Virotherapy for Breast Cancer

by

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Abstract

Breast cancer (BrCa) is the most common cancer and the leading cause of cancer-related deaths in women worldwide. Current therapies for BrCa are insufficient to cure metastatic disease and are often associated with debilitating side effects that severely deteriorate the quality of patients' lives. Therefore, novel therapeutic approaches for the treatment of BrCa are being sought. Virotherapy, a strategy to use live viruses as therapeutics, is an emerging field that holds promise for better treatment of cancer. Cancer virotherapy could be broadly categorized into two sub-classes: (i) use of non-replicating viruses as vectors in cancer gene therapy, and (ii) use of replicating viruses as oncolytic agents. In this study, we have studied both aspects of virotherapy. First, we have determined the feasibility of using non-replicating adenovirus (Ad) encoding transcriptionally targeted interleukin-2 (IL-2) for the treatment of BrCa. Second, we have studied the oncolytic activities of genetically-engineered replication-competent vaccinia virus (VACV) in BrCa.

IL-2 has been approved for the treatment of some malignancies, however severe toxicities related to systemically administered IL-2 limit the therapeutic usefulness of this cytokine. Based on previous findings that restricting the high concentrations of IL-2 within the tumor could both reduce the toxicities and enhance its anti-tumor activities, we aimed to study the safety and anti-tumor efficacy of human IL-2 expressed specifically from the tumor cells. We constructed Ad vectors encoding hIL-2 under the control of either the BrCa-specific mammaglobin promoter/enhancer sequence (Ad-MPE2-IL-2) or the constitutively active immediate early promoter of murine CMV (MCMV) (Ad-MCMV-IL-2). Our data show that the MPE2 promoter is highly efficient in restricting expression of a transgene (luciferase or hIL-2) to BrCa cells, and that while the Ad-

MCMV-IL-2 is highly toxic, the Ad-MPE2-IL-2 is safe in mice. Furthermore, while both the vectors were able to significantly delay growth of breast tumors in a syngeneic mouse model, complete tumor regressions were seldom observed in the treated animals. Additionally, attempts to enhance the anti-tumor efficacy of the Ad-MPE2-IL-2 by combining with a pro-apoptotic drug, PAC1, were unsuccessful.

Previous studies from our group have shown that deletion of the VACV *F4L* gene, homolog of cellular RRM2 which is a rate limiting factor in the synthesis of dNTPs, highly attenuates virus growth in normal cells and renders it avirulent in mice. In the study presented here, we determined the oncolytic properties of VACV lacking either the *F4L* alone (ΔF4L) or lacking *F4L* together with *J2R* (ΔF4LΔJ2R). Our results show that replication of ΔF4L VACV is dependent on cellular RRM2; notably, BrCa cells with high levels of RRM2 supported high levels of replication of these viruses. Both ΔF4L and ΔF4LΔJ2R VACVs showed better specificity for BrCa *in vitro* compared to the VACV with a deletion of J2R (ΔJ2R) only. All three mutant viruses were able to completely control tumor growth in a xenograft mouse model of BrCa. In a syngeneic mouse model, all mutant viruses significantly delayed tumor growth and increased the overall survival of treated mice, although the anti-tumor effect was less profound compared to that in the xenograft model. Furthermore, the oncolytic activity of ΔF4L VACV towards the stem-like ($CD44^{+}CD24^{-/low}$) population isolated from SUM-149 cells was similar to that towards the differentiated cell population in that triple-negative human BrCa cell line. Finally, all mutant viruses were found to grow to high titers in endothelial cells *in vitro* and they increased hypoxia in xenograft tumors in mice upon intra-tumoral injection. Taken together, this study shows that virotherapy is a promising strategy for the treatment

of breast cancer but would likely require additional interventions to completely clear tumors.

DEDICATION

I dedicate this thesis to my late mother *Sunaina Devi Chaurasiya* (1952-1986 A.D)

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List of Abbreviations and Acronyms Used in this Dissertation

ΔF4L	vaccinia virus lacking *F4L* gene
ΔF4LΔJ2R	vaccinia virus lacking *F4L* and *J2R* genes
ΔJ2R	vaccinia virus lacking *J2R* gene
2D	2-dimensional
3D	3-dimensional
7-AAD	*7*-aminoactinomycin D
Ad	adenovirus
bp	base pairs
BCA	bicinchoninic acid assay
BrCa	breast cancer
CAR	coxsackievirus adenovirus receptor
CD	cluster of differentiation
cDNA	complementary DNA
CEV	cell-associated virus
CMV	cytomegalovirus (immediate early promoter)
CSC	cancer stem cell
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAPI	4', 6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dNTP	deoxy-ribonucleoside triphosphate
E1A	adenovirus early region 1A
EBV	Epstein-Barr virus
EEV	extra-cellular enveloped virus
ER	estrogen receptor
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FDA	U.S. Food and Drug Administration

GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
gusA	β-glucuronidase
HEK293	human embryonic kidney 293 cells (AdE1-transformed)
HER2	human epidermal growth factor receptor 2
hIL-2	human interleukin-2
HPβCD	hydroxypropyl-β-cyclodextrin
HSV	herpes simplex virus
hTERT	human telomerase reverse transcriptase
IEV	intracellular enveloped virus
IHC	immunohistochemistry
IL-2	interleukin-2
IL-2R	interleukin-2 receptor
IMV	intra-cellular mature virus
i.t	intra-tumoral
ITR	inverted terminal repeats
i.v	intravenous
kb	kilobases
LAK	lymphokine-activated killer cell
MCMV	murine cytomegalovirus (immediate early promoter)
MEM	minimum essential medium
MGB	mammaglobin
MHC	major histocompatibility complex
MLP	major late promoter
MMTV	mouse mammary tumor virus
MOI	multiplicity of infection
MPE2	mammaglobin promoter/duplicated enhancer sequence
mRNA	messenger RNA
miRNA	microRNA
MVA	modified vaccinia virus Ankara
NCI	National Cancer Institute

Neo	neomycin
NK	natural killer
NYCBH	New York City Board of Health
PAC1	procaspase activating compound 1
PDX	patient-derived xenograft
pfu	plaque forming units
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PyMidT	polyoma virus middle T antigen
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD1	programmed death-1
Pexa-Vec	pexastimogene devacirepvec
PAGE	polyacrylamide gel electrophoresis
rIL-2	recombinant IL-2
RIPA	radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute medium
RR	ribonucleotide reductase
RRM1	ribonucleotide reductase large sub-unit
RRM2	ribonucleotide reductase small sub-unit
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of mean
shRNA	small (or short) hairpin RNA
siRNA	small (or short) interfering RNA
TK	thymidine kinase
TMK	thymidylate kinase
TNBC	triple-negative breast cancer
TNF	tumor necrosis factor
TRAIL	TNF-alpha related apoptosis inducing ligand

