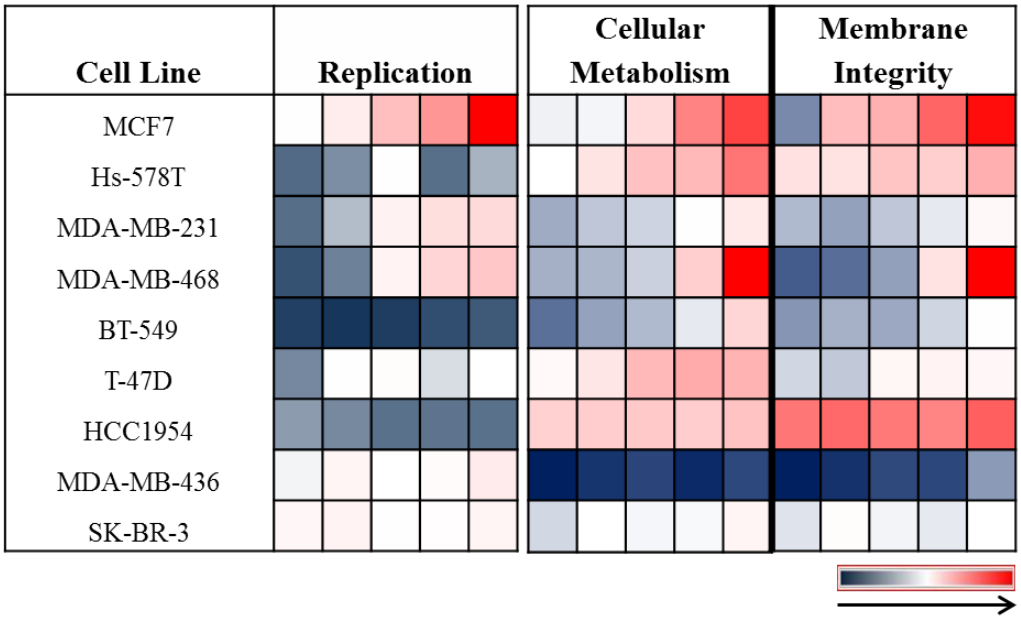

Figure 3.1. Heat map showing BHV-1gfp replication on breast cancer cell panel. Cells were infected at MOIs of 0.5, 1.0, 2.5, 5.0 and 10.0 for 1 hour at 37ºC. Virus replication as a function of GFP fluorescence was quantified two days pi using a Typhoon Bioanalyzer and is represented in absolute fluorescence units. Cellular viability, in terms of cellular metabolism and membrane integrity, was quantified two days pi as measured by alamarBlue and CFDA-AM fluorescence, respectively. Data were analyzed as a fold change over background and minimum and maximum fold change values were set at dark blue and red respectively. White represents median values. Replication and cytotoxicity are arranged in order of increasing MOI.

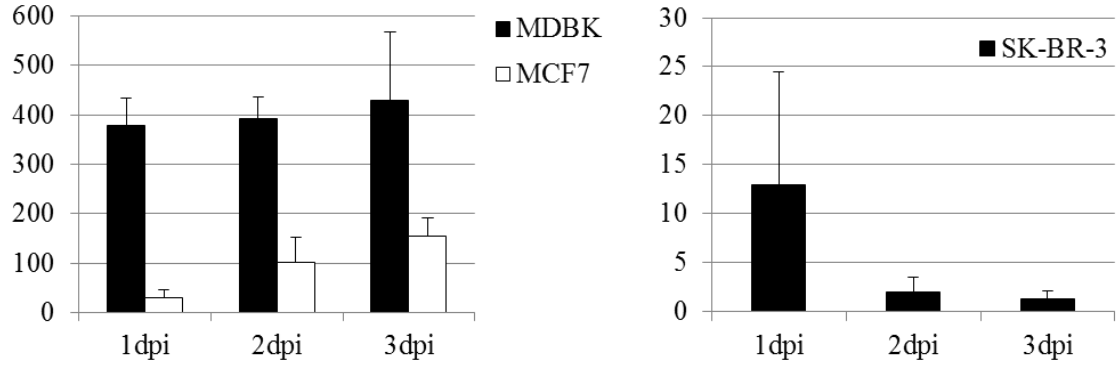

Figure 3.2. Despite initiation of viral replication, BHV-1gfp only produces an appreciable burst on MCF7 and SK-Br-3 cells. Cells were infected with BHV-1gfp at MOI 5 for 1 hour at 37ºC. Triplicate samples of viral supernatants and cell-associated virus particles were collected one, two and three days pi and titrated on naïve MDBK monolayers. Error bars represent mean + SEM, n=3.

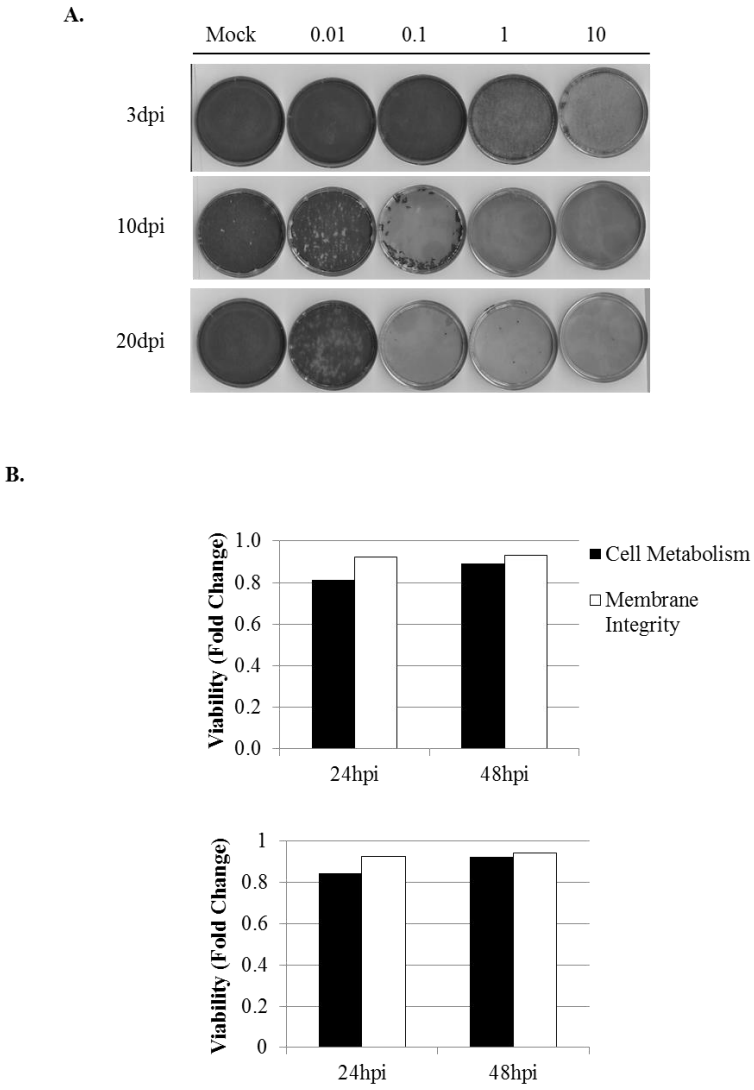

Figure 3.3. BHV-1gfp infected Hs-578T cultures show significant cell loss in long term culture following low multiplicity infection. Hs-578T cells were infected with BHV-1gfp at MOI 0.01, 0.1, 1 and 10 for 1 hour at 37ºC. Cells were fixed and Giemsa stained 3, 10 and 29 days pi (**A**). Hs-578T cell death is not mediated by a soluble bi-product of BHV-1gfp infection. Hs-578T cells were infected with BHV-1gfp at MOI 3 for 1 hour at 37ºC and supernatants were harvested at 24 and 48 hours pi and applied to

naïve MDBK and Hs-578T cells. Cellular viability, in terms of cellular metabolism and membrane integrity, was quantified two days pi as measured by alamarBlue and CFDA-AM fluorescence, respectively (**B**).

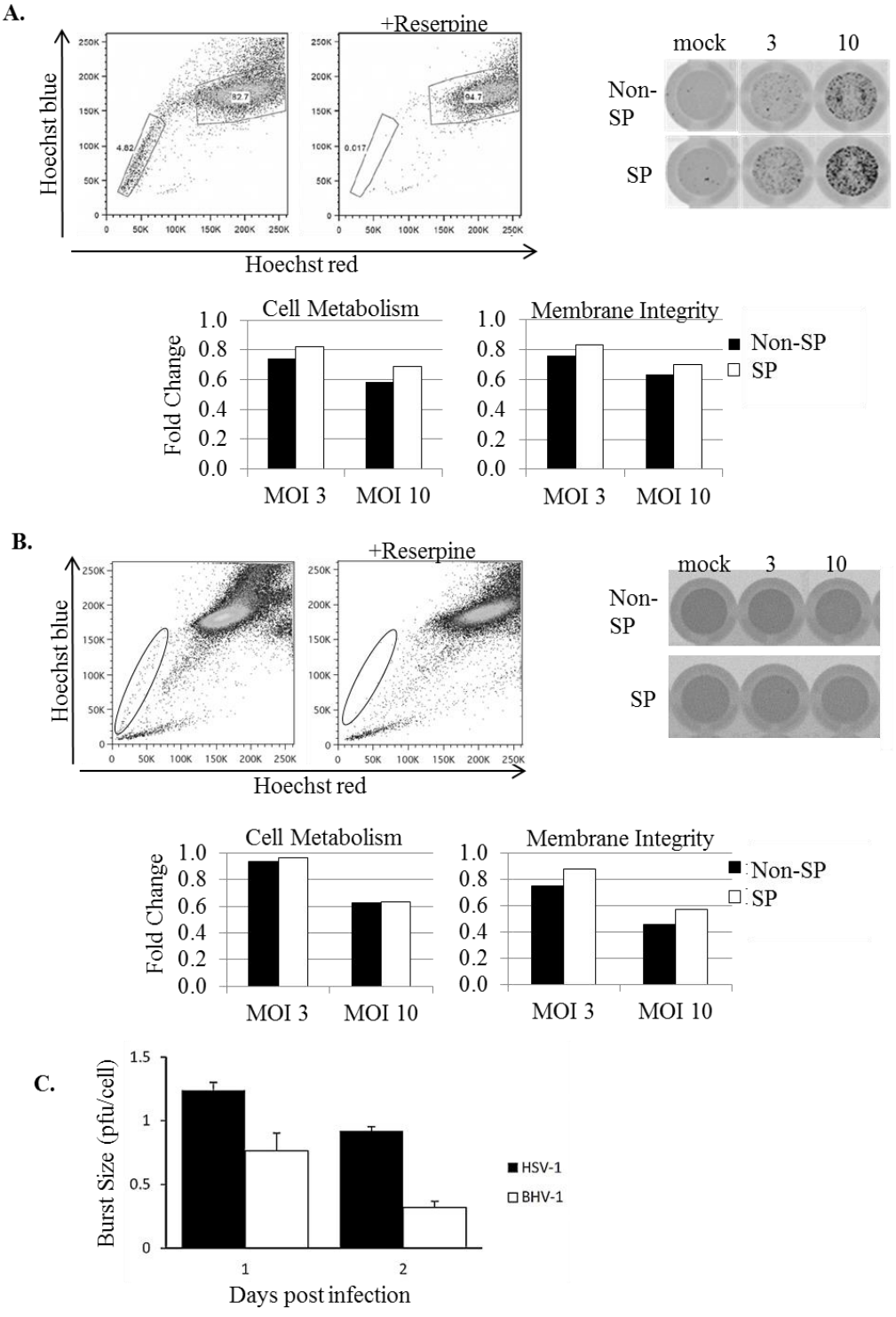
A.
+Reserpine
Hoechst blue
Hoechst red
82.7
4.82
94.7
0.017
mock
3
10
Non-SP
SP
Cell Metabolism
Membrane Integrity
Fold Change
1.0
0.8
0.6
0.4
0.2
0.0
MOI 3
MOI 10
Non-SP
SP
B.
+Reserpine
Hoechst blue
Hoechst red
mock
3
10
Non-SP
SP
Cell Metabolism
Membrane Integrity
Fold Change
MOI 3
MOI 10
Non-SP
SP
C.
Burst Size (pfu/cell)
1.5
1
0.5
0
1
2
Days post infection
HSV-1
BHV-1

Figure 3.4. Bulk BCCs and SP are susceptible to BHV-1gfp infection. MCF7 cells were stained with Hoechst 33342 in the absence or presence of 50 μM reserpine and analyzed by flow cytometry. Gates indicate the percentage of cells from the parent gate (top left). Sorted MCF7 non-SP and SP cells were immediately plated and infected with BHV-1gfp at MOI 3 and 10 for 1 hour at 37^{o}C. Virus replication, as a function of GFP fluorescence, was visualized two days pi using a Typhoon Bioanalyzer and is shown as a scanned plate (top right). Cellular viability, in terms of cellular metabolism (bottom, left) and membrane integrity (bottom, right), was quantified two days pi as measured by alamarBlue and CFDA-AM fluorescence, respectively, n=3 (**A**). MDA-MB-436 cells were stained with Hoechst 33342 in the absence or presence of 50 μM reserpine and analyzed by flow cytometry. Gates indicate the percentage of cells from the parent gate (top left). Sorted MDA-MB-436 non-SP and SP cells were immediately plated and infected with BHV-1gfp at MOI 3 and 10 for 1 hour at 37^{o}C. Virus replication, as a function of GFP fluorescence, was visualized two days pi using a Typhoon Bioanalyzer and is shown as a scanned plate (top right). Cellular viability, in terms of cellular metabolism (bottom, left) and membrane integrity (bottom, right), was quantified two days pi as measured by alamarBlue and CFDA-AM fluorescence, respectively, n=3 (**B**). MCF7 SP cells were infected with BHV-1gfp at MOI 5 for 1 hour at 37^{o}C. Triplicate samples of viral supernatants and cell-associated virus particles were collected one and two days pi and titrated on naïve MDBK cells. Error bars represent mean + SEM, n=3 (**C**).