T-Vec	Talimogene laherparepvec
UTR	untranslated region
UV	ultraviolet
VACV	vaccinia virus
VLS	vascular leak syndrome
WR	Western reserve

Chapter 1: Introduction

1.1 Breast Cancer

1.1.1 Background

Breast cancer (BrCa) is the most common cancer among women, accounting for approximately 25% of total cancers and 15% of cancer-related deaths in women worldwide (Jemal, Center et al. 2010; Anampa, Makower et al. 2015; Torre, Bray et al. 2015). In Canada, 25,200 new cases of BrCa and 5000 BrCa-related deaths were estimated in 2015 (Canadian Cancer Society, 2015). Major advancements in the diagnosis and treatment of BrCa made over the last 3 decades have improved the quality of life and overall survival of patients. However, despite improvements in diagnostic methods, 20-50% of patients develop metastatic disease which remains difficult to treat and accounts for the majority of the BrCa related deaths (Higgins and Wolff 2008; Lu, Steeg et al. 2009). While BrCa is very common in women, men can also develop BrCa although the incidence rate is at least 100 times lower than that in women.

BrCa is a highly heterogeneous disease and the extensive intra- and inter-tumoral heterogeneity is thought be the result of the distinct cells of origin and specific alterations at genetic and epigenetic levels (Polyak 2007). BrCa has been classified in many different ways and the classification is ever evolving. Traditional classifications of BrCa were based on the histology and biology of the tumors whereas recent classification schemes are mainly based on molecular differences (Malhotra, Zhao et al. 2010; Cancer Genome Atlas 2012). Traditionally, breast tumors have been classified into a number of benign or malignant types depending on several morphological characteristics such as growth patterns, types of cells involved, cytological and nuclear characteristics, and stromal responses (Mallon, Osin et al. 2000). Benign tumors are thought to originate from

epithelial and myoepithelial cells, whereas malignant tumors are thought to originate from transformation of luminal epithelial cells (Mallon, Osin et al. 2000).

Comprehensive gene expression profiling by different independent groups using a large set of breast tumors has led researchers to categorize BrCa into five major molecular sub-types which vary in the expression levels of the estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). The 5 molecular sub-types are: normal breast-like, luminal A, luminal B, $HER2^{+}/ER^{-}$, and basal-like or triple-negative (Perou, Sorlie et al. 2000; Hu, Fan et al. 2006; Sorlie, Wang et al. 2006). Furthermore, a recent review by Reis-Filho and Pusztai classified BrCa into 7 subtypes which are: normal breast-like, luminal A, luminal B, HER2-enriched, basal-like, claudin-low and molecular apocrine (Reis-Filho and Pusztai 2011). These subtypes differ not only in their ER and HER2 status but also in several other important proliferation-related genes such as Ki67 and cyclin B1 (Reis-Filho and Pusztai 2011; Eroles, Bosch et al. 2012). Treatments for BrCa are dictated to some extent by the molecular subtypes. Prognosis varies among the subtypes, but in general, patients with basal-like (or triple-negative) tumors have the worst prognosis, whereas patients with luminal A tumors have the best prognosis (Sorlie, Perou et al. 2001; Polyak 2007).

1.1.2 Breast Cancer Stem Cells

The cancer stem cell (CSC) hypothesis postulates that CSCs, capable of self-renewal and differentiation, are important for initiation and maintenance of tumors (Clarke, Dick et al. 2006; McDermott and Wicha 2010). CSCs are believed to share multiple features with normal stem cells such as self-renewal, pluripotency, drug resistance and relative quiescence (Clarke 2006). Although the CSC hypothesis

underscores the involvement of CSCs in the initiation and maintenance of tumors, the origin of CSC is not clear from this hypothesis. However, it is possible that CSCs result from transformation of stem cells or progenitor cells residing in tissue (McDermott and Wicha 2010). CSCs are thought to reside at the top of the hierarchies which differentiate into non-stem populations in a unidirectional way creating tumor heterogeneity, with the bulk of the tumor composed of differentiated cells (Chaffer, Brueckmann et al. 2011).

The first evidence for the presence of CSC in cancer came from a study of acute myeloid leukemia in 1994 (Lapidot, Sirard et al. 1994). In the context of solid tumors, the presence of CSC was first demonstrated in breast tumors by Al-Hajj and colleagues in 2003 (Al-Hajj, Wicha et al. 2003). They showed that a subset of BrCa cells with high levels of the cluster of differentiation (CD) molecule CD44, and low levels of CD24 (*i.e.*, $CD44^{+}CD24^{-/low}$) were tumorigenic with as few as 10^2 cells capable of initiating tumor growth in NOD/SCID mice. Upon serial passaging in mice, this subset of cells was able to generate new tumors with both a $CD44^{+}CD24^{-/low}$ population as well as phenotypically diverse populations of non-tumorigenic cells, recapitulating the complexity of the initial tumor (Al-Hajj, Wicha et al. 2003). Different surface markers have been used to isolate CSCs from different malignancies and the frequency of CSCs differs even within the same type of tumor (Klonisch, Wiechec et al. 2008).

Besides cell surface markers, properties of stem cells such as high activities of drug efflux pumps have also been used to identify cancer stem-like cells (Hiraga, Ito et al. 2011). For example, cells with the ability to exclude Hoechst 33342 dye, called the "side population" based on their staining pattern during fluorescent-activated cell sorting, have been isolated from breast and other cancers (Hiraga, Ito et al. 2011; Xiong, Ma et al.

2014). This population has been found to be enriched for cells with CSC properties such as self-renewal and high tumorigenicity.

Conventional therapies which often target highly proliferating cells are prone to miss the CSCs that are slow-dividing and can maintain quiescence for a long time (Phillips, McBride et al. 2006; Creighton, Li et al. 2009; McDermott and Wicha 2010). Furthermore, killing of only differentiated cells in the tumor may allow for enrichment of CSCs that can cause disease recurrence with a worse prognosis (Li, Lewis et al. 2008). Therefore, targeting of CSCs is important to achieve long term success in cancer therapy. It is for this reason that most of the novel therapeutics are tested for their effect on CSCs in preclinical studies. As in tumors *in vivo,* a subset of cells in cancer cell lines *in vitro* appears to have a CSC phenotype, allowing candidate drug testing *in vitro* on CSCs without having to isolate CSCs from animals/humans (Klonisch, Wiechec et al. 2008).

1.2 Breast Cancer Therapies

1.2.1 Current Therapies

Surgery is commonly used to remove primary breast tumors while radiation and chemotherapies are used as adjuvants in the treatment of BrCa (Higgins and Wolff 2008; Burstein and Morrow 2015). For most of the 20th century, the Halsted radical mastectomy, an aggressive form of surgery that includes removal of breast together with muscles of the chest wall, was the standard form of surgery for the treatment of BrCa (Fisher, Jeong et al. 2002; Cotlar, Dubose et al. 2003). This approach was based on the premise that metastasis in BrCa occurs through locoregional spread and thus could be cured by aggressive surgery. However, studies in the 1970's showed that the radical mastectomy has no benefit over less aggressive surgeries in terms of either disease

recurrence or overall survival (see reviews (Fisher, Jeong et al. 2002; Anampa, Makower et al. 2015). Later, less aggressive breast conservation surgery became more common for local therapy combined with a wide variety of systemic adjuvant therapies (White, Achuthan et al. 2011). Adjuvant therapies given systemically are aimed at eradicating disseminated cancer cells in order to minimize the probability of metastatic growth (Jin and Mu 2015). Like many other types of cancer, metastases are the main cause of death in BrCa patients, accounting for more than 90% of total mortality (Gupta and Massague 2006).

Many studies have shown that use of adjuvant radiation-, chemo-, endocrine and HER2-directed therapies significantly reduces the risk of disease recurrence and improves the overall survival of patients (Ciccone, Viale et al. 1988; Hudis and Dang 2004; Clarke 2006). Radiation is usually applied to the tumor bed and regional lymph nodes after the resection of the primary tumor (Vallis and Tannock 2004). The most common chemotherapy adjuvant regimens include the combination of cyclophosphamide with doxorubicin and/or docetaxel, or methotrexate with 5-fluorouracil (an excellent review on this topic is provided by (Anampa, Makower et al. 2015)). However, use of radiation- and chemotherapies is limited by off-target toxicities and development of resistance to these therapies (Nabholtz and Gligorov 2005; Jones 2008). Off-target toxicities resulting from chemotherapies substantially affect the quality of patients' lives and approximately 40% of patients suffer from disease recurrence and die of metastases (Early Breast Cancer Trialists' Collaborative 2005). More recent therapies for BrCa have been developed to target the expression of ER, PR and HER2 expression. About 75% of human BrCas express ER, and endocrine therapy is used for the treatment of ER-positive

BrCa (Zhao and Ramaswamy 2014). Tamoxifen, an ER modulator, is the most commonly used endocrine therapy for ER/PR$^+$ BrCa. Likewise, fulvestrant, an ER degrading agent, and aromatase inhibitors such as anastrozole and letrozole are also commonly used for such types of BrCa (Early Breast Cancer Trialists' Collaborative 2005; Gibson, Lawrence et al. 2009; Ciruelos, Pascual et al. 2014). These endocrine therapies are used to treat women with both early and advanced BrCa, however, long-term success of such therapies is often limited by relapse of disease and development of resistance (Higgins and Baselga 2011). Development of resistance to endocrine therapy is thought to result from multiple mechanisms including loss of ER expression, post-translational modification of ER, and de-regulation of ER co-activators (Zhao and Ramaswamy 2014).

HER2, a receptor tyrosine kinase that plays an important role in cell proliferation and malignant growth, is over-expressed in approximately 25-30% of all BrCa (Wolff, Hammond et al. 2014; Zhao and Ramaswamy 2014). Patients with HER2$^+$ BrCa are treated with trastuzumab, a monoclonal antibody against HER2. Trastuzumab is usually given in combination with adjuvant chemotherapy either concurrently or sequentially (Slamon, Eiermann et al. 2011). Implementation of trastuzumab in the front-line therapy has substantially increased the 5-year disease-free survival and overall survival of HER2$^+$ BrCa patients (Jelovac and Wolff 2012). However, trastuzumab has been shown to induce some degree of cardiac toxicity, and approximately half of treated patients experience disease relapse within 3 years (Baselga, Perez et al. 2006; Jelovac and Wolff 2012).

Basal-like or triple-negative breast cancer (TNBC) is the most aggressive sub-type with the highest relapse rate and high incidence of brain metastasis (Anders and Carey 2008). TNBCs are not amenable to currently available targeted therapies as they lack these molecular targets. Anthracyclines and taxanes are usually used in the treatment of TNBC, however with very limited success (Anders and Carey 2008). Although favorable responses to chemotherapy are initially observed in TNBC patients, they quickly develop resistance to the drugs and disease relapse is very common with the median survival of only 13 months (Carey, Dees et al. 2007; Andre and Zielinski 2012). Therefore, there is a dire need of novel therapeutics to improve the treatment of TNBC.

1.2.2 Experimental Therapies

Because the existing therapies are not very effective in the treatment of metastatic BrCa, novel therapeutics are being sought. Increased understanding about the molecular and cellular etiology of BrCa has facilitated the proposal of several new therapeutics and the list is ever growing. However, despite a huge number of novel drugs entering clinical trials each year, only a handful of them are approved. In fact, oncologic drugs have the lowest rate of approval: only 1 out of 15 drugs that enter phase I trials gets regulatory approval (Hay, Thomas et al. 2014), a rate almost 3 times lower than that of non-oncologic drugs (Hay, Thomas et al. 2014). Model systems for preclinical evaluation of therapeutic drugs play an important role in determining the likelihood of success of a particular drug in the clinical settings. Poor or inadequate selection of preclinical model systems not only allows many drugs to enter into clinical trials and fail after spending huge amount of money and time (it can cost 1 billion dollar and 15 years of time to bring a drug to the market (Kola and Landis 2004)); but it can also block many potential drugs

from clinical testing which would otherwise be successful (Zips, Thames et al. 2005). Preclinical models routinely used in laboratories for the evaluation of experimental drugs are briefly discussed below.

1.3 Preclinical Models for Evaluating Experimental Drugs

1.3.1 In Vitro Cell Monolayer Cultures

There has been a steady increase in the number of cell lines from different types of human and animal cancers since the HeLa line was first established in the early 1950s (Zips, Thames et al. 2005). Presently, *in vitro* cell cultures are used as the first step for the evaluation of most, if not all, candidate drugs in the field of cancer research (Zips, Thames et al. 2005). Use of established cancer cell lines allows for high-throughput screening of large number of potential drugs in a short time period. In the early 1990s, the U.S National Cancer Institute (NCI) introduced a "disease-oriented" approach for screening drugs using a panel of 60 cancer cell lines which represent 9 distinct tumor types (leukemia, melanoma, cancers of the colon, lung, brain, kidney, ovary, breast, and prostate) (Wilding and Bodmer 2014; Kim, Sung et al. 2015). This approach was designed to filter candidate therapeutics such that only a fraction of the candidate drugs would be selected for further evaluation in xenograft models. While the NCI panel has been proven beneficial and is still routinely used in screening novel drugs, it includes a small number (<7) of cell lines from each type of cancer, hence the probability of identifying a responder subset for any novel drug is low (Wilding and Bodmer 2014). Furthermore, the use of established cancer cell lines as models for evaluation of candidate drugs is surrounded by controversies. It has been reported that after long term culture, cancer cell lines undergo genetic and epigenetic changes that are no longer representative

of the original tumor. Also, it is clear that cell lines neither contain the relevant components of the tumor microenvironment nor retain the heterogeneity present in the original tumor (Wilding and Bodmer 2014). In contrast, Barretina *et al.* have reported strong correlations between primary tumors of different cancer types and established cancer cell lines, in terms of mutation status, transcription profiles, and changes in DNA copy number (Barretina, Caponigro et al. 2012). In this study, the authors compared the genomic data and mRNA expression profiles from 947 cancer cell lines, derived from 36 different tumor types, with those of primary tumors available on public databases. They also tested the pharmacological profiles of 24 known anti-cancer drugs in 479 of the cancer cell lines. From this large scale study the authors concluded that cell lines "may provide representative genetic proxies for primary tumors in many cancer types" (Barretina, Caponigro et al. 2012). Despite controversies, cell lines have made significant contributions in the development of some of the existing anti-cancer drugs, and are unlikely to be completely replaced by other models in foreseeable future.