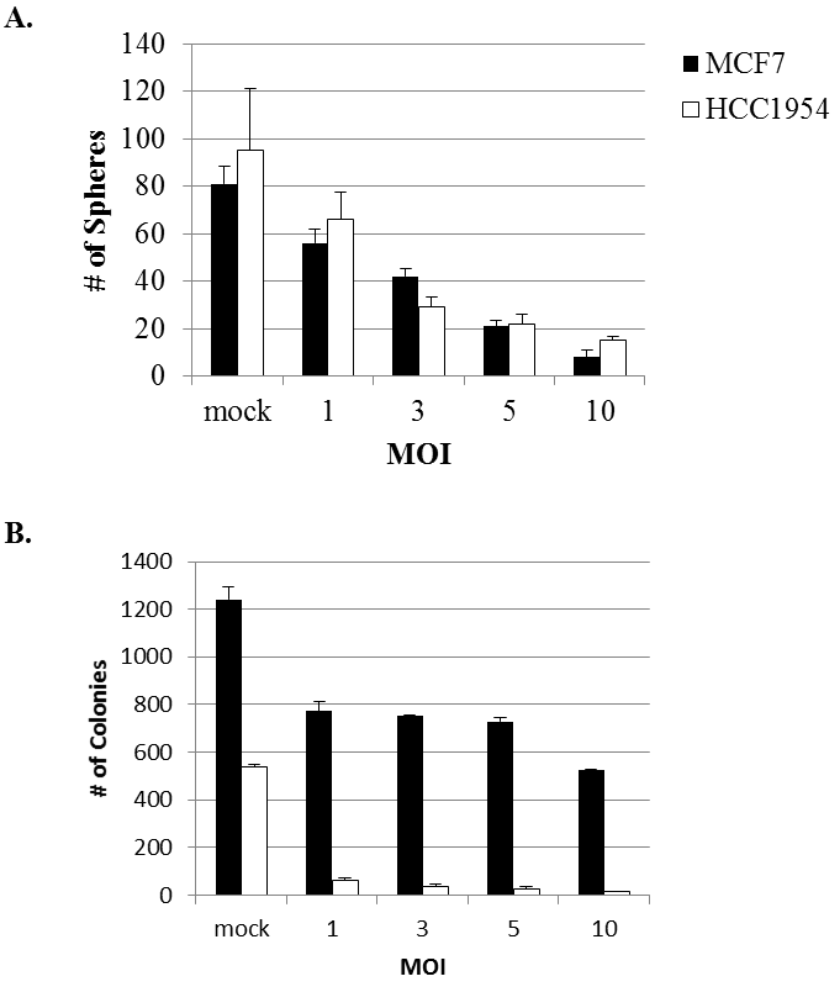

Figure 3.5. BHV-1gfp reduces the self-renewal and differentiation of BCICs. MCF7 and HCC1954 tumorspheres were dissociated and $3x10^4$ cells were mock or BHV-1gfp-infected at MOI 1, 3, 5 and 10 for 1 hour at 37ºC. Spheres were counted at 4 days pi (sphere-forming assay) and colonies at 10 days pi (colony-forming assay) and compared between mock and BHV-1gfp-infected groups.

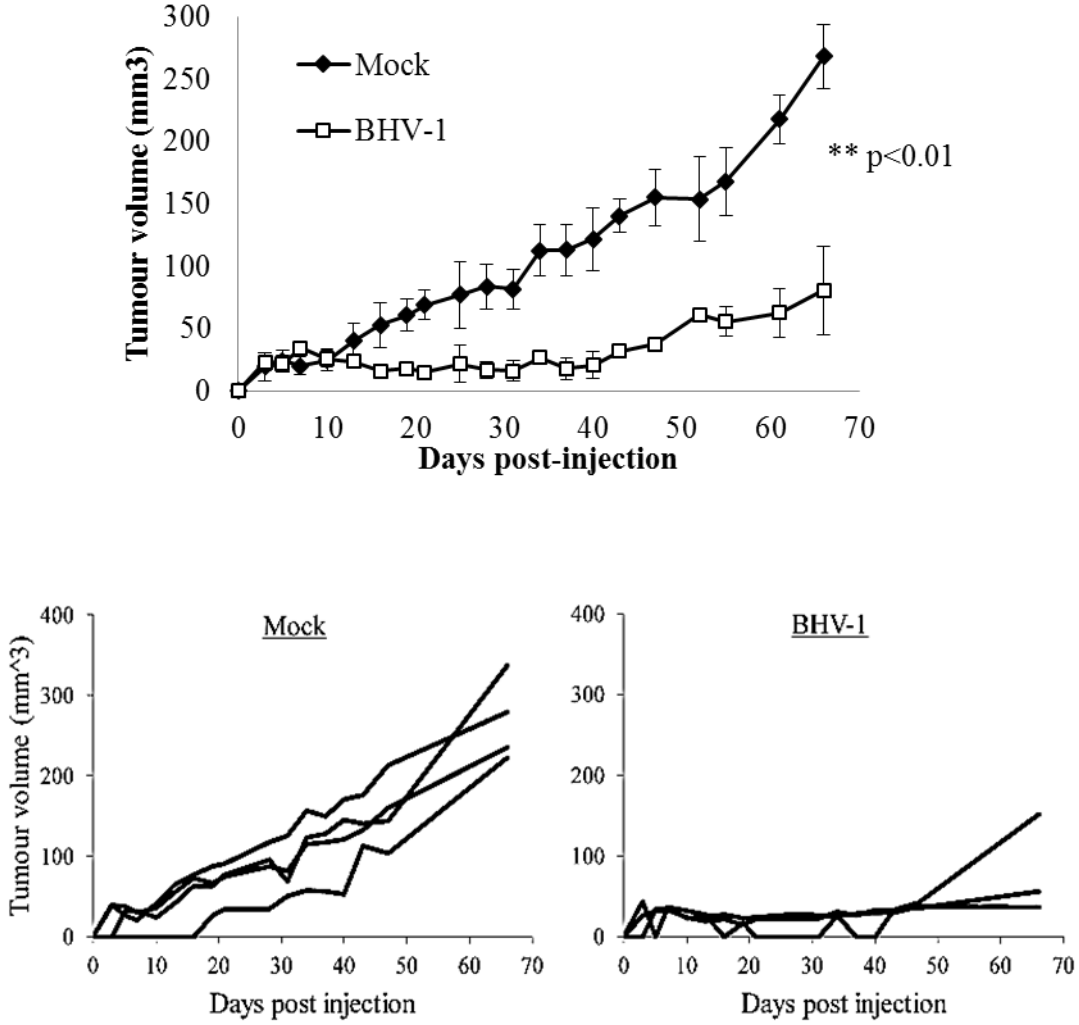

Figure 3.6. BHV-1gfp-infected MCF7 cells have reduced tumor forming ability in vivo. NSG mice were injected with 10^6 mock-infected (n=4) or BHV-1gfp-infected (n=4) MCF7 cells one day following SC injection of 17β-estradiol pellets. Tumour growth was measured twice per week for up to 66 days pi. **, $p<0.01$.

Chapter 4: Combination therapy with 5-Azacytidine and oncolytic bovine herpesvirus type 1 shows improved efficacy compared to monotherapy in an aggressive, tolerized cotton rat model of breast adenocarcinoma

This chapter consists of an author-generated version of the manuscript entitled "Combination therapy with 5-Azacytidine and oncolytic bovine herpesvirus type 1 shows improved efficacy compared to monotherapy in an aggressive, tolerized cotton rat model of breast adenocarcinoma", prepared and submitted to Molecular Therapy in July 2014. As this manuscript is in submission, no copyright permission is required.

For this paper, I performed all cell culture, virus propagation and purification for *in vitro* and *in vivo* studies, viral titers, drug preparation, western blots and measurements of virus replication and cellular viability. Furthermore, I performed Chou-Talalay analysis on *in vitro* combination treatment data and all statistical analysis contained within the manuscript. Analysis of histology data was aided by Dr. Jean-Claude Cutz (McMaster University). Invaluable assistance for cotton rat (CR) experiments was provided by Dr. Meghan Verschoor. All figures, with the exception of figures 5 and 6, were created by myself and I wrote the manuscript. Dr. Karen Mossman and Dr. Meghan Verschoor provided intellectual direction and aided in revising the manuscript.

Context and background information

Based on the observation that BHV-1 can infect and kill human BC cells and BCICs, and that tumor cell death can occur in the absence of a productive infection (Chapter 4), I was interested in evaluating the *in vivo* antitumor efficacy of BHV-1. However, BHV-1 does not efficiently bind and enter murine cells (Hushur, Takashima et al. 2004), ruling out the use of conventional murine models in experiments. Attempts to pseudotype BHV-1 with HSV-1 gB and gD to make entry into murine cells more efficient failed due to an inability to isolate and purify clones, which successfully expressed these two proteins (Hushur, Takashima et al. 2004). Unfortunately, the laboratory which had successfully performed BHV-1 pseudotyping and purification has since disbanded and we were unable to contact any former members.

Syngeneic and tumor-tolerized models represent more relevant models with which to evaluate the pre-clinical efficacy of OVs as they take into account the role of the immune system, which has been shown to play an important role in OVT (Sobol, Boudreau et al. 2011, Workenhe, Simmons et al. 2014). The CR (*Sigmodon hispidus*) is commonly used in anti-BHV-1 vaccination research, as viral-induced pathology is similar to that seen in cattle and infection is immunogenic (Papp, Babiuk et al. 1998). Furthermore, CRs are used as a pre-clinical model to study the antitumor efficacy of oncolytic adenovirus vectors as they are susceptible to infection (Toth, Spencer et al. 2007). Cell lines derived from spontaneous fibrosarcomas of the breast (LCRT) and osteosarcomas of the bone (CCRT and VCRT) allowed for the development of syngeneic tumor models (Prince 1994).

Initially, I evaluated the ability of BHV-1 to initiate replication and induce cellular cytotoxicity in LCRT cells; however, a significant decrease in viability was not observed. Recent studies in BC have shown that combination therapy with OVs has resulted in improved antitumor responses (Chung, Advani et al. 2002, Zeng, Li et al. 2013). I chose to assess the combination of BHV-1 with 5-Aza, a DNMTi, because methylation has been shown to play a role in the development and prognosis of BC (as reviewed in (Byler, Goldgar et al. 2014)). Furthermore, a recent study described improved antitumor responses in a murine model of malignant glioma after combination therapy with an oncolytic herpesvirus vector and 5-Aza (Okemoto, Kasai et al. 2013). First, I determined the safety of combining BHV-1 with 5-Aza in normal primary human cells, as the drug may alter cellular sensitivity to the virus. From these data a maximum tolerated concentration of drug was determined, below which decrease in cellular viability did not occur. Subsequently, to determine whether the combination of BHV-1 with 5-Aza resulted in enhanced LCRT cell death, cells were pretreated with the drug for 14 hours at a range of concentrations and then infected with the virus. The initiation of virus replication and decreases in cellular viability was measured two days pi. I found that pretreatment increased virus replication and significantly decreased tumor cell viability in comparison to either treatment alone. This effect was absent when both treatments were given simultaneously. Furthermore, although a significant increase in viral titers was observed with 5-Aza treatment, this was not considered biologically significant relative to virus input. By generating Chou-Talalay plots and calculating the combination index for

each dose-effect combination using Compu-Syn software, I ascertained that BHV-1 strongly synergizes with 5-Aza to elicit LCRT cell death *in vitro*.

Next, I wanted to determine whether combination therapy with BHV-1 and 5-Aza had improved antitumor efficacy when compared to monotherapy in a CR model of breast adenocarcinoma. Preliminary dose escalation studies were performed to determine the safety and efficacy of BHV-1 monotherapy in CRs. Once tumors reached treatable size, they were injected intratumorally (i.t) once daily for five consecutive days and tumor measurements were taken every other day. In comparison to $5x10^6$ plaque-forming unit (pfu) BHV-1, which was well tolerated but showed no antitumor efficacy, $5x10^7$ pfu BHV-1 caused severe lung pathology which limited long term survival. Given these data and considering that 5-Aza increases BHV-1 replication in LCRT cells, I chose the lower $5x10^6$ pfu BHV-1 dose for combination therapy studies. Once tumors reached treatable size, CRs were treated with either phosphate buffered saline (PBS), 5-Aza (1 dose, 2 mg/kg intraperitoneally (i.p.)), BHV-1 (5 doses, $5x10^6$ pfu i.t.) or 5-Aza plus BHV-1. For the combination therapy group, 5-Aza was delivered i.p one day prior to the first BHV-1 injection. Although I observed a delay in tumor growth, combination therapy did not significantly increase survival. Interestingly, combination therapy significantly decreased the incidence of secondary lesions located in the armpit on the lateral side (with respect to the primary tumor) and the number and grade of lesions in the lungs. During necropsy I observed that the majority of the primary tumor in 38% (3/8) of CRs in the combination therapy group was comprised of a fluid-filled space and not a solid tissue mass. The contents of the fluid was not determined. Histologically, I found that combination therapy

induces increased vascular disruption and tumor cell clearance in comparison to either treatment in isolation.

The use of relevant *in vivo* models are required to properly evaluate the antitumor efficacy of OVs. The use of immunodeficient models, while convenient, fail to take into account the role of the immune system and are therefore less indicative of clinical outcomes (Sobol, Boudreau et al. 2011, Workenhe, Pol et al. 2013). The treatment of metastatic disease remains a major hurdle to successful cancer therapy. The CR model of breast adenocarcinoma is highly aggressive with high rate of metastasis to the lymph nodes and lungs, which is also common in human breast cancer patients (Disibio and French 2008, Berman, Thukral et al. 2013). Overall, combination therapy with BHV-1 and 5-Aza significantly reduced the incidence of metastasis, perhaps through the induction of an antitumor immune response. Further studies are needed to understand the interplay between BHV-1 and 5-Aza; however, these data are encouraging considering that metastatic disease remains a significant obstacle to successful cancer therapy.