1.3.2 Spheroid Culture Models

Spheroids are multi-cellular 3-dimensional (3D) structures that are considered miniature tissue analogs. Compared to monolayer cultures, spheroid cultures offer a better model system for testing novel oncologic drugs as they are more representative of the 3D complexity of tumors *in vivo* (Kim 2005; Hirschhaeuser, Menne et al. 2010). Furthermore, mRNA expression profiles in spheroid cultures have been found to more closely reflect that of *in vivo* tumors compared to the classical 2D cell culture (Hirschhaeuser, Menne et al. 2010). Thus, the spheroid models serve as a bridge between the 2D cell culture models and animal (murine) models (Edmondson, Broglie et al. 2014).

Many potential drugs that were effective in killing cancer cells in the simplistic monolayer culture models were found to be ineffective in spheroid culture models, thus spheroid cultures are often thought to be a tool for negative selection of candidate drugs in order to reduce the cumbersome *in vivo* testing (Hirschhaeuser, Menne et al. 2010; Huh, Hamilton et al. 2011). However, it has also been documented that some potential targets which may not be expressed by cancer cells in monolayer cultures may be expressed in 3D spheroid cultures. In this case the spheroid models could serve as primary tools for positive selection (Dardousis, Voolstra et al. 2007; Howes, Chiang et al. 2007).

Many different techniques have been used by different investigators to generate spheroids of different sizes. Spheroids can be generated either from a single cell line or by combination of multiple cell lines. Co-spheroids generated by cancer cells together with fibroblasts have been previously used to test cancer specificity of drugs (Friedrich, Ebner et al. 2007). Taken together, spheroid cultures provide a simple, quick and cost-effective platform of intermediate complexity relative to monolayer and *in vivo* models, for preclinical evaluation of potential drugs.

1.3.3 Xenograft Models

Among the *in vivo* models for preclinical evaluation of drugs, human tumor xenograft models are the most commonly used. The vast majority of preclinical efficacy studies for novel drugs are carried out in xenograft models (Hollingshead 2008; Jung 2014). Human cancer cells are implanted in immune-compromised mice at ectopic sites such as subcutaneous sites or the renal capsule, or at orthotopic sites to generate tumor xenografts (Jung 2014). Human cancer cells implanted into immune-compromised mice

grow readily to form tumors and such tumors have been found to retain important characteristics of the donor patient's tumor such as histologic appearance and genetic profiles (Rofstad and Lyng 1996; Sausville and Burger 2006). Moreover, cells isolated from tumor xenografts show much similarity with cells isolated from donor patient's tumor in terms of sensitivity to drugs *in vitro* (Rofstad 1995). In addition, human xenografts sometimes show organ-specific metastatic patterns similar to those of the donor's tumor, allowing study of the effect of novel drugs not only on primary tumors but also on metastatic tumors in xenograft models (Rofstad 1995; Mi, Pezzuto et al. 2009). Despite the similarities between a tumor xenograft and the donor patient's tumor, data from clinical trials suggest that mouse xenograft models are inadequate in predicting the outcome of cancer therapeutics in human trials (Johnson, Decker et al. 2001; Sausville and Burger 2006; Siolas and Hannon 2013).

More recently, use of patient-derived xenograft (PDX) models is gaining interest in preclinical evaluation of drugs as they may more accurately predict the drugs' effects in cancer patients. PDX models are generated by grafting fresh human tumor specimens subcutaneously, orthotopically or under the kidney capsule of immune-deficient mice (Garber 2009; Tentler, Tan et al. 2012). One reason that these models are thought to be more realistic for preclinical evaluation of drugs is that the tumor contains stromal components from the donor patients, retaining the original tumor microenvironment, at least initially. Furthermore, the PDX maintains histology and genetic expression profiles of the original tumor (Garber 2009; Choi, Lin et al. 2014). The rate of engraftment of patient tumor tissue is lower than that of cancer cell lines, and varies between 23 to 75% depending on the tumor type (Siolas and Hannon 2013). Interestingly, the rate of

engraftment of tumors derived from breast cancer patients has been shown to correlate with the aggressiveness of the original tumors and survival of the donor patients (DeRose, Wang et al. 2011). Based on the strong co-relation between rate of engraftment and prognosis, investigators have suggested that the course of disease could be predicted from the rate of engraftment (DeRose, Wang et al. 2011). In general, the PDX model is better representative of patients' tumors compared to the traditional xenograft model and is expected to more reliably predict the outcome of cancer therapeutics in humans (Siolas and Hannon 2013).

1.3.4 Syngeneic Models

One serious limitation of the xenograft models is the lack of a functional immune system in the animal. The lack of a functional immune system prohibits the study of the interaction between candidate drugs and the immune system and the ultimate effect of such interactions on the tumors (HogenEsch and Nikitin 2012). In addition, a functional immune system is important for assessing whether anti-tumor immune responses are induced by direct effects of the drug on the tumor. Transgenic mice which spontaneously develop tumors provide a platform for preclinical evaluation of drugs in the presence of intact immune system (Politi and Pao 2011). Mammary-specific promoters have been used to drive expression of many known oncogenes specifically in the mammary epithelium in order to generate transgenic mouse models of BrCa (Fantozzi and Christofori 2006). For example, the oncogenes polyoma middle T antigen (PyMT) (Guy, Cardiff et al. 1992), erbB2/neu (Guy, Webster et al. 1992) and C-Myc (Schoenenberger, Andres et al. 1988) have been placed under control of the mouse mammary tumor virus (MMTV) long terminal repeat sequence to generate transgenic mice that develop BrCa.

MMTV-PyMT mice develop multifocal mammary carcinomas and metastatic tumors in the lymph nodes and lungs (Guy, Cardiff et al. 1992). In this transgenic model, the loss of estrogen/progesterone receptors during late-stage metastasis co-relates with over-expression of erbB2 and cyclin D1 similar to what is observed in breast tumors in humans (Maglione, Moghanaki et al. 2001). Furthermore, PyMT has been shown to activate many pathways that are also activated by erbB2 (HER2/neu), an oncogene overexpressed in human BrCa (Toneff, Du et al. 2010; Zhao and Ramaswamy 2014). The similarities between the tumors of MMTV-PyMT mice and human tumors make this model useful in BrCa research.