Paper: Combination therapy with 5-Azacytidine and oncolytic bovine herpesvirus type 1 shows improved efficacy compared to monotherapy in an aggressive, tolerized cotton rat model of breast adenocarcinoma

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Running Title: 5-Azacytidine synergizes with Bovine herpesvirus type 1

Key words: oncolytic virus, breast cancer therapy, epigenetic modifiers, secondary lesions

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Abstract

Oncolytic viruses selectively replicate in tumor cells by exploiting biochemical differences between normal and tumor cells. Treatment with epigenetic modifiers such as 5-Azacytidine, a DNA methyltransferase inhibitor, has been shown to increase the replication and cytotoxicity of oncolytic viruses *in vivo* and *in vitro*. The cotton rat is an attractive animal to study oncolytic viruses, as syngeneic models of breast adenocarcinoma and osteosarcoma are well established, and many features of primary and secondary tumor growth recapitulate human disease. Treatment of LCRT breast cancer cells with 5-Azacytidine increases BHV-1-mediated cytotoxicity *in vitro*. Chou-Talalay analysis indicates a very strong synergism between BHV-1 and 5-Azacytidine. *In vivo*, BHV-1 monotherapy delayed tumor growth but did not improve survival of cotton rats with subcutaneous breast adenocarcinomas. BHV-1 and 5-Azacytidine combination therapy decreased the number of secondary lesions, with enhanced tumor cell clearance compared to BHV-1 monotherapy. Overall, these studies suggest that combination therapy improves therapeutic efficacy compared to oncolytic viral montherapy in cotton rats with subcutaneous breast adenocarcinomas and significantly decreases the incidence of secondary lesions. Together, these results warrant further investigation of BHV-1 combination therapy with epigenetic modifiers for the treatment of breast cancer, particularly in the context of the prevention and treatment of secondary lesions.

Introduction

Oncolytic viruses (OV) selectively replicate in tumor cells by exploiting the biochemical differences between normal and tumor cells [1,2]. OVs can elicit tumor cell death directly through lysis or by stimulating an antitumor immune response. The efficacy of oncolytic herpes simplex virus type 1 (oHSV-1) has been well characterized in preclinical and clinical studies [3]. Although the induction of antitumor immunity has been demonstrated following intratumoral (i.t) administration of oHSV-1 [4,5], systemic delivery will be required for treating metastatic lesions. However, the high incidence of pre-existing immunity to HSV-1 may limit systemic delivery of the virus. Such obstacles warrant the development of non-human viruses for OV therapy (OVT).

Bovine herpesvirus type 1 (BHV-1) is a member of the *Herpesviridae* family that initiates bovine respiratory disease complex in cattle through transient immunosuppression [6]. Despite the prevalence of BHV-1 in cattle, no human infections or sero-conversions have been reported [6]. Normal human cells are susceptible to BHV-1 binding and entry, but are not permissive for BHV-1 replication [7]. In contrast, human immortalized, transformed and tumor-initiating cells are permissive to infection [7,8]. Interestingly, the ability of BHV-1 to kill human bulk and tumor-initiating breast cancer cells does not depend on the initiation of virus replication or the production of a viral burst [8]. However, BHV-1 does not efficiently bind and enter murine cells [9], making the use of conventional mouse models problematic.

The cotton rat (CR; *Sigmodon hispidus*) is commonly used in anti-BHV-1 vaccination research, as viral-induced pathology resembles that seen in cattle [10]. BHV-1 infection of

CRs is immunogenic, inducing mucosal and systemic immune responses, particularly following intranasal inoculation [10]. The CR is an attractive syngeneic, immune competent model to study OVs, as cell lines derived from spontaneous fibrosarcomas of the breast (LCRT) and osteosarcomas of the bone (CCRT and VCRT) have been developed [11]. CRs are used to evaluate the antitumor efficacy of oncolytic adenoviruses, including neutralization studies to predict human responses to intravenous (i.v) injection [12].

Antitumor responses from OVT differ significantly when comparing tolerized and non-tolerized tumor-associated antigen (TAA) models. Central and peripheral tolerance can dampen TAA-specific cytotoxic lymphocyte responses, resulting in poor therapeutic outcomes [13,14]. TAA-specific CD8+ T cells, essential for mediating tumour regression, are produced in non-tolerized syngeneic tumour models, but not in tolerized tumour models [14,15]. These observations highlight the importance of evaluating OVs using tolerized animal models, which better recapitulate the human immune landscape, allowing for enhanced understanding of the features that determine therapeutic success.

Aberrant DNA methylation events frequently occur in cancer and include global DNA hypomethylation and CpG island hypermethylation, leading to the silencing of tumour-suppressor genes or the expression of oncogenes [16]. Recent studies have used DNA methyltransferase inhibitors (DNMTi) such as 5-Azacytidine (5-Aza) to study the role of methylation in the development and prognosis of breast cancer [17,18]. While treatment of breast cancer cells with 5-Aza induced differential expression of tumor suppressor genes and oncogenes, it was unable to induce tumor cell death on its own [19,20]. OVs have been combined with chemo and radiotherapy to exploit differences in the mechanism of tumor

cell death elicited by each treatment, resulting in enhanced anti-tumour responses [3,21,22]. Therefore, combining 5-Aza with agents that increase tumor cell death by counteracting the pro-survival effects of oncogenes may be efficacious [20,23].

Results

BHV-1 replication and cytotoxicity in LCRT cells.

BHV-1 is able to initiate replication and/or induce cellular cytotoxicity in human breast cancer cells from a variety of subtypes [8]. The cytotoxicity of BHV-1 on the CR breast carcinoma cell line LCRT was evaluated. Cells were infected with BHV-1 at a range of MOIs and the initiation of replication, ascertained by visualizing GFP fluorescence, and cellular viability were analyzed 2 days pi. The expression of GFP fluorescence indicates the initiation of BHV-1 replication, not full permissivity to the virus. Henceforth, we will refer to the initiation of BHV-1 replication, indicated by GFP fluorescence, as virus replication. Induction of BHV-1 replication in LCRT cells was observed at MOIs greater than 2.5 (Fig. 1a). However, a significant decrease in cellular viability, defined as a decrease greater than 20%, did not occur at any of the MOIs examined (Fig. 1b).

5-Aza does not alter the permissivity of normal primary cells to BHV-1 infection.

As normal primary CR cell lines do not exist, normal primary human cell lines were used to test 5-Aza toxicity. While BHV-1 is unable to induce cytotoxicity in normal human cells [7], epigenetic modifiers may alter cellular sensitivity to the virus. The IC_{50} values of 5-Aza were 18 μM and 3.3 μM on normal human Ventressca and HEL fibroblasts, respectively (Data not shown). Treatment with 3 or 10 μM 5-Aza did not increase BHV-1 replication, as determined using measures of GFP fluorescence, on normal human Ventressca or HEL fibroblasts (Data not shown). A significant increase in cytotoxicity in Ventressca cells was observed when 5-Aza (10 μM) was combined with BHV-1 (MOI 3 or 5) in comparison to BHV-1 only infected samples (Fig. 2a). This effect was also

detected in HEL cells when 5-Aza (10μM) was combined with BHV-1 (MOI 1 and 5) (Fig. 2b). However, the combination of 5-Aza with BHV-1 did not increase cytotoxicity compared to that seen with 5-Aza alone in either cell line (Fig. 2). Therefore, only concentrations below the IC50 value for HEL were used in further experiments.

BHV-1 and 5-Aza act synergistically to kill LCRT cells.

Studies combining OVs with epigenetic modifiers such as 5-Aza have shown synergistic or additive effects that enhance tumor cell death [23-25]. To make sure that 5-Aza was functional in LCRT cells, the expression of Dmnt1 was evaluated as a control. When incorporated into DNA, 5-Aza forms a covalent bond with DMNTs, such as Dmnt1 [26]. This bond is irreversible and results in degradation of Dmnt1, reducing cellular levels of the enzyme [26]. Western blot analysis indicated that 14 hour treatment with 1 and 3 μM of 5-Aza is sufficient to reduce Dmnt1 expression in LCRT cells (Fig. 3a). These data verify 5-Aza activity in LCRT cells. We then determined whether 5-Aza was able to enhance the replication or cytotoxicity of BHV-1. LCRT cells were treated with 5-Aza at 0.5, 1 or 3 μM for 14 hours and subsequently infected with BHV-1 at MOI 3 or 5. Regardless of the concentration, treatment with 5-Aza increased initiation of BHV-1 replication (Fig. 3b). Likewise, combination treatment was more effective in reducing cellular viability, with 3 μM 5-Aza significantly reducing cellular viability to 60% and 45% of untreated cells following infection with BHV-1 at MOI 3 or 5, respectively (Fig. 3c). For reference, using this assay we routinely observed a decrease to 20% cellular viability in MDBK cells (BHV-1 MOI 3), which are fully permissive to BHV-1 infection (Data not shown).

To evaluate whether the interaction between BHV-1 and 5-Aza is synergistic or additive, we generated Chou-Talalay plots using CompuSyn software (Data not shown) [27]. The combination index (CI) for each treatment was calculated and the dose-effect combinations (cellular viability) of 5-Aza and BHV-1 on LCRT were determined. Table 1 shows that there is very strong synergism (CI < 1) between 5-Aza and BHV-1 regardless of the concentration of 5-Aza or MOI of virus used. These results suggest that BHV-1 and 5-Aza act synergistically to kill LCRT cells.

5-Aza increases de novo production of BHV-1 in LCRT cells.

To determine whether increased GFP expression is indicative of productive virus replication, the viral burst was determined. LCRT cells were treated with 0, 1 or 3 µM 5-Aza and subsequently infected with BHV-1 at MOI 5. Cells and supernatant were collected 1, 2 and 3 days pi and virus titrated on naïve MDBK cells. A minimal viral burst was detected in untreated samples at the time points examined (Fig. 4). However, a statistically significant increase in viral titers was detected 2 days pi between untreated cells and cells treated with 1 and 3 µM 5-Aza (Fig. 4). Although the increase in virus output with 5-Aza treatment was statistically significant, this increase is not considered biologically significant relative to virus input. For reference, we routinely observe a viral burst of 400 pfu/cell 2 days pi in MDBK cells (BHV-1 MOI 3), which are fully permissive to BHV-1 infection. Additionally, the apparent drop in virus output with 3 µM 5-Aza at 3 dpi is likely due to cellular cytotoxicity induced by the combination therapy at these concentrations (Fig. 3c).

BHV-1 monotherapy does not increase the survival of cotton rats bearing subcutaneous LCRT tumors.

Recent studies have shown that *in vitro* assays do not always predict *in vivo* outcomes [15,28]. Tumors are complex organs that employ a multitude of mechanisms to influence tumor cell survival, proliferation and spread. These mechanisms impact the success of OVT by affecting virus replication, spread and recruitment of the immune system to the tumor microenvironment [29,30]. As a major barrier to effective OVT is central and peripheral tolerance, we evaluated whether BHV-1 possesses antitumor capabilities in a tolerized CR model of breast carcinoma. The CR LCRT model is extremely aggressive; PBS treated tumors reached endpoint within 10 days on average. Tumor growth was highly variable with increases in volume from the beginning of treatment to endpoint varying between 11 and 30 fold (PBS controls).

Preliminary dose-escalation studies were performed to investigate the safety and efficacy of BHV-1 in CRs bearing subcutaneous LCRT tumors. Tumors were treated i.t. with $5x10^6$ or $5x10^7$ pfu BHV-1 once daily for five days and monitored for tumor growth and survival. No survival advantage or tumor regression was observed in animals treated with $5x10^6$ pfu BHV-1 (Fig. 5). The $5x10^6$ pfu BHV-1 dose was well tolerated with no adverse effects observed. Hemorrhagic centers that turned necrotic appeared on large tumors several days post treatment (Fig. 1Sb); however, this was not exclusive to the BHV-1 group, suggesting that this phenomenon may be associated with tumor size.

Animals treated with $5x10^7$ pfu BHV-1 displayed significantly increased survival (Fig. 2), however, all animals reached endpoint due to respiratory distress or tumour burden.

Histologically, the lungs contained multiple high-grade tumors that were mostly found around the bronchioles and in the pleura (Fig. 3Sc,d). Diffuse alveolar damage and pulmonary hemorrhage was also evident. Extensive damage and oedema in the lungs, in conjunction with the secondary lesions in the armpit, significantly contributed to respiratory distress. Pathological analysis suggested that the CRs developed lymphangitic carcinomatosis, which is common in breast adenocarcinoma and is caused by dissemination of tumor cells through the lymphatics in the lung [31]. The absence of significant immune cell infiltration suggests that lung pathology was not due directly to BHV-1 infection, but was caused by tumor invasion and growth. Furthermore, hemorrhagic centers formed on large tumors, and severe ulceration was common in BHV-1 treated tumors that eventually turned necrotic (Fig. 1Sc).

BHV-1 and 5-Aza combination therapy improved therapeutic efficacy and decreased the incidence of secondary lesions.

Although we observed a synergistic effect between 5-Aza and BHV-1 that enhanced cytotoxicity in LCRT cells *in vitro*, we wanted to determine whether this effect was maintained *in vivo*. Due to the extensive lung pathology observed in CRs treated with $5x10^7$ pfu BHV-1 and given that 5-Aza increases BHV-1 replication in vitro, we chose the lower $5x10^6$ pfu BHV-1 dose for combination therapy studies. As BHV-1 monotherapy ($5x10^6$ pfu) did not significantly increase CR survival, we wanted to determine the differences in the antitumor effect between mono and combination therapy with BHV-1 with 5-Aza. Approximately two weeks post subcutaneous implantation of LCRT cells, CRs were treated with either PBS, 5-Aza (1 dose, 2 mg/kg i.p.), BHV-1 (5

doses, $5x10^6$ pfu i.t.) or 5-Aza plus BHV-1. For the combination therapy group, 5-Aza was delivered one day prior to commencing daily BHV-1 injections. Combination therapy delayed tumor growth in 38% (3/8) of animals, but did not significantly increase survival compared to BHV-1 treated controls (Fig. 6). However, it is important to note that unlike murine models for which tumor growth is relatively uniform, these tumors were highly varied and the conventional method of taking measurements (length and width by caliper measurement) did not provide an accurate means by which to estimate tumor volume. During necropsy procedures, we found that 38% (3/8) of primary tumors harvested from CRs treated with combination therapy (indicated by an * in tumor volume graphs in Fig. 6) were mainly comprised of fluid filled space and not solid tumour mass (Fig. 4S).