Unlike transplanting human cancer cells into mouse, cancer cells from one mouse can easily be transplanted into another mouse of the same immune-competent strain (syngeneic transplantation) without causing a host-versus-graft reaction. Thus syngeneic models allow one to investigate the role of the intact immune system in tumor progression and therapy (Gravekamp, Sypniewska et al. 2004; Fantozzi and Christofori 2006; Ottewell, Coleman et al. 2006). MTHJ, a cell line derived from a MMTV-PyMT transgenic mouse tumor, readily forms tumors when implanted into the mammary fat pad of FVB mice (Desilva, Wuest et al. 2012) and has been previously used to study anti-tumor immunity induced by an oncolytic virus (Hummel, Safroneeva et al. 2005). Moreover, the triple-negative murine BrCa cell line 4T1 is commonly used to generate tumors in immune-competent mice (Kaur, Nagaraja et al. 2012). This cell line was derived from a mammary tumor that arose spontaneously in a wild-type BALB/c mouse. 4T1 cells grow aggressively when injected into the mammary fat pad of syngeneic mice and form metastatic lesions in the brain, bones, lungs and liver (Aslakson and Miller

1992; Yang, Mani et al. 2004). Hence, 4T1 is a useful model for studying metastatic progression, the effect of candidate drugs on primary and metastatic tumors, and the role of the immune system in cancer progression and therapy. Another murine BrCa cell line that is used to generate tumors in mice is EMT6. This cell line was derived from a tumor that arose in a BAL/c mouse as a result of implanting a hyperplastic mouse mammary alveolar nodule (Rockwell, Kallman et al. 1972). EMT6 cells grow aggressively in mouse and efficiently form metastases in lungs and liver (Gorczynski, Chen et al. 2013).

1.4 Gene Therapy

Gene therapy is defined as an approach of correcting genetic abnormalities or treating a human disease through the use of nucleic acid-based drugs. The concept of gene therapy was conceived in the 1960s when it was discovered that viruses could cause transformation by integrating their genetic material into the genome of infected cells (Temin 1961; Das, Menezes et al. 2015). In 1966, Tatum proposed that viruses could be used for genetic manipulation of somatic cells in order to achieve therapeutic benefits (Tatum 1966; Das, Menezes et al. 2015). In 1970s, the first gene therapy in human was performed using Shope papilloma virus encoding a viral arginase to treat hyperarginemia in two young girls (Rogers, Lowenthal et al. 1973; Terheggen, Lowenthal et al. 1975). While no severe adverse effects were observed, the treatment failed to achieve a therapeutic effect (Terheggen, Lowenthal et al. 1975). In 1990, a retroviral vector encoding adenosine deaminase was used to treat two children suffering from severe combined immunodeficiency. One of the two children exhibited a positive response; this was the first evidence that gene therapy can be used to treat genetic disorders (Blaese, Culver et al. 1995; McCrudden and McCarthy 2014). The advent of recombinant DNA

technology greatly facilitated the advancement of gene therapy between 1963 and 1990 (Cotrim and Baum 2008).

According to the European Commission 2003/63 directive, "a gene therapy medicinal product is essentially a piece of nucleic acid that is delivered either *in vivo* or *ex vivo* and that can have prophylactic, diagnostic, or therapeutic values" (McCrudden and McCarthy 2014). This broad definition encompasses traditional gene therapy focused on delivering a normal gene to replace an abnormal gene or include cytotoxic genes to kill abnormal cells (*e.g.,* cancer cells), as well as more recent approaches to induce RNA interference (siRNA, shRNA and microRNA) or immune modulation (DNA vaccines) (McCrudden and McCarthy 2014).

1.5 Cancer Gene Therapy

The concept of gene therapy logically arose from the observation that certain diseases such as adenosine deaminase deficiency (Epstein, Cox et al. 1983), sickle cell anemia (Driss, Asare et al. 2009) and hyperarginemia (Epstein, Cox et al. 1983) are caused by defects in a single gene, thus could be treated and potentially cured by replacing the defective gene (Baltimore 1978; Porteus, Connelly et al. 2006). Although first conceived as a strategy for curing monogenic disorders, the field of gene therapy evolved in an unanticipated direction and cancer, a disease characterized by a variety of genetic alterations, became the main focus of gene therapy (Roth and Cristiano 1997). More than two-thirds of all gene therapy clinical trials approved to date have involved cancer (http://www.abedia.com/wiley/indications.php; accessed April, 2016). Unlike gene augmentation therapy where the goal is to restore a defective gene, the goal of many cancer gene therapies is to kill the cancer cells. Many different strategies have been

studied for direct or indirect killing of cancer cells through gene therapy including: introduction of a toxic gene, destruction of tumor vasculature, introduction of tumor-suppressor genes, functional deletion of an oncogene, and direct or indirect enhancement of the host immune recognition of tumor cells (Rosenfeld and Curiel 1996; Hughes 2004). Cancer gene therapy in general has three components: (i) a vector for delivering the therapeutic gene, (ii) regulatory sequences to control expression of the transgene if needed, and (iii) the therapeutic gene. Overall success of a cancer gene therapeutic is determined by the combination of efficiency of the vector in delivering therapeutic gene, activity and possibly specificity of the targeting strategy, and robustness of the therapeutic gene.

1.5.1 Vectors

Vectors used in gene therapy are either viral or non-viral. The non-viral vectors can be cellular such as dendritic cells (Chen, Emtage et al. 2001), tumor associated lymphocytes (Narayanan, Jaramillo et al. 2004) and autologous fibroblasts(Suminami, Elder et al. 1995), or non-cellular such as naked plasmid DNA (Nafissi, Sum et al. 2014) and liposomes (Roder, Keil et al. 2003). The advantages of using non-viral vectors are: they are less immunogenic, non-pathogenic, relatively easy to manufacture, and can carry genes of large sizes (Foldvari, Chen et al. 2015). Despite these advantages, non-viral vectors are less commonly used in gene therapy because they are fairly inefficient in transferring transgenes and the expression of transgenes delivered by non-viral vectors is more transient (Nishikawa and Huang 2001). Less than 30% of all gene therapy clinical trials to date have utilized non-viral vectors (Wiley 2016; http://www.abedia.com/wiley/vectors.php). In contrast, viral vectors are highly

efficient in transferring genes both *in vitro* and *in vivo*, and the expression of the transgene could be long-lasting depending on the type of virus (Hitt and Gauldie 2000; Nayerossadat, Maedeh et al. 2012). Most commonly used viral vectors are adenovirus, retrovirus, adeno-associated virus, herpes simplex virus, and vaccinia virus (Nayerossadat, Maedeh et al. 2012). The choice of vector is dictated by several factors including the type of target cells, the size of the therapeutic gene, and the desired duration of therapeutic gene expression (Table 1.1). Among 2210 gene therapy clinical trials approved to date, 506 used adenovirus, 420 used retro viruses, 165 used vaccinia virus, 137 used adeno-associated virus, 73 used herpes simplex virus, and the rest used other viral and non-viral vectors (http://www.abedia.com/wiley/vectors.php; accessed: April 2016).

Table 1.1: Comparisons of Viral Vectors for Gene Therapy.

	Adenovirus	Adeno-associated virus	Herpes Virus	Retro/lentivirus	Vaccinia virus
Genome	dsDNA	ssDNA	dsDNA	ssRNA (+)	dsDNA
Capsid	icosahedral	icosahedral	icosahedral	icosahedral	complex
Coat	protein	protein	protein	enveloped	enveloped
Virion diameter	70-90 nm	18-26 nm	150-200 nm	80-130 nm	170-200 nm
Genome size	36-40 kb	5 kb	120-200 kb	3-9 kb	130-280 kb
Infection/ tropism	dividing and quiescent cells	dividing and quiescent cells	dividing and quiescent cells	dividing	dividing and quiescent cells
Integration	no	no	no	yes	no
Transgene expression	transient	potentially long-lasting	potentially long-lasting	long-lasting	transient
Cloning capacity	8 kb	4.5 kb	>30 kb	8 kb	25 kb

Modified from: http://www.genetherapynet.com/viral-vectors.html

1.5.2 Adenovirus (excerpts from Chaurasiya and Hitt, 2016, Appendix)

1.5.2.1 Adenovirus: Origin and History

Rowe and colleagues first discovered adenovirus (Ad) in 1953 while trying to culture human adenoid tissue in the laboratory (Rowe, Huebner et al. 1953). Following the discovery of human Ad, nonhuman Ads have been isolated from a number of species including dog, mouse, chimpanzee, pigs as well as other mammalian and avian species (Wadell, Hammarskjold et al. 1980; SHENK 1996). After its discovery, Ad was extensively studied as a model system to understand basic eukaryotic cellular processes such as DNA replication, transcription, RNA splicing, and translation (McConnell and Imperiale 2004). The study of Ad led Sharp and colleagues to discover the existence of introns and the process of mRNA splicing (Berget, Moore et al. 1977). Human Ads belong to the genus *Mastadenovirus* which can cause mild illness in humans. Approximately 5—10% of all respiratory diseases in children are caused by Ads (Berk 2007). Ad infection may also result in conjunctivitis and gastroenteritis in children (Mautner, Steinthorsdottir et al. 1995; O'Brien, Jeng et al. 2009). Occasionally, Ad infection may cause severe complications especially in individuals with compromised immune systems such as patients with acquired immune deficiency syndrome and patients who have undergone organ transplants (La Rosa, Champlin et al. 2001; Kojaoghlanian, Flomenberg et al. 2003).

1.5.2.2 Adenovirus: Classification and Life-Cycle

More than 100 serotypes of Ad are known, among which 51 are isolated from humans. Based on sequence homology and their ability to agglutinate red blood cells, the

51 serotypes of human Ads have been classified into 6 groups: A to F (Wadell, Hammarskjold et al. 1980; Berk 2007). The serotypes most widely studied and most commonly used as vectors for gene therapy are Ad2 and Ad5, both of which belong to group C (SHENK 1996; Douglas 2007). The adenovirion is a non-enveloped icosahedral particle about 70-90 nm in size containing a linear double-stranded DNA genome of approximately 36 kilobase pairs (kb) (Horwitz 1990). The facets of the icosahedral capsid of the virion are composed mainly of trimers of hexon protein, and some other minor proteins. The vertices of the capsid are composed of penton bases anchoring the fiber proteins that are responsible for the primary attachment of the virion to the cell surface (Horwitz 1990).

The first event in virus infection is the binding of fiber protein to the coxsackievirus adenovirus receptor (CAR) on the cell surface (Bergelson, Cunningham et al. 1997). This is followed by a secondary interaction between virion penton and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins leading to internalization of the virion by clathrin-dependent endocytosis (Defer, Belin et al. 1990; Wang, Guan et al. 2000). The levels of primary (CAR) and secondary (integrins) receptors present on the cell surface determine the efficiency with which the cell will be infected by adenovirus (Ambriovic-Ristov, Gabrilovac et al. 2004). After internalization, the acidic environment of the endosome leads to escape of the virion to the cytoplasm. Here the virion is trafficked by dynein along microtubules towards the nucleus (Kelkar, Pfister et al. 2004). During translocation towards nucleus, the virion undergoes sequential disassembly and the viral genome is ultimately imported to the nucleus through the nuclear pore complex. Viral DNA

replication begins 6-8 hours post-infection and it takes 24-36 hours for the virus to complete its life cycle (Liu, Naismith et al. 2003).

The viral genome is flanked by inverted terminal repeats (ITRs) of 90-140 base pairs which are required in *cis* for the replication of the viral genome (Garon, Berry et al. 1972; Wolfson and Dressler 1972). The ITRs are covalently bound by terminal protein(McConnell and Imperiale 2004). In addition to the ITRs, the packaging signal (ψ) is also required in *cis* for proper folding and packaging of the viral genome into the capsid (Ostapchuk and Hearing 2003). The viral genome is divided into non-contiguous, overlapping early and late transcription regions: E1A, E1B, E2, E3 and E4 are early genes whereas L1 to L5 are late genes (Horwitz 1990; SHENK 1996). The products of early genes as well as the replication of viral DNA are prerequisites for the expression of late genes (Thomas and Mathews 1980).

E1A, the first transcription unit to be expressed, produces two major proteins following differential mRNA processing. These proteins are required for the transcriptional activation of other early genes (E1B, E2, E3 and E4) and also to induce an S-phase like state in the infected cells (Moran and Mathews 1987). The E1A proteins bind to retinoblastoma protein allowing the release of E2F, ultimately forcing the infected cells to enter into S-phase (Goran Akusjarvi 1986). Because of the crucial role of E1A in viral replication, E1A is often deleted in order to make the virus replication-deficient. The two major products of the E1B transcription unit are involved in blocking host mRNA transport, promoting viral mRNA transport and blocking E1A-induced apoptosis to prevent premature death of the infected cells (Pilder, Moore et al. 1986; Moran 1993). The E1B product (E1B-55kDa) directly binds to the p53 protein to block E1A-induced

apoptosis. E1A and E1B are considered oncogenes as they have the ability, when used in combination, to transform human and rodent cells *in vitro* (Graham 1984; SHENK 1996).