LCRT tumors grow quickly and are very invasive with a high probability of developing lung and lymph lesions [11,32]. In this study, secondary lesions were found in the armpit on the lateral side in all CRs in the BHV-1 (3/3) and 5-Aza (4/4) monotherapy groups. The incidence of secondary lesions in the armpit was significantly lower ($p=0.03$) in combination therapy treated CRs, in which lesions were detected in only 38% (3/8) of animals. In addition, secondary lesions were also frequently detected posterior to the primary tumor. The incidence of these lesions was 67% in BHV-1 (2/3) and 75% in 5-Aza (3/4) treated CRs, in comparison to only 13% (1/8) in combination therapy treated animals, although these differences were not statistically significant ($p=0.06$). Furthermore, lesions found in the lungs of combination treated CRs were fewer and of lower grade when compared to those in the lungs of CRs treated with BHV-1 alone (Fig.

3Se,f). Importantly, we did not detect any respiratory distress in these animals that limited survival.

Combination therapy with BHV-1 and 5-Aza induces vascular destruction and tumor cell clearance in subcutaneous LCRT cotton rat tumors.

Recent studies have described pro- and anti-angiogenic effects from OVT, depending on the model and OV used. oHSV-1 has been shown to infect and lyse endothelial cells and to alter the expression of angiogenic factors to increase or decrease microvessel density [33,34]. Here, tumors were harvested from non-responders from BHV-1 and 5-Aza treatment groups and responders from the combination therapy group at endpoint for histological analysis. A responder was defined as an animal that had slower tumor growth in comparison to monotherapy controls (5-Aza or BHV-1), had increased survival, or for which a fluid filled space was present in the primary tumor at endpoint. It should be noted that the histology for the PBS treated control was obtained from a previous experiment, but is representative of this group. Histologically, CRs treated with combination therapy had large areas of tumor cell clearance that were more extensive in comparison to the tumors of animals treated with BHV-1 monotherapy (Fig. 5S). Interestingly, we observed a comparable amount of vascular disruption in CRs treated with combination therapy relative to BHV-1 treated controls (Fig. 5Sc), despite observing fewer secondary lesions in animals treated with combination therapy. Together these data suggest that 5-Aza may sensitize LCRT cells to BHV-1-mediated oncolysis and decrease the rate of secondary lesions by this or another mechanism.

Discussion

Clinical studies have demonstrated that OVs are an effective and novel cancer therapeutic with unique tumor-targeting mechanisms. However, it has become apparent that a combination therapy approach will be necessary to achieve sustained antitumor responses. Current efforts focused on understanding OV-host interactions have revealed novel agents for combination therapy [35]. Epigenetic modifiers enhance OV replication and cytotoxicity by modifying viral gene expression and antiviral immune responses [23-25]. In addition to upregulating genes associated with innate and adaptive immunity, 5-Aza increases the sensitivity of tumor cells to T cell mediated cytotoxicity by increasing expression of TAAs [36,37]. Enhanced immune activation following 5-Aza treatment may serve to break central and peripheral tolerance to TAAs [36,38]. Furthermore, clinical data support the use of epigenetic modifiers, such as 5-Aza, in the treatment of breast cancer as they improve patient responses to therapy [17,18,39].

In this study, we showed that BHV-1 infection was inefficient in LCRT cells and a significant decrease in cellular viability was not observed. However, when cells were treated with 5-Aza prior to BHV-1 infection, we detected a 55% decrease in cellular viability (Fig. 3c). This coincided with an increase in virus replication and bovine infected cell protein 0 (bICP0) expression (Fig. 3b and Data not shown), but only a modest increase in viral titers (Fig. 4). We have previously reported that BHV-1 is able to induce cytotoxicity in human breast cancer cells in the absence of a productive infection [8]. These data suggest that the presence of a fully permissive cellular environment is not required for BHV-1 to elicit oncolytic activity.

Animal models play an important role in the pre-clinical evaluation of OVs. However, the relevancy of these models has come under scrutiny as their ability to predict the anticancer efficacy of OVT has been limited [3]. Antitumor responses from OVT differ significantly when comparing tolerized and non-tolerized TAAs [13,14,28]. Although we did not achieve complete responses to BHV-1 mono or combination therapy, by using a tolerized model of breast adenocarcinoma we were able to evaluate the oncolytic capacity of BHV-1 in the presence of natural local and systemic immunosuppression. This better recapitulates the human immune landscape and is therefore a more accurate predictor of therapeutic efficacy. In contrast to small RNA OVs for which intratumoral viral replication and spread determines the success of therapy [40], this does not seem to be the case for large DNA viruses such as HSV-1, where the involvement of the host immune system plays a critical role in antitumor efficacy [14,15,28]. We observed an increase in BHV-1 replication in vitro following treatment of LCRT cells with 5-Aza; however, it seems unlikely that combination therapy functions via increased BHV-1 replication in vivo given that combination treatment (5-Aza and BHV-1 $5x10^6$ pfu) did not result in the extensive pathology seen with $5x10^7$ pfu BHV-1, despite the permissivity of CRs to BHV-1 infection [11]. These data are consistent with reports that direct virus-mediated effects do not dictate the success of OVT using herpesvirus vectors [14,15,28]. Studies from our group have shown that HSV vectors with the greatest oncolytic effect in multiple murine models of breast carcinoma were those which had a lower viral burst and were rapidly cleared from the tumor [15]. Treatment with 5-Aza may increase permissivity of LCRT cells to BHV-1 by dampening the antiviral immune response, resulting in

increased virus replication and cytotoxicity. However, as a significant increase in the viral burst was not observed it seems likely that combination therapy with 5-Aza elicits its *in vivo* effects by modulating the host antitumor immune response as is also the case for several other HSV vectors [15]. Unfortunately, due to the sensitivity of CRs to anesthetics we were unable to use in vivo imaging techniques such as the Spectrum IVIS system to visualize the biodistribution of BHV-1.

The treatment of secondary lesions remains a major hurdle to successful cancer therapy. The induction of self-perpetuating and sustained systemic antitumoral protection is important in the treatment of non-resectable disease [41-43]. LCRT tumors grow quickly and are very invasive with a high probability of developing lung and lymph lesions [11,32]. High incidence of secondary lesions to the lymph nodes and lungs is also common in human breast cancer patients [44,45]. Treatment of CRs with $5x10^7$ pfu BHV-1 caused regression of the primary tumor in 40% (4/10) of animals and significantly increased survival (Fig. 2S). However, this dose was not well tolerated and long-term survival was not achieved as animals reached endpoint due to respiratory distress caused by significant lesions in the lungs. In contrast, CRs treated with combination therapy (5-Aza and BHV-1 $5x10^6$ pfu) showed a significant decrease in the number of secondary lesions in the armpit ($p=0.03$). Both the number and grade of tumors in the lungs of combination therapy treated animals was decreased relative to BHV-1 monotherapy treated CRs (Fig. 3Sa,b,e,f). These data suggest that combination therapy may induce antitumor systemic immune responses to limit the formation of secondary lesions. Moreover, these data highlight the recurring

theme that, at least for herpesvirus based OVs, increased virus replication does not equate with increased efficacy.

The disruption of epigenetic processes during virus infection can lead to altered cellular and viral gene expression and function which influence cell status [46,47]. Herpesviruses have been shown to induce global cellular epigenetic modification using virus encoded proteins and miRNA species which regulate virus replication and pathogenesis [48,49]. Recent studies have described using epigenetic modifiers such as 5-Aza in combination with OVs to improve antitumor responses [23]. It is plausible that epigenetic reprogramming by 5-Aza may synergize with that induced by BHV-1 to alter susceptibility of tumor cells to BHV-1 infection and modulate immune mediated antitumor effects. Further studies are required to unravel the relationship between BHV-1 and 5-Aza. How 5-Aza modulates the antiviral response and/or the tumor microenvironment to enhance therapeutic efficacy remains to be elucidated. Understanding the mechanism by which 5-Aza modulates BHV-1 infection can ultimately be exploited to inform further development of novel combination therapy strategies.

Material and Methods

Cell Lines

Cell lines were maintained at 37ºC + 5% CO_2 in medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. HeLa and human embryonic lung (HEL) cells (American Type Culture Collection [ATCC], Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The CR cell line LCRT was obtained from Dr. Ann Tollefson (St. Louis University) and were cultured in DMEM supplemented with 10% FBS. Madin-Darby bovine kidney (MDBK) cells were obtained from Vikram Misra (University of Saskatchewan) and were maintained in DMEM supplemented with 5% horse serum. Ventressca (primary human adult lung fibroblasts) were obtained from Dr. Jack Gauldie (McMaster University) and were maintained in MEM/F15 with 15% FBS.

Viruses

BHV-1 expressing GFP was a kind gift from Dr. Günther Keil (Friedrich-Loeffler-Institut, Germany) and was propagated and titrated on MDBK cells. Virus preparations were sucrose cushion purified [8]. Purified virus was resuspended in phosphate buffered saline (PBS) and stored at -80ºC.

Drug Preparation

5-Aza (Sigma-Aldrich, St. Louis, MO) stock powder was stored at -20°C and dissolved in complete DMEM to obtain a working solution. Drug was freshly prepared for each experiment.

Western Blot Analysis

Cells were treated with 1 or 3 µM 5-Aza for 14 hours 24 and whole cell lysates were immediately collected (Dmnt1 blots). Whole cell lysates were collected in 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, St. Louis, MO]) and lysed on ice for 30 min. Lysates were centrifuged at 1,000 rpm for 10 min at 4ºC and the supernatants were collected. Protein was quantified using a Bradford assay kit (Bio-Rad Laboratories, Mississauga, ON). Whole cell extracts were boiled in sample buffer containing sodium dodecyl sulfate (SDS) and β-mercaptoethanol and run on a 7.5% for Dmnt1 expression analysis. Gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) with a wet transfer apparatus at 100 V for 1 hour. All blots were blocked in 5% non-fat milk in Tris-buffered saline (TBS) at room temperature for 2 hours. Blots were probed with primary antibodies specific for Dmnt1 ((C-17); 1:400, Santa Cruz Biotechnology, Dallas, TX) diluted in TBS-Tween (0.1%), overnight at 4ºC. Blots were probed with anti-goat secondary antibodies conjugated to horseradish peroxidise (HRP; Sigma) diluted 1:2000 in 5% non-fat milk in 0.1% TBS-Tween. Blots were visualized by chemiluminescence.

Measurement of Virus Replication and Cellular Viability

LCRT cells ($1x10^5$ cells/mL) were seeded into 96-well plates and treated for 14 hours 24 with 1 or 3 µM 5-Aza prior to infection with BHV-1 at MOI 3 or 5 for 1 hour at 37ºC. Two days post infection (pi) plates were scanned on a Typhoon BioAnalyzer (GE

Healthcare, Piscataway, NJ) to visualize virus replication as a function of GFP fluorescence. Two days pi cellular viability was assessed using the SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). Measures of cellular metabolism were assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO). Cells were incubated with MTT (10% v/v) for 4 hours at 37^{o}C, after which fluorescence was read. Samples were analyzed in triplicate with a total of three independent experiments performed. Data were analyzed relative to uninfected controls and corrected for background fluorescence. The combination index (CI) for each concentration of 5-Aza with each MOI of BHV-1 was calculated using CompuSyn software (ComboSyn Inc., Paramus, NJ) and used to evaluate pharmacological synergy.

Determination of IC_{50} Values on Normal Primary Cells

Human normal primary cells ($1x10^5$ cells/mL) were seeded into 96-well plates and treated for 14 hours 24 with 0, 0.25, 0.5, 1, 3, 5, 7 or 10 µM 5-Aza. Two days pi cellular viability was assessed using the SpectraMax i3 Multi-Mode Microplate Reader. For both cell lines survival was measured relative to untreated controls. Median-effect plots were generated using CompuSyn software to determine the IC_{50} values of 5-Aza.

Viral Burst

LCRT cells were seeded into 6-well plates and treated for 14 hours 24 with 1 or 3 µM 5-Aza prior to infection with BHV-1 at MOI 3 or 5 for 1 hour at 37^{o}C. One, two and three days pi viral supernatants and infected cells were collected. Samples were freeze/thawed three times and sonicated for 1 min prior to centrifugation at 1,000 rpm for 10 min at 4^{o}C.

Supernatant was collected and titrated by serial dilution in serum-free DMEM. Dilutions were applied to MDBK cells for 1 hour at 37°C. MDBK monolayers were maintained in DMEM supplemented with 0.5% horse serum in 1% methylcellulose. At 2 days pi cells were scanned on a Typhoon BioAnalyzer (GE Healthcare, Piscataway, NJ) and plaque-forming units (pfu) were counted and viral burst calculated.

Cotton Rat *in vivo* experiments

Cotton Rats were maintained at the McMaster University Central Animal Facility and all the procedures were performed in full compliance with the Canadian Council on Animal Care and approved by the Animal Research Ethics Board of McMaster University. Six to seven week old CRs were subcutaneously implanted with $5x10^5$ LCRT cells. Tumors reached treatable size within two weeks post injection. Tumors were treated by injecting $5x10^6$ or $5x10^7$ total pfu BHV-1 [14,28] (50 µL total) once daily for five consecutive days, or $5x10^6$ total pfu BHV-1 (50 µL total) for five consecutive days one day following a single intraperitoneal (i.p) injection of 5-Aza (2 mg/kg) [50]. Tumors were measured every two days and fold changes in tumor volume were calculated relative to the volume at the start of treatment (d=0). Animals were considered to be at endpoint when tumors reached 10% of their total body weight, tumor ulceration occurred in non-BHV-1 treated animals or when breathing difficulties were observed due to metastases. Tumors were resected from animals at endpoint and fixed in 10% neutral buffered formalin for 2-5 days depending on the size of the tumor (5 days for large tumors) and transferred to 70% ethanol for preservation. Tumor tissue was embedded in paraffin and 4 µm sections were prepared.

Sections were stained with hematoxylin and eosin (H&E) and subsequently analyzed using a Leica DM IRE2 microscope (light source EXFO X-Cite 120).