The two transcription units in the E2 region encode proteins required for the replication of viral DNA (Jared D. Evans 2002). E2a encodes the 72-kDa DNA-binding protein whereas E2b encodes the viral DNA polymerase and terminal protein precursor (pTP). The E3 region encodes at least seven proteins most of which are involved in subversion of the host immune system to allow a more robust infection. For example, E3-gp19K blocks the presentation of viral antigens by MHC class I thus preventing lysis of the infected cells by cytotoxic T lymphocytes (Jared D. Evans 2002). The E3 region is non-essential for virus replication *in vitro*. At least six proteins are encoded by the E4 region. The products of the E4 region have diverse functions including facilitation of viral DNA replication, enhancement of late gene expression, and down-regulation of host protein synthesis (Jared D. Evans 2002). This region can also play a role in promoting the transforming ability of E1A (Moore, Horikoshi et al. 1996).

All the late region genes (L1-L5) are expressed from a common promoter called major late promoter (MLP). The primary major late transcript undergoes alternative splicing to produce individual transcripts. The products of late genes are mainly structural in function (Jared D. Evans 2002).

1.5.2.3 Adenovirus Vectors

During the late 1960s it was found that adenoviruses can recombine during growth in culture. This finding ultimately set the stage for the use of Ad as a vector for gene delivery to cells both *in vitro* and *in vivo* (Lewis, Baum et al. 1966; Pierce, Rosenbaum et al. 1968; Lewis and Rowe 1970). Ads have many features that make them

a suitable vector for gene therapy including: i) the viral genome is relatively easy to manipulate by recombinant DNA technology; ii) scaling up and purification of the recombinant virus for use in the clinic is relatively easy; iii) the virus infects both quiescent and dividing cells with high efficiency; iv) recombinant viruses are fairly stable as the viral genome does not undergo rearrangement at a high rate; v) in permissive cells the virus replicates to high levels producing up to 10,000 plaque forming units (pfu) per infected cell; and vi) high levels of transgene expression are achieved (Hitt and Gauldie 2000; Sadeghi and Hitt 2005). Moreover, the viral genome is maintained as an episome in the infected cell and rarely integrates into the cellular genome. This increases the safety of adenoviral vectors as the risk of insertional mutagenesis is quite low. However, because of the episomal nature of the vector genome, transgene expression is transient in dividing cells (Sadeghi and Hitt 2005). These features have made Ad a vector of choice for gene therapy which is evident from the fact that adenoviral vectors have been used in almost a quarter of all the gene therapy clinical trials performed to date (Wiley, 2016; http://www.abedia.com/wiley/vectors.php).

Different regions of the viral genome can be replaced with transgene(s) to generate mammalian gene transfer vectors. As described above, E1A-encoded proteins are crucial for the expression of both early and late viral genes and hence for replication of the virus. Deletion of the E1A region not only makes the virus replication-deficient but also increases the cloning capacity of the vector. The packageable viral genome is limited in length to 105% of the wild-type genome size, thus one can insert only up to 1.8 kb in the vector without deletion of any viral sequences (Bett, Prevec et al. 1993). However, deletion of the E1 region allows insertion of transgenes up to 5.1 kb in size. Because E3-

encoded proteins are non-essential for virus replication *in vitro*, the E3 region is often removed from Ad vectors. Deletion of E3 together with E1 can further increase the cloning capacity, accommodating insertion of foreign genes up to 8.2 kb in size. Ad vectors deleted in E1, both with and without E3 deletion, are referred to as first generation vectors (Danthinne and Imperiale 2000). First generation vectors are the most commonly used Ad vectors for the purpose of gene therapy.

1.5.3 Targeting Modality

Targeting of specific types of cell by viral vectors can be achieved at 3 different levels: (i) transductional targeting (ii) transcriptional targeting and (iii) post-transcriptional targeting. Transductional targeting refers to modifications of the virus in order to enhance infection of a particular type of cell or to reduce infection of non-target cells. For example, adenovirus transduces cells by binding to CAR (Bergelson, Cunningham et al. 1997), however, many types of cancer cells lack CAR (Li, Pong et al. 1999; Kim, Zinn et al. 2002). Furthermore, adenovirus has high liver tropism; systemic injection of Ad (or dissemination from local injection) in animals results in rapid uptake by the liver (Huard, Lochmuller et al. 1995; Hiltunen, Turunen et al. 2000). This can lead to severe liver toxicity. Different modifications, especially in the fiber protein have been shown to successfully target cancer cells and/or de-target liver and other normal cells (Goldman, Rogers et al. 1997; Dmitriev, Krasnykh et al. 1998; Nicklin, Dishart et al. 2003).

Transcriptional targeting is the second level of targeting that comes into effect after the virus has already infected the cells. Transcriptional targeting is aimed at restricting the expression of therapeutic gene to the target cells. This could be achieved

by using tissue/tumor specific promoters to control the transgene (Sadeghi and Hitt 2005). Many types of cancers express high levels of certain transcripts that are not expressed, or expressed to very low levels in normal cells. The regulatory elements (promoters/enhancers) of such genes, when used to drive a therapeutic gene, could restrict the expression of the gene to cancer cells. One such promoter is the human telomerase reverse transcriptase (hTERT) promoter that is highly active in 85-95% of primary cancers and 100% of tumor derived cell lines (Kim, Piatyszek et al. 1994; Shay 1995). This promoter has been widely studied as a means for transcriptional targeting of toxic genes to cancer cells (Pan and Koeneman 1999; Koga, Hirohata et al. 2001; Gu, Andreeff et al. 2002). Other examples of tissue/tumor specific promoters that have been studied for their utility in transcriptional targeting of therapeutic genes include promoter elements from the prostate specific antigen, survivin, hypoxia-inducible factor-1, alpha-fetoprotein and alpha-lactalbumin (reviewed in(Sadeghi and Hitt 2005)). Interestingly, some viral promoters are active only in certain types of cancer but not in normal cells. For example, Epstein-Barr virus (EBV) promoter BamC has been shown to be very specific for EBV-related B-cell lymphomas (Franken, Estabrooks et al. 1996; Harada, Yalamanchili et al. 1998). The high specificity of this EBV promoter is due to the fact that transcription factors for EBV promoters are present in those cells (Franken, Estabrooks et al. 1996).

Transcriptional targeting is not only important for targeting therapeutic genes to cancer cells but is also an important strategy for rescuing vectors encoding toxic genes. High levels of transgene expression is usually desirable in the target cells, however, construction and propagation of Ad vectors encoding such toxic genes are challenging as

the transgene expression can induce toxicity in the packaging cells, reducing vector yield (Rubinchik, Norris et al. 2002). In some cases the toxicity in the packaging cell line is so severe that the cells die after transfection with the vector DNA resulting in total failure to obtain a live viral vector. In other cases the toxicity places a strong selective pressure on the resulting viral vector to reduce or completely eliminate transgene activity. This selective pressure may give rise to revertants or to mutations within the transgene expression cassette leading to reduction or complete ablation of transgene expression. The replicative advantage of these revertant/mutant viruses over the desired vector would reduce the feasibility of large scale vector production (Rubinchik, Norris et al. 2002). The most common approaches to address this situation involve differential regulation of transgene expression at the transcriptional level in the packaging and target cells (Figure 1.1).

Post-transcriptional targeting is seldom used on its own, usually being employed in addition to transcriptional targeting in order to ensure that the transgene is not expressed in non-target cells due to leakiness in the tumor/tissue specific promoters. MicroRNAs (miRNA) that are downregulated in cancer cells are generally exploited for post-transcriptional targeting of therapeutic genes in cancer gene therapy (Suzuki, Sakurai et al. 2008; Cawood, Chen et al. 2009). For example, insertion of the recognition sequence for the liver specific miRNA, miR-122, in the 3'UTR of a suicide gene has been shown to reduce hepatotoxicity by detargeting liver (Suzuki, Sakurai et al. 2008).

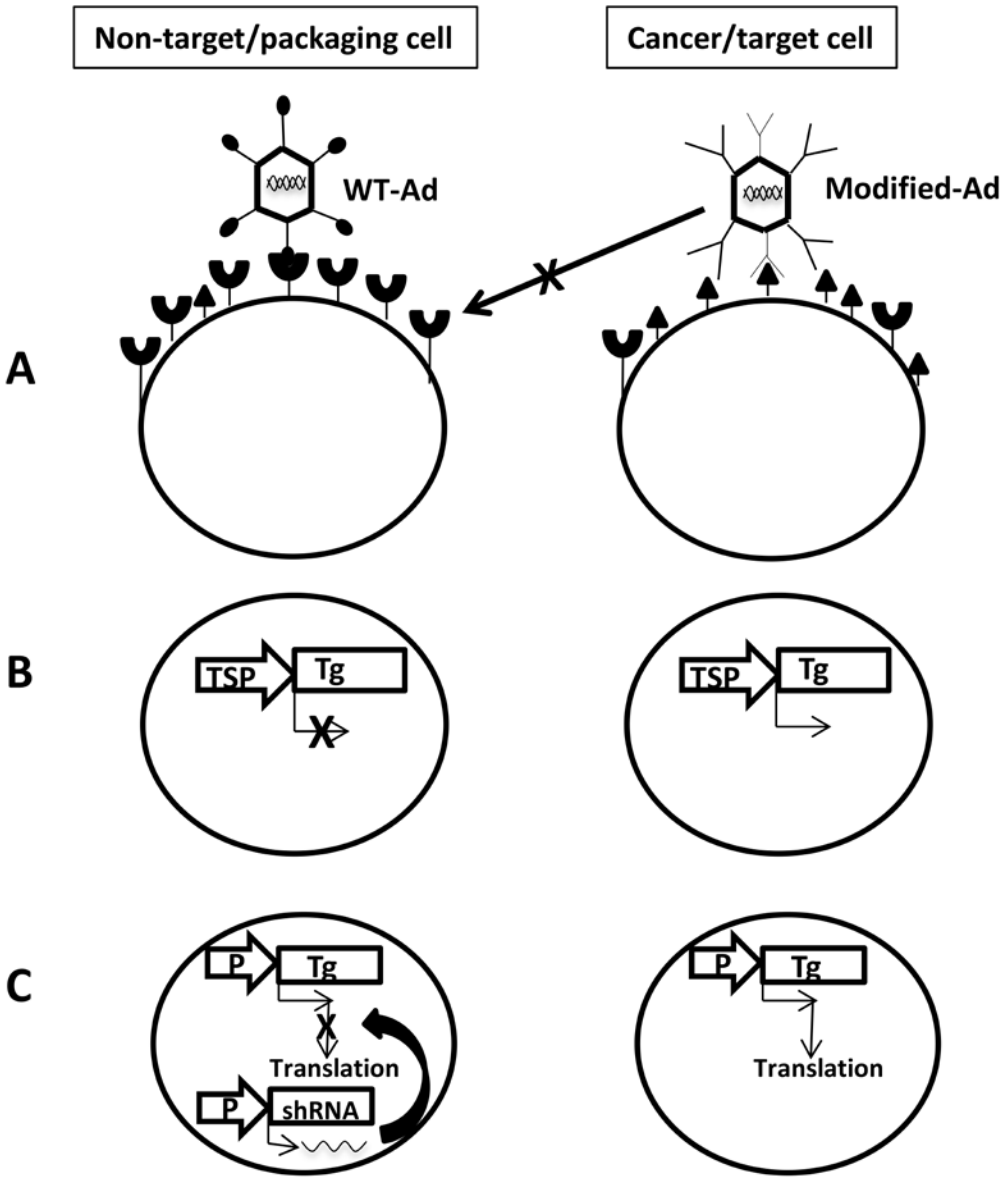

Non-target/packaging cell
Cancer/target cell
WT-Ad
Modified-Ad
A
B
TSP
Tg
TSP
Tg
C
P
Tg
Translation
P
shRNA
P
Tg
Translation

Figure 1.1: Targeting modalities. (A) Transductional targeting. Targeting of cancer cells and de-targeting of normal cells can be achieved by modifying the fiber knob of the virus such that it can bind only to receptors that are abundant on cancer cells but not on normal cells. (B) Transcriptional targeting. Tissue/tumor specific promoter can be used to control expression of transgene, *e.g.,* toxic genes, which facilitate rescue of the virus and also increase cancer specificity. (C) Translational targeting by the use of shRNA. Expression of shRNA targeting the transgene, from a different vector, can block the transgene expression and facilitate rescue of virus. (WT-Ad, wild type adenovirus; TSP, tissue/tumor specific promoter; Tg, Transgene; P, promoter; shRNA, short-hairpin RNA). Adapted and modified from (Chaurasiya and Hitt 2016).

1.5.3.1 The Mammaglobin Promoter/Enhancer for Transcriptional Targeting of Therapeutic Genes to Breast Cancer

Mammaglobin-1 (MGB), encoded by *SCGB2A2*, belongs to the secretoglobin family of polypeptides, which consists of small secreted polypeptides (Watson and Fleming 1996). Although the genes in the secretoglobin family are regulated by steroid hormones, expression of MGB has been shown to be independent of steroid hormones (Watson, Darrow et al. 1998). MGB is a 93 amino acid polypeptide with a molecular mass of 23.4 (glycosylated form) or 16.2 (deglycosylated form) kDa. The gene was originally identified by Watson and Fleming in 1996 while searching for genes with altered expression in primary BrCa compared to normal breast tissue (Watson and Fleming 1994). In humans, the MGB gene maps to chromosome 11 band q13, a genomic region that is frequently amplified in BrCa (Watson, Darrow et al. 1998). Previously MGB was thought to be exclusively expressed in mammary tissue (Goedegebuure, Watson et al. 2004), however, recent studies have shown that MGB is expressed in gynecologic carcinomas, and over-expression of MGB is linked to tumor progression in ovarian cancer (Hagemann, Pfeifer et al. 2013; Fischer, von Brunneck