Statistical Analysis

One way ANOVA was used to analyze the significance of the differences in viral burst with a Bonferroni post-hoc test to compare the pairs of data within the distribution. The log-rank Mantel-Cox test was used to determine statistical significance for the difference in Kaplan-Meier survival between treatments. The chi-squared test was used to determine the statistical significance of the incidence in secondary lesions between treatments. The null hypothesis was rejected for p-values less than 0.05. Survival analysis was carried out using GraphPad Prism (LaJolla, CA, USA) and all other analysis were performed using Microsoft Excel (Redmond, WA, USA).

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Dose 5-Aza (μM)	Dose BHV-1 (MOI)	Effect	CI
0.5	1.0	0.87685	0.17848
0.5	3.0	0.80107	0.06363
0.5	5.0	0.56613	0.00338
1.0	1.0	0.74678	0.02872
1.0	3.0	0.79427	0.07894
1.0	5.0	0.56373	0.00503
3.0	1.0	0.59519	0.01460
3.0	3.0	0.60882	0.01785
3.0	5.0	0.43979	0.00343

Table 4.1. Combination Index for BHV-1 with 5-Aza on LCRT cells. Abbreviations: 5-Aza, 5-Azacytidine; MOI, multiplicity of infection; CI, Combination index. Data were collected in triplicate and expressed as means, n=3. Effect represents cellular viability relative to untreated controls. CI values were calculated using CompuSyn software (Version 1; Cambridge, MA). Synergism (CI < 1), antagonism (CI = 1), additive effect (CI > 1).

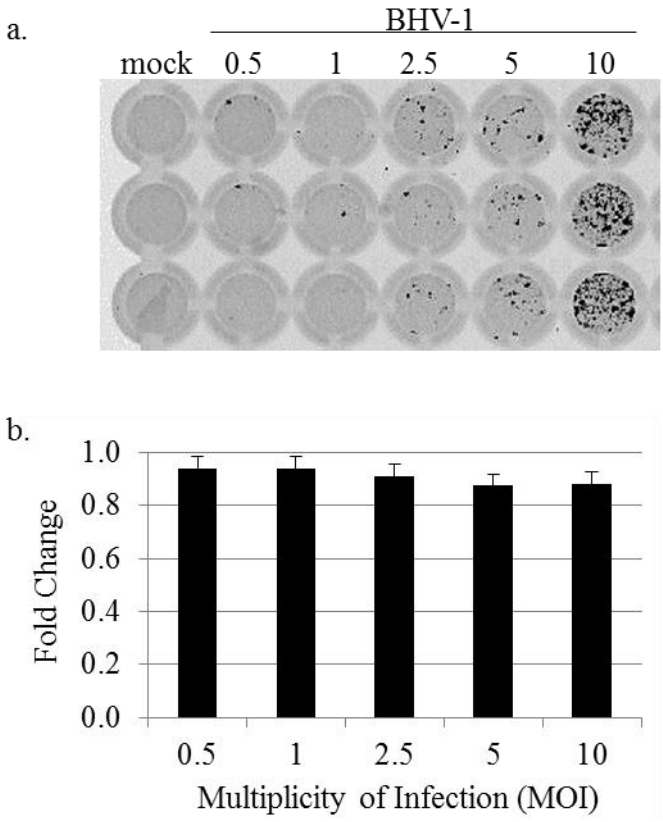
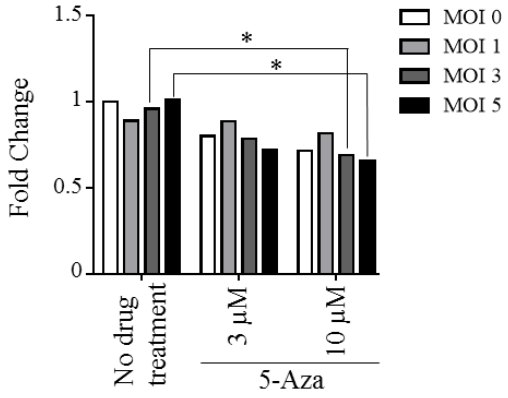


Figure 4.1. BHV-1 replication and cytotoxicity on LCRT. LCRT cells in 96-well plates were mock or infected with BHV-1 at the indicated MOIs for 1 hour at 37°C. **(a)** GFP expression, as a marker of virus replication, was detected using a Typhoon Bioanalyzer (Amersham Biosciences, Piscataway, NJ) at 2 days pi. **(b)** Cell metabolism, as a measure of cell viability, was assessed using MTT at 2 days pi. Fluorescence was detected using a SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) and the fold change in fluorescence relative to untreated, uninfected controls was calculated. Data were collected in triplicate and are represented as the mean.

a.



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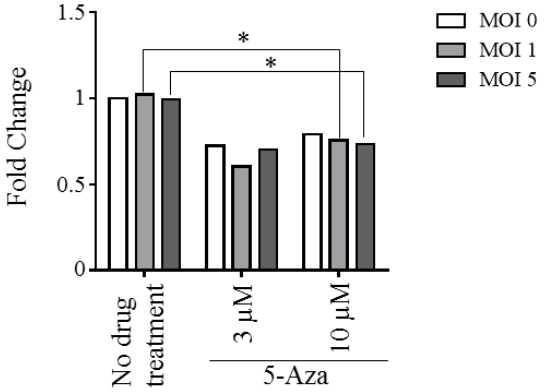


Figure 4.2. BHV-1-mediated cytotoxicity with and without 5-Aza treatment on normal primary human cell lines Ventressca and HEL. (a) Ventressca or **(b)** HEL cells were seeded into 96-well plates and were mock or infected with BHV-1 at the indicated MOIs for 1 hour at 37°C. Cell metabolism, as a measure of cell viability, was assessed using MTT at 2 days pi. Fluorescence was detected using a SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) and the fold change in fluorescence relative to untreated, uninfected controls was calculated. Data were collected in triplicate and are represented as the mean, $n = 2$. * $p<0.05$

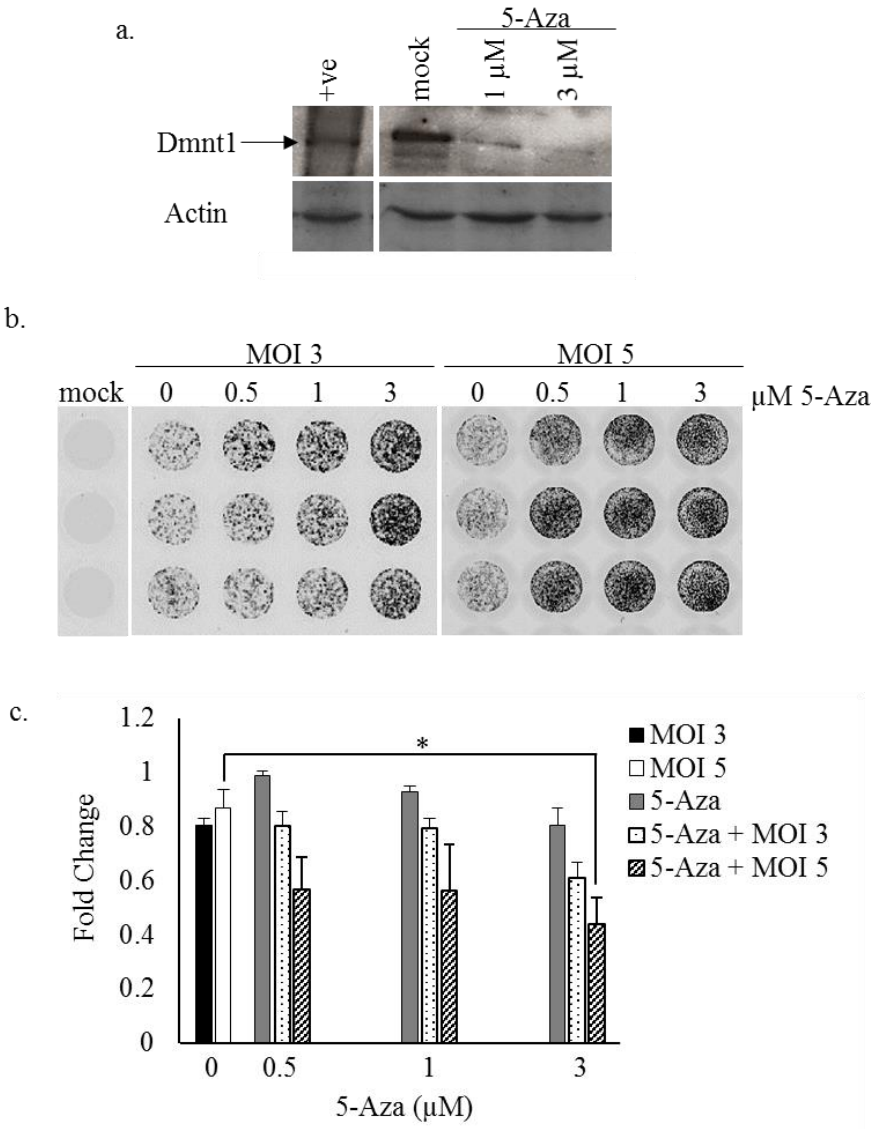


Figure 4.3. 5-Aza enhances BHV-1 replication and cytotoxicity on LCRT. (a) LCRT cells were treated with 5-Aza at 1 or 3 µM. After 14 hours, whole cell lysates were harvested for Western blot analysis with Dnmt1 primary antibody. Actin served as a loading control. Postive (+ve) control HeLa whole cell lysate. LCRT cells in 96-well plates were treated with 5-Aza at 0.5, 1 or 3 µM for 14 hours, then mock or infected with

BHV-1 at MOI 3 or 5 for 1 hour at 37°C. **(b)** GFP expression, as a marker of virus replication, was detected using a Typhoon Bioanalyzer (Amersham Biosciences, Piscataway, NJ) 2 days pi and **(c)** cell metabolism, as a measure of cell viability, was assessed using MTT 2 days pi. Fluorescence was detected using a SpectraMax i3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA) and the fold change in fluorescence relative to untreated, uninfected controls was calculated. Error bars represent mean + SEM, n=3. *$p<0.05$

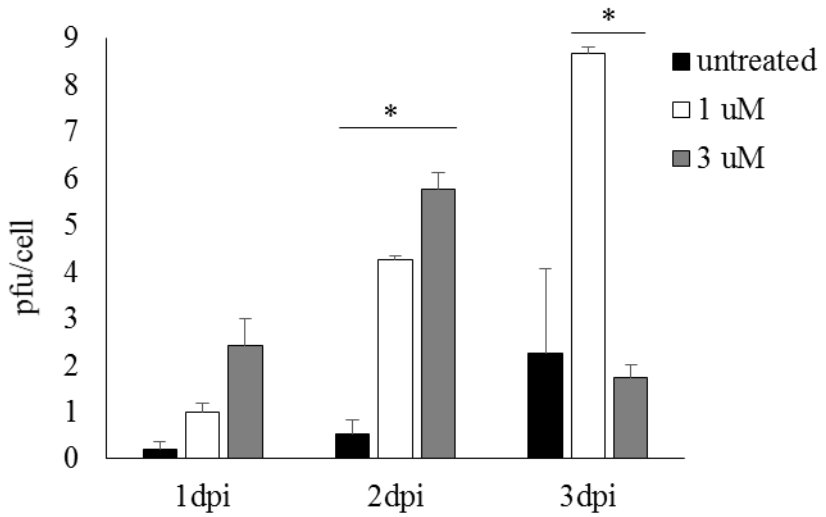


Figure 4.4. BHV-1 viral burst increases with 5-Aza treatment in LCRT cells. Cells were infected with BHV-1 at MOI 5 for 1 hour at 37ºC. Triplicate samples of viral supernatants and cell-associated virus particles were collected one, two and three days pi and titrated on naïve MDBK monolayers. Error bars represent mean + SEM, n=3. *$p<0.05$

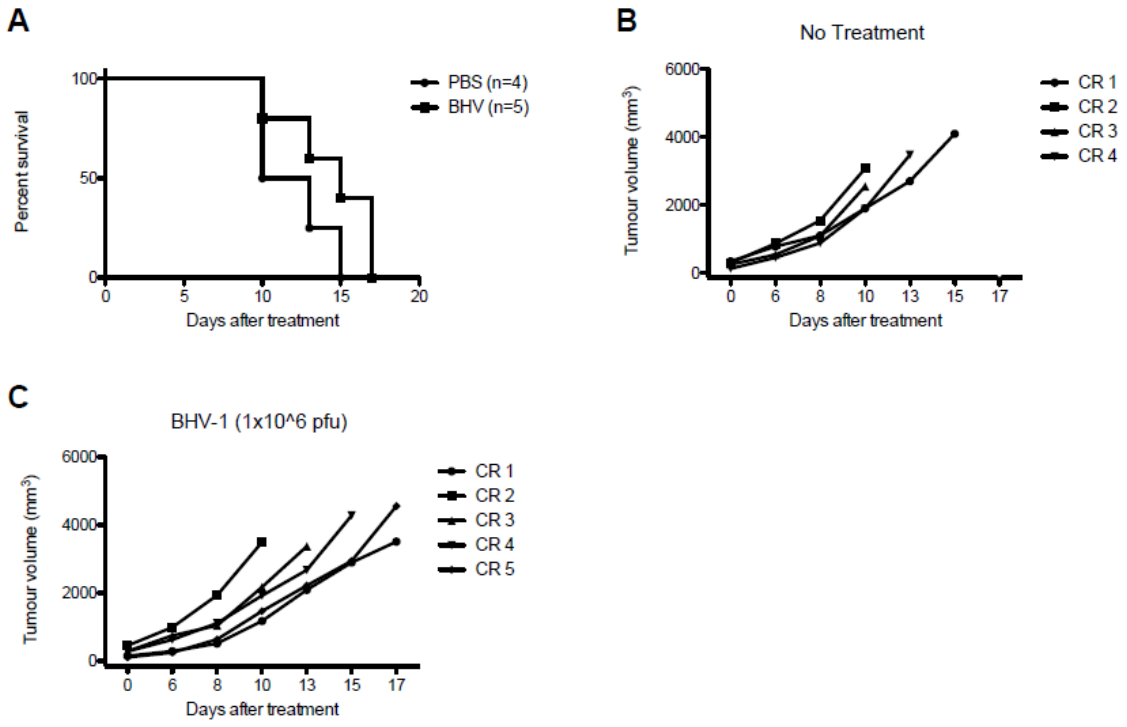


Figure 4.5. Kaplan-Meier survival and tumor volumes for cotton rats treated with $5x10^6$ pfu BHV-1. $5x10^5$ LCRT cells were implanted into cotton rats by subcutaneous injection. When tumors reached treatable size they were treated with $5x10^6$ pfu BHV-1 intratumorally, one dose daily for 5 days. Tumors were measured every two days until animals reached end point. **(a)** Kaplan-Meier estimates of survival and **(b)** tumor volumes of cotton rats treated with PBS and **(c)** $5x10^6$ pfu BHV-1 are shown.

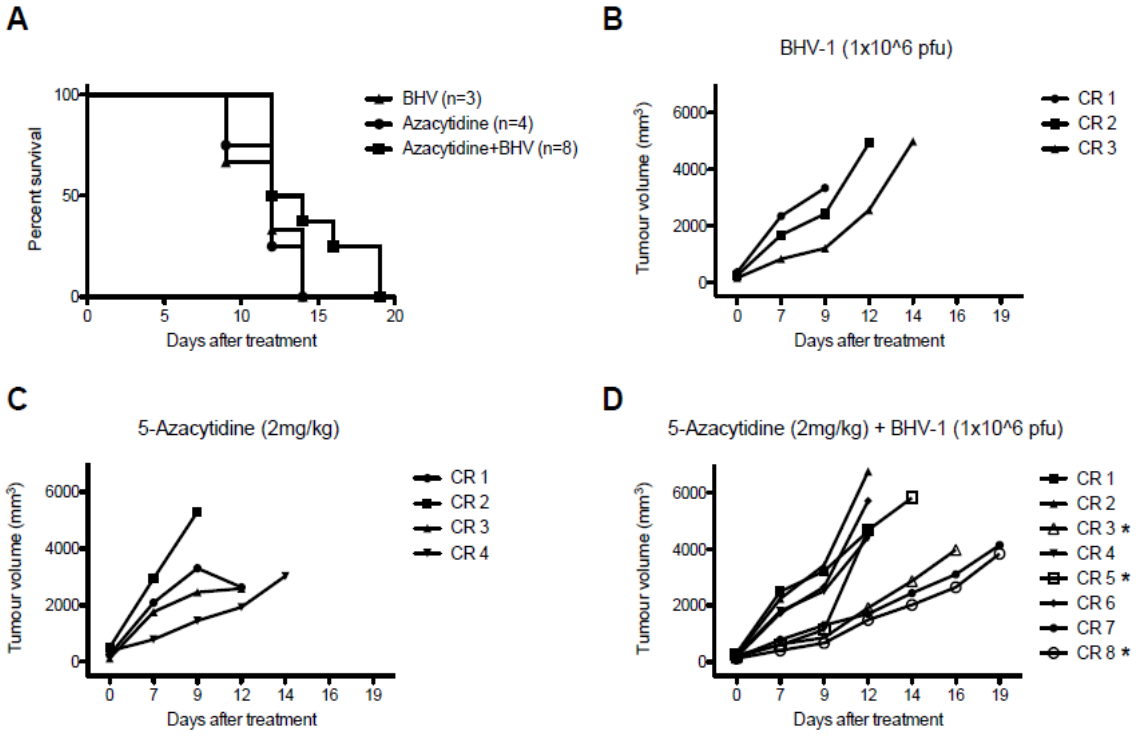
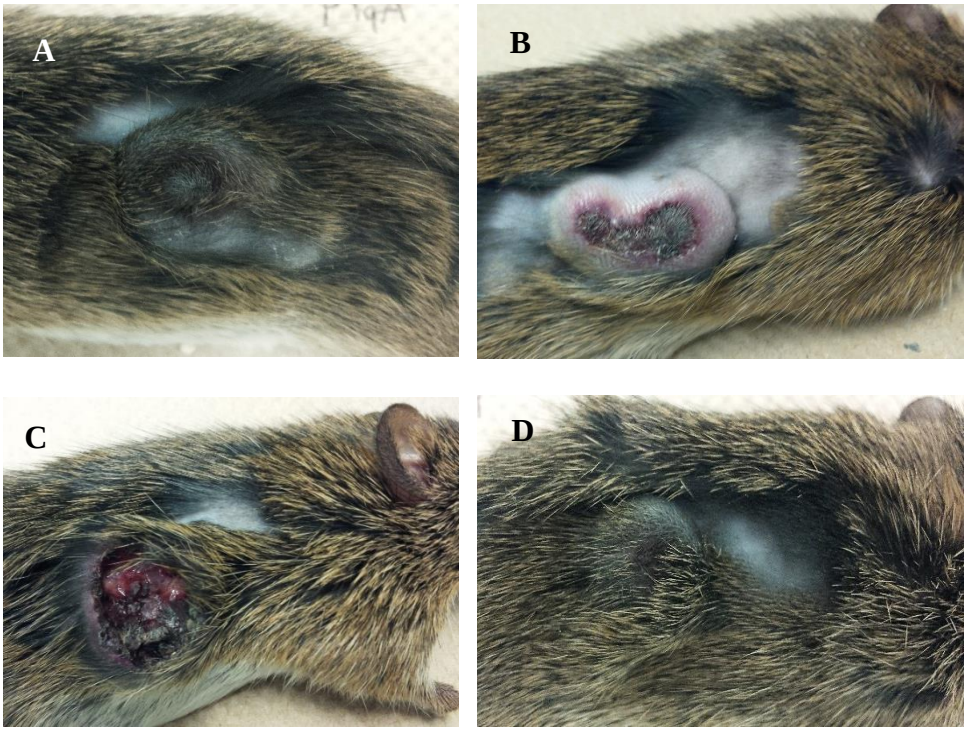
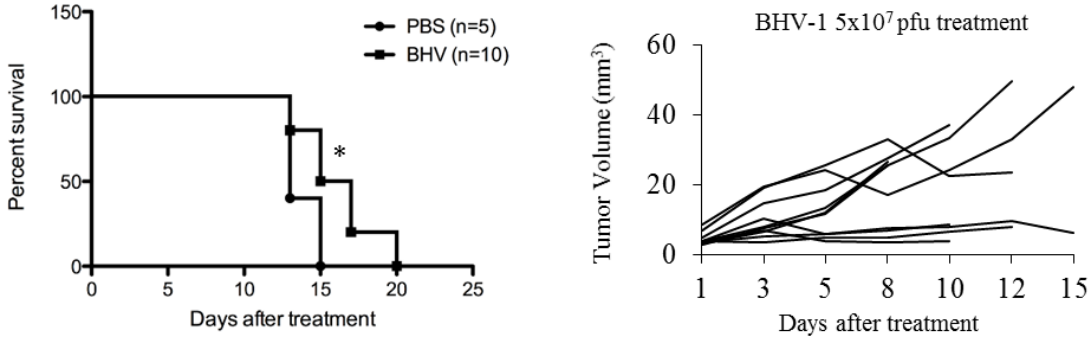


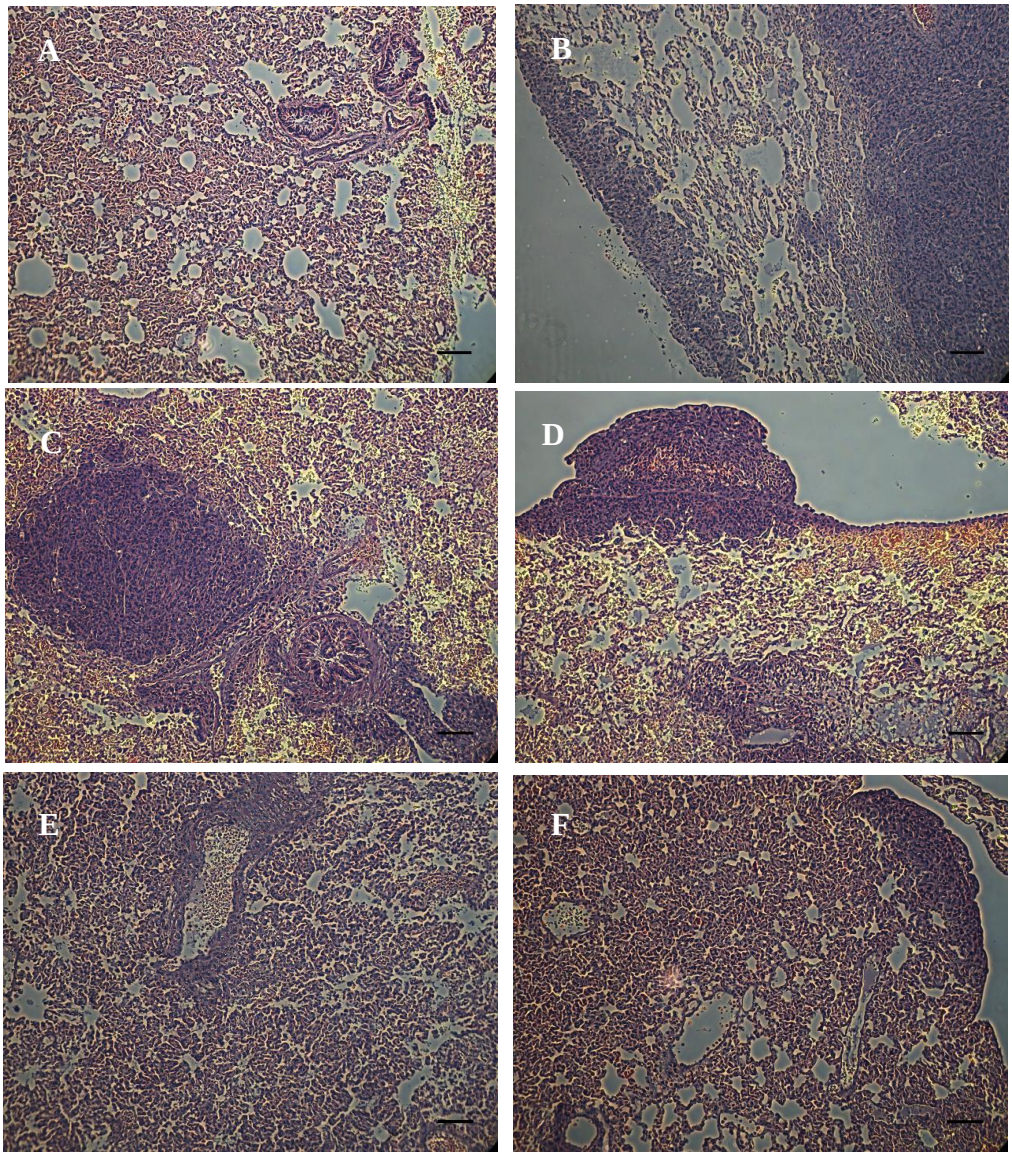
Figure 4.6. Kaplan-Meier survival curve and tumor volumes for cotton rats treated with $5x10^6$ pfu BHV-1, 2 mg/kg 5-Aza, or $5x10^6$ pfu BHV-1 and 2 mg/kg 5-Aza combination therapy. $5x10^5$ LCRT cells were implanted into cotton rats by subcutaneous injection. When tumors reached treatable size they were treated with $5x10^6$ pfu BHV-1 intratumorally one dose daily for 5 days, one dose 5-Aza (2mg/kg) intraperitoneally, or pre-treated with 5-Aza (2 mg/kg) intraperitoneally 1 day prior to intratumoral injection of $5x10^6$ pfu BHV-1, one dose daily for 5 days. Tumors were measured every two days until animals reached end point. **(a)** Kaplan-Meier estimates of survival and tumor volumes of cotton rats treated with **(b)** $5x10^6$ pfu BHV-1 **(c)** 2 mg/kg 5-Aza or **(d)** $5x10^6$ pfu BHV-1 and 2 mg/kg 5-Aza are shown. * indicates animals for which vacuous cavities were found in primary tumors upon necropsy.



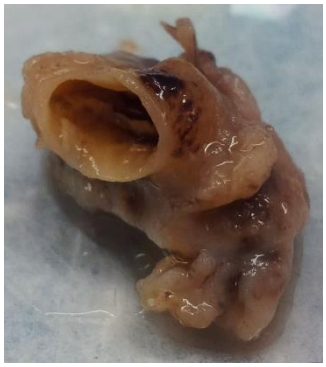
Supplementary Figure 4.1S. Subcutaneous LCRT breast adenocarcinomas on the right flank of cotton rats. $5x10^5$ LCRT cells were implanted into cotton rats by subcutaneous injection. When tumors reached treatable size they were treated with **(a)** PBS; **(b)** $5x10^6$ pfu BHV-1 intratumorally, one dose daily for 5 days; **(c)** $5x10^7$ pfu BHV-1 intratumorally, one dose daily for 5 days; **(d)** 5-Aza (2 mg/kg) intraperitoneal one day before intratumoral BHV-1 ($5x10^6$ pfu) injections, one dose daily for 5 days. Tumors were measured every two days until animals reached end point.



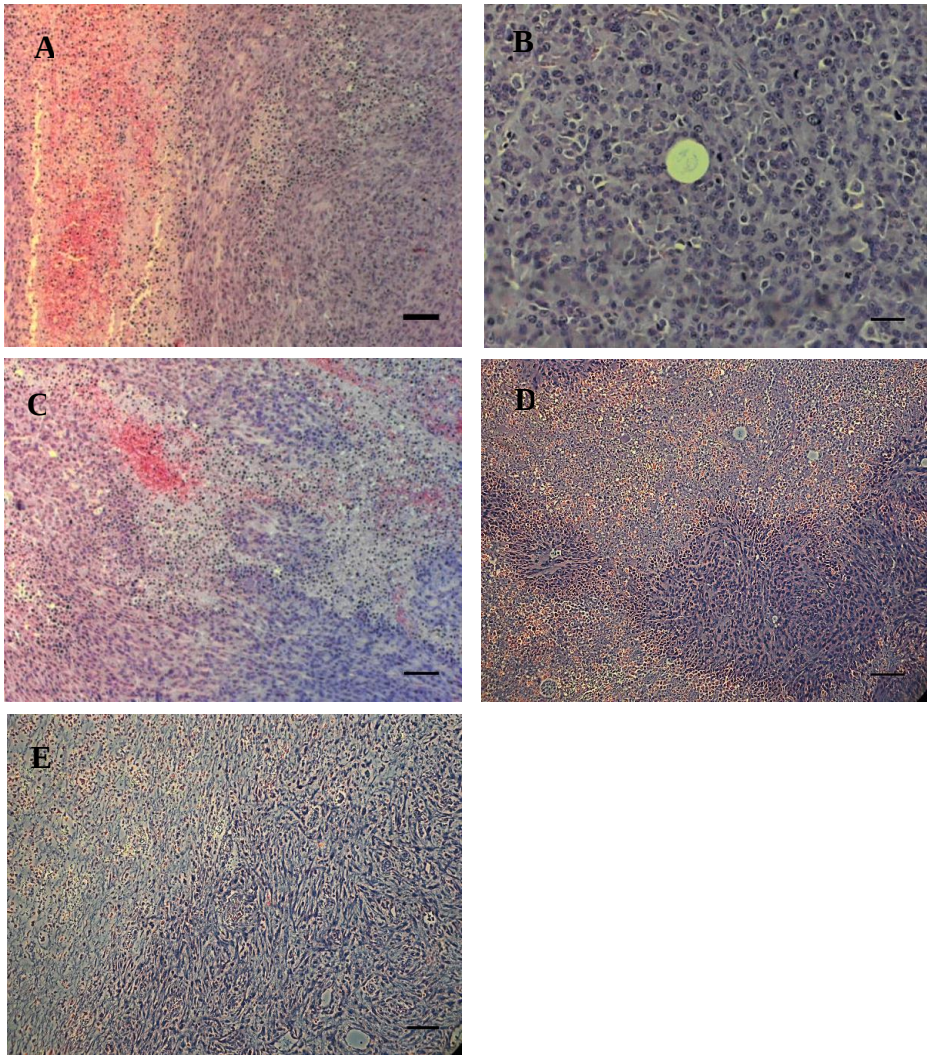
Supplementary Figure 4.2S. Kaplan-Meier survival curves and tumor volumes of cotton rats bearing subcutaneous LCRT tumors. $5x10^5$ LCRT cells were implanted into cotton rats by subcutaneous injection. When tumors reached treatable size they were treated with $5x10^7$ pfu BHV-1 intratumorally, one dose daily for 5 days. Tumors were measured every two days until animals reached end point. Kaplan-Meier estimates of survival (left) and tumor volumes (right) of cotton rats for each treatment regimen are shown.



Supplementary Figure 4.3S. Representative histologic analysis of lung metastases from BHV-1 treated cotton rats. Lungs were excised from (**a and b**) BHV-1 ($5x10^6$ pfu), (**c and d**) BHV-1 ($5x10^7$ pfu) and (**e and f**) combination therapy (5-Aza and BHV-1 $5x10^6$ pfu) treated animals at endpoint, fixed and H&E stained for histological analysis. All images were captured at 10x magnification with a Leica DM IRE2 microscope. Scale bars = 1 mm.



Supplementary Figure 4.4S. Subcutaneous LCRT breast adenocarcinoma harvest from a combination therapy treated cotton rat. $5x10^5$ LCRT cells were implanted into cotton rats by subcutaneous injection. When tumors reached treatable size they were treated with 5-Aza (2 mg/kg) pre-treatment 1 day prior to intratumoral injection of $5x10^6$ pfu BHV-1, one dose daily for 5 days. Tumors were excised at endpoint and preserved in 10% neutral buffered formalin for histology.



Supplementary Figure 4.5S. Representative histologic analysis of primary tumors from BHV-1, 5-Aza and combination therapy treated cotton rats. Tumors were excised from PBS (**(a)** vasculature; **(b)** bulk tumor), BHV-1 ($5x10^6$ pfu) (**(c)** vasculature; **(d)** tumor cell clearance) and combination treated (5-Aza (2 mg/kg), BHV-1 ($5x10^6$ pfu)) (**(e)** tumor cell clearance) animals at endpoint, fixed and H&E stained for histological analysis. All images were captured at 10x magnification with the exception of (**b**) taken at 20x with a Leica DM IRE2 microscope. Scale bars = 1 mm (a, c-e), 0.5 mm (b).

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Chapter 5: General Discussion and Conclusions

Advancements in cell and tissue culture methods in the 1970s allowed for more comprehensive studies of the effects of viral infections (Russell, Peng et al. 2012). Since then, numerous OVs have been described with a handful progressing to clinical trials (Russell, Peng et al. 2012, Pol, Bloy et al. 2014). Oncolytic HSV-1 vectors are among the most studied and consequently most successful OVs, with T_{vec} in phase III clinical trials for the treatment of unresected Stage III B/C or Stage IV melanoma (NCT00769704). Despite recent progress in the implementation of oncolytic HSV-1 vectors for the treatment of multiple malignancies, our understanding of the biological interplay at hand is still in its infancy. The safety of oncolytic HSV-1 has been demonstrated in multiple clinical trials at current maximal feasible doses (Russell, Peng et al. 2012, Pol, Bloy et al. 2014). However, it is speculated that because results continue to fall short of projected outcomes, higher doses may be required to improve patient responses, which may also be accompanied by dose-limiting toxicities. Furthermore, the majority of clinical trials completed to date involve intratumoral administration of oncolytic HSV-1 (Geevarghese, Geller et al. 2010, Russell, Peng et al. 2012). Although amplification of antitumor immune responses has been observed following intratumoral administration, systemic delivery will be required for the treatment of metastatic disease (Senzer, Kaufman et al. 2009, Kaufman, Kim et al. 2010). In fact, it wasn't until recently that the only case of complete remission in a patient following systemic administration of an OV was documented (Russell, Federspiel et al. 2014). Neutralization by serum factors, due to the

high incidence of pre-existing immunity to HSV-1, may limit systemic delivery of the virus. Moreover, majority of preclinical and clinical studies with oncolytic HSV-1 vectors are performed in models of glioblastoma due to the neurotropic nature of the virus. These obstacles warrant the development of wt non-human viruses for OVT.

The objectives of this thesis were to investigate the oncolytic breadth of BHV-1 in human tumor cells, to determine the cellular factor(s) which dictate cellular sensitivity to the virus and to evaluate the *in vivo* antitumor efficacy of BHV-1 in immunocompetent tumor models.

5.1 BHV-1 and Ras Signalling

The heterogeneity that exists both within and between tumor types warrants the development of novel OVs with distinct mechanisms of tumor cell targeting. The ability of BHV-1 to infect and kill a wide variety of human tumor cell types suggests its applicability as a broad spectrum OV. However, knowledge of the factor(s) which dictate cellular sensitivity to BHV-1 will allow for more efficient tumor cell targeting and improve the antitumor response to BHV-1 OVT.

The ability of Ras proteins to signal through multiple pathways with roles in cell cycle regulation make them important regulators of cell proliferation (as reviewed in (Coleman, Marshall et al. 2004)). The role of cell cycle status and its impact on HSV-1 infection have been well elucidated. For instance, the activity of CDKs have been shown to stimulate HSV-1 IE gene transcription and viral replication (Schang, Phillips et al. 1998).

The IE protein ICP0 plays an important role in regulating cell cycle progression by co-localizing with and stabilizing cyclin D3 (Kawaguchi, Van Sant et al. 1997). Data on cell cycle regulation by BHV-1 are beginning to surface, although exact mechanistic details have yet to be elucidated. In a study by Winkler and colleagues, acute infection of the trigeminal ganglia of calves induced the expression of cyclin D1, E and A facilitating productive infection (Winkler, Schang et al. 2000). Furthermore, during latency the association of cyclin-CDK complexes is regulated by latency gene products to keep cells in G_1 phase cell cycle arrest (Jiang, Hossain et al. 1998). Recent studies implicate E2F proteins as important factors in regulating BHV-1 infection. The E2F family of transcription factors regulate gene expression, including those involved in cell cycle regulation. Transactivation of BHV-1 promoters by E2F1 have been shown to enhance productive infection in multiple cell types (Shin, Tevosian et al. 1996, Workman and Jones 2010). Conversely, knockdown of E2F1 inhibits BHV-1 productive infection (Workman and Jones 2010).

The results presented as part of this dissertation demonstrate the ability of BHV-1 to infect and kill a wide variety of human tumor cell types (Chapter 2 and 3). Preliminary data failed to correlate type I IFN signalling with susceptibility to BHV-1 infection, as is the case for other species-specific OVs (Stojdl, Lichty et al. 2000, Wang, Ma et al. 2004, Krishnamurthy, Takimoto et al. 2006, Rodrigues, Cuddington et al. 2010). Furthermore, the ability of BHV-1 to infect and kill immortalized cell types suggested that mutations in p53 or retinoblastoma (RB) may play a role in sensitivity to BHV-1 as they are commonly deregulated during immortalization. However, mutation of p53 and/or RB did

not correlate with increased sensitivity to BHV-1. In contrast to HSV-1, which counteracts the anti-viral effects of PKR through the expression of ICP34.5, BHV-1 does not encode a homolog of this protein. This led us to speculate that BHV-1 may selectively replicate in tumor cells with constitutively active Ras signalling. A rudimentary screen of the mutation status of common oncogenes and tumor suppressors in NCI panel cell lines using the Sanger Institute COSMIC online database indicated that mutations in KRAS correspond to high levels of BHV-1 replication (Chapter 2).

Until recently, very little was known about the signalling pathways involved in BHV-1 infection. The infection of Madin Darby Bovine Kidney (MDBK) cells with BHV-1 has been shown to induce biphasic activation of the PI3K and mitogen activated protein kinase (MAPK) pathways, determined by assessing the phosphorylation status of downstream effectors Akt (also known as PKB) and extracellular signal-regulated kinase (ERK)1/2, respectively (Zhu, Ding et al. 2011). Given our data showing that KRAS contributes to, but is not the sole factor dictating sensitivity to BHV-1 infection, and considering that Ras signalling can induce activation of the MAPK and PI3K pathways, we conducted studies to determine the activation status of Akt and ERK1/2 in human tumor cells following BHV-1 infection. Unfortunately, our data failed to delineate changes in the phosphorylation status of these signalling effectors. While this was surprising, it was not unexpected as tumor cells contain a plethora of mutations and the complex interactions between pathways downstream of Ras make the study of independent factors difficult. Future studies should focus on the dissection of the MAPK

and PI3K pathways in normal primary cells which overexpress oncogenic KRAS to decrease background from pre-existing mutations.

The observation that lung and colon tumor cell types support the highest levels of BHV-1 replication but also commonly have mutations in KRAS, raised the question as to whether this was cause or effect (Chapter 2). However, our data showing that the overexpression of KRAS in normal primary cells elicits susceptibility to BHV-1 infection suggests that it is in fact the expression of mutated KRAS, not the histological origin of the tumor that dictates sensitivity to BHV-1 (Chapter 2). What remains unclear is whether it is the mutation of KRAS itself, or the resultant changes in the stimulation of downstream signaling pathways and altered expression of related signaling effectors that is directly responsible for mediating cellular sensitivity to BHV-1. Overall, we suspect that the bottom line is the induction of E2F and its positive regulation of bICP0. Our data indicates that as cellular permissivity to BHV-1 infection increases so does the protein expression of bICP0 (Chapter 2). Given the ubiquitous role of bICP0 during productive infection this observation is not surprising. Although the effects of E2F knockdown and overexpression have been studied in bovine cells (Workman and Jones 2010, Workman and Jones 2011), similar experiments will be necessary to discern the role of the E2F family of transcription factors in BHV-1 infection of human tumor cells. To date, we've shown that the knockdown of E2F1 in the mammary tumor cell line MCF7 decreases viral titers (Chapter 2). However, the effect of E2F1 knockdown varied between different tumor cell types and combination knockdown with KRAS did not have an additive effect as might be expected. It is possible that different E2F family members have varied effects

depending on the cell type (Geiser and Jones 2003), or that the cumulative effect of mutations in E2F regulatory proteins and pathways in tumor cells differentially effect the result of E2F knockdown/overexpression. Further study of the relative involvement of KRAS and downstream signaling pathways/effectors in cellular permissivity to BHV-1 will be required to develop the virus as an effective cancer therapeutic. Knowledge of the molecular mechanisms which govern OV infectivity will allow for more efficient tumor cell targeting and specific application for maximal efficacy in the clinic.

5.2 BHV-1 and Breast Cancer Initiating Cells

Our ability to detect and resect primary breast tumors has significantly improved patient survival. However, drug resistance and relapse remain formidable problems in achieving long term cures. Although it remains controversial, CICs are thought to be responsible for cancer recurrence and have been shown to be refractory to radiation and chemotherapeutics. Given their presumed roles in drug resistance and relapse, the development of BC therapies which are able to target and kill BCICs is paramount. The use of OVs to kill BCICs is appealing due to their unique mechanisms of tumor cell targeting.

The second paper presented in this dissertation examined the ability of BHV-1 to infect and kill BCICs, which were enriched by sorting for the SP based on efflux of the DNA-binding dye Hoechst 33342 (Chapter 3). We would argue that sorting based on Hoechst dye efflux ability is more reliable than the conventional method of sorting for

$CD44^{+}CD24^{-/low}$ cells as the expression of these markers is inconsistent between BC subtypes and within BC cell lines and patient tumors (Liu, Nenutil et al. 2014, Zhong, Shen et al. 2014). Due to mounting controversy in the field, secondary methods to validate the identity of the presumed enriched population of BCICs should be used.

BCICs enriched from both luminal and basal BC cell lines were used for a more comprehensive analysis of the range of infectivity of BHV-1 in this dynamic cell type (Chapter 3). BHV-1 is able to initiate replication in BCICs with cytotoxicity observed independent of a productive infection (Chapter 3). While this could limit effective targeting and clearance of BCICs *in vivo*, it seems unlikely due to recent studies highlighting the importance of indirect mechanisms of tumor cell destruction, such as antitumor immune responses (Sobol, Boudreau et al. 2011, Workenhe, Pol et al. 2013, Workenhe, Simmons et al. 2014). We have shown that BHV-1 infection of BCICs limits their ability to form tumors *in vivo*; however, future studies should evaluate the antitumor efficacy of BHV-1 against established BCIC-derived tumors (Li, Zeng et al. 2012, Wang, Chen et al. 2012, Zeng, Hu et al. 2013).

To our knowledge, only one study has examined the factors dictating sensitivity of CICs to OV infection (Ahtiainen, Mirantes et al. 2010). Defects in type I IFN signaling were found to mediate the sensitivity of BCICs to oncolytic adenovirus (Ahtiainen, Mirantes et al. 2010). Our data indicates that the mutation of KRAS confers cellular sensitivity to BHV-1 (Chapter 2). Recently, studies have emerged aimed at elucidating the role of KRAS signaling in CICs. Data indicates that KRAS mutation increases CIC self-renewal, differentiation and tumor-initiating ability (Fearon and Wicha 2014, Moon, Jeong et al.

2014, Okada, Shibuya et al. 2014). Furthermore, Moon and colleagues observed an increase in the expression of CD44 in colorectal CICs with KRAS mutations (Moon, Jeong et al. 2014). Although controversial, the expression of CD44 has been linked with poor prognosis in multiple cancers (Chen, Zhou et al. 2014, Dan, Hewitt et al. 2014, Luo, Wu et al. 2014, Ozawa, Ichikawa et al. 2014). Further studies involving the knockdown/overexpression of oncogenic KRAS in BCIC-enriched mammosphere populations will allow us to determine the role of KRAS in BCIC sensitivity to BHV-1 infection. Analyses should include systematic evaluation of the effects of the inhibition of KRAS downstream signalling factors, including the MAPK and PI3K pathways (Donovan, Pommier et al. 2013, Okada, Shibuya et al. 2014). These data will provide information on the KRAS-mediated mechanisms of BCIC maintenance, allowing for the development of BHV-1 as a more effective therapeutic with the ability to target BCICs.

5.3 BHV-1 Mechanism of Cell Death

There are two generally accepted mechanisms of apoptosis, intrinsic (mitochondrial) and extrinsic (death receptor), which participate in extensive cross-talk to mediate cellular stress responses. The mechanism by which BHV-1 induces cellular death is largely unknown and appears to be cell type dependent. Studies indicate the ability of BHV-1 to induce apoptosis in multiple cells types; however, whether the induction of cellular death requires BHV-1 penetration and replication varies by cell type (Hanon, Meyer et al. 1998, Devireddy and Jones 1999, Winkler, Doster et al. 1999, Xu, Zhang et al. 2012).

The entry of *alphaherpesviruses* into certain cell types is mediated by HVEM, a novel member of the tumor necrosis family (TNF) of receptors (Montgomery, Warner et al. 1996, Whitbeck, Peng et al. 1997). The gD-HVEM interaction has been shown to mediate both pro- and anti-apoptotic signalling events, which can occur in the absence of a productive infection (Rooney, Butrovich et al. 2000, Lasaro, Tatsis et al. 2008, Sciortino, Medici et al. 2008). Furthermore, danger signals which are activated in response to viral attachment and entry serve to prepare cells against viral infection. For instance, gD-HVEM binding alters the early innate anti-viral immune response by affecting proinflammatory cytokine production (MacLeod and Minson 2010).

There is an increasing body of literature implicating the involvement of virus-host cell interactions in dictating cellular sensitivity to infection. It is tempting to speculate that BHV-1 is able to induce cellular death in human tumor cells in the absence of virus entry and replication; however, experiments using ultraviolet (UV)-inactivated virus should be used to confirm this hypothesis. We suspect a range of results depending on tumor type due to the cell type specific mechanisms of BHV-1-induced cellular death. However, by examining the effects of BHV-1 attachment, particularly within the context of a non-productive infection, we can dissect the role of viral and cellular receptors in the response of human tumor cells to BHV-1 infection. Studies by Hanon and colleagues have determined that the deletion of gD (but not gC, gE, gG, gH, gI) abolishes the ability of BHV-1 to induce penetration-independent apoptosis in peripheral blood mononuclear cells and bovine lymphoma cells (Hanon, Meyer et al. 1998). Data also indicate that affinity purified gD is unable to induce apoptosis in peripheral blood mononuclear cells

on its own (Hanon, Keil et al. 1999). This suggests the involvement of an additional viral component or interacting partner at the cell surface in the cooperated induction of apoptosis. The role of gD in non-permissive tumor cell death can be studied by evaluating changes in cytotoxicity following infection with BHV-1 that expresses mutated gD, making it unable to bind HVEM but retaining the ability to form functional interactions with other cellular receptors (Hanon, Keil et al. 1999, Yoon, Kopp et al. 2011). These experiments should be repeated with gH-null BHV-1 mutants (Hanon, Meyer et al. 1998). Furthermore, studying cytotoxicity mediated by UV-inactivated BHV-1 with intact or mutated gD/gH will further discern the role of virus attachment and replication in cellular death responses in human tumor cells.

Throughout productive infection bICP0 acts as a potent transactivator of viral genes and is heavily involved in counteracting the host anti-viral immune response (Fraefel, Zeng et al. 1994, Henderson, Zhang et al. 2005, da Silva, Gaudreault et al. 2011). Due to its extensive participation in productive infection, it isn't surprising that bICP0-null BHV-1 mutants have decreased infectivity and do not plaque efficiently in MDBK cells (Geiser, Zhang et al. 2005). Interestingly, bICP0 protein expression indirectly activates caspase 3-mediated cellular death when transfected into cells (Henderson, Zhang et al. 2004). We observed a correlation between bICP0 protein expression and cellular sensitivity to BHV-1 (Chapter 2). Although the expression of tegument bICP0 (pre-IE bICP0) has not been described, ICP0 is a well-established component of the HSV-1 inner viral tegument (Yao and Courtney 1992). We propose that collaboration between mechanisms initiated by virion-cell attachment and pre-IE bICP0 brings about cellular death in the absence of a

productive infection. In resistant cell lines where bICP0 protein expression was not detected but decreases in cellular viability occurred, it is possible that pre-IE bICP0 activates replication-independent apoptosis. Alternatively, BHV-1 may establish a persistent-like infection in these cells with low levels of viral gene expression which may ultimately lead to delayed cellular cytotoxicity, especially in cases of low multiplicity infections. Given our data, in addition to that in the literature, it seems likely that novel virus-host interactions are important for BHV-1-initiated cellular death in different cell types.

5.4 BHV-1 in Combination Therapy

The pre-clinical evaluation of OVs typically begins with high throughput screening techniques to determine the breadth of infectivity in tumor cells. This approach is followed by assessment of the *in vivo* antitumor efficacy of the virus in animal tumor models. However, recent studies have shown that *in vitro* assays do not always predict *in vivo* outcomes (Workenhe, Pol et al. 2013, Workenhe, Simmons et al. 2014). Overall, pre-clinical studies aim to characterize virus-host interactions in the hopes of improving therapeutic efficacy.

Several considerations are vital in order to produce data that is as clinically relevant as possible, perhaps the most important being the selection of a relevant animal tumor models The importance of the immune system and the role of central and peripheral tolerance as a barrier to successful OVT has made the study of OV antitumor efficacy

within the context of an immunocompetent host an absolute must (Sobol, Boudreau et al. 2011, Russell, Peng et al. 2012, Workenhe, Pol et al. 2013, Workenhe, Simmons et al. 2014). Engagement of the immune system in OVT achieves prolonged antitumor responses by breaking immune tolerance, even when the virus has long been cleared. This circumvents the requirement for extensive OV spread within the tumor, which is thought to play a minor role in the success of HSV-1 OVs (Sobol, Boudreau et al. 2011, Ayala-Breton, Russell et al. 2014, Workenhe, Simmons et al. 2014). Furthermore, due to the importance of the immune system in OVT *in vivo* studies should include immunological readouts (antitumoral and antiviral) in addition to those traditionally performed. Optimization of immunological responses could very well hold the key to the efficacy of OVT.

A tumor certainly does not consist solely of tumor cells and the associated vasculature. The heterogeneity of a tumor includes the presence of multiple cell types under varying cellular conditions including necrosis, hypoxia and inflammation, with underlying deviations in gene mutations. Ultimately, the tumor microenvironment plays a pivotal role in dictating OVT efficacy. Targeting it disrupts tumor integrity by impeding tumor-microenvironment crosstalk and modulates immune cell infiltration and function (as reviewed in (Wojton and Kaur 2010)). The cascade of changes from the disruption of tumor vasculature can impede or improve OV efficacy on a case by case basis. OVs themselves can infect vascular endothelial cells leading to vascular shutdown or induce changes in the production of anti/pro angiogenic factors (Kurozumi, Hardcastle et al. 2007, Wojton and Kaur 2010). In each case the antitumor effects vary, highlighting the

requirement for precise study of the *in vivo* molecular effects during OVT. These data can be used to optimize the treatment schedule and potential agents for use in combination therapy with OVs.

An increasing number of studies involve the use of OVs in combination therapy. The combination of OVs with conventional (chemo and radiation) and immunotherapeutics exploit the interaction between the OV, tumor microenvironment and host immune system to improve therapeutic efficacy. Increased interest in the area of epigenetics and its role in tumor development and prognosis has made the use of epigenetic modifiers, such as 5-Aza, an attractive alternative to conventional anticancer therapies. Recent studies have described improved antitumor responses after combination therapy with OVs and 5-Aza (Okemoto, Kasai et al. 2013).

The third paper presented in this dissertation evaluates the antitumor potential of BHV-1 in combination with 5-Aza for the treatment of BC (Chapter 4). BHV-1 does not efficiently bind and enter murine cells (Hushur, Takashima et al. 2004), ruling out the use of conventional murine tumor models. The CR model of breast adenocarcinoma is highly aggressive with high rate of metastasis to the lymph nodes and lungs, which is also common in human BC patients (Disibio and French 2008, Berman, Thukral et al. 2013). *In vitro*, the synergism between 5-Aza and BHV-1 enhanced virus replication and cytotoxicity in CR BC cells pretreated with 5-Aza, but did not significantly increase viral titers (Chapter 4). *In vivo,* BHV-1 mono and combination therapy delayed tumor growth but did not prolong survival of CR bearing subcutaneous breast tumors (Chapter 4). Interestingly, combination therapy significantly decreased the number of secondary

lesions compared to BHV-1 monotherapy (Chapter 4). Despite the increase in virus replication *in vitro*, it's likely that combination therapy functions via an indirect mechanism *in vivo*. This hypothesis is based on the absence of an increase in pathology, specifically pertaining to that observed in the lungs when the viral dose was increased in combination treatment. This was surprising given the permissivity of CRs to BHV-1 infection (Prince 1994). In addition, histological analysis of lung samples did not indicate that pathology was virally induced even at the highest dose of BHV-1 (Chapter 4). These data highlight the disparity between *in vitro* and *in vivo* results and suggest the involvement of the immune system in antitumor effects of BHV-1 combination therapy. Unfortunately, the lack of reagents to evaluate the immune response in CRs makes their continued use in *in vivo* studies problematic as immune readouts are essential in pre-clinical OVT studies (Sobol, Boudreau et al. 2011, Workenhe, Pol et al. 2013, Workenhe, Simmons et al. 2014). Furthermore, CRs are sensitive to anesthesia so we were unable to use *in vivo* imaging techniques to visualize the biodistribution of BHV-1. Due to these obstacles we are only able to speculate as to the antitumor mechanism of BHV-1 combination therapy with 5-Aza. The availability of reagents and ease of handling in alternative non-murine models such as Syrian hamsters may present an attractive alternative to CRs (Wold and Toth 2012). The inaccessibility is unfortunate because the CR model of breast adenocarcinoma recapitulates several characteristics which are similar when compared to what happens in human BC patients (Disibio and French 2008, Berman, Thukral et al. 2013). Furthermore, the high rate of metastasis in this model

makes it clinically relevant as metastatic disease remains a significant hurtle to sustained patient cures.

5.5 Conclusions

The use of OVs in cancer therapy is promising due to their specificity and lack of debilitating side effects which go in hand with conventional chemotherapeutics and radiation. There is excitement in the field over promising clinical data and novel OVs with unique therapeutic targets continue to be described. Despite the advances made, challenges remain in optimizing delivery, overcoming physical barriers to dissemination within the tumor as well as systemically, and achieving sustained host antiviral and antitumoral immune responses. Manipulation of immunological outcomes using combination therapy has become an attractive method used to mediate the efficacy of OVT. While it is tempting to combine an OV with the therapeutic agent of the hour, an in-depth understanding of virus-host interactions will be required to unleash the full potential of OVT.

Our results indicate that BHV-1 is a broad spectrum OV with a unique mechanism of tumor targeting in comparison to other species-specific viruses. The majority of work presented in this dissertation focuses on the application of BHV-1 for BC therapy. In contrast to current treatments which are largely contingent upon receptor expression status, BHV-1 is able to infect and kill BC cells and BCICs from luminal and basal

subtypes. Taken together, these data are exciting as the majority of OVs have a selective range of infectivity dictated by the mutation expressed by the virus. Although the precise mechanism dictating cellular sensitivity to BHV-1 was not determined, our data suggests that KRAS mutations play a significant role. It will be interesting to unravel the relative roles of downstream signaling effectors in BHV-1 infection. Furthermore, the ability of BHV-1 to elicit cellular death in the absence of a productive infection and at low multiplicity holds promise for clinical efficacy at low doses. The method by which BHV-1 elicits death in human tumor cells is unknown, but the role of KRAS signaling in cell cycle regulation may provide a possible mechanism. Finally, BHV-1 monotherapy did not significantly impact tumor growth or survival of CR bearing subcutaneous breast tumors. However, combination therapy with 5-Aza significantly decreased the number of secondary lesions compared to BHV-1 monotherapy. These data indicate the antitumor efficacy of BHV-1 in an aggressive and highly metastatic model of BC. Overall, the data presented in this dissertation endorse further study of BHV-1 for application in OVT with efficacy against multiple tumor types, particularly BC.

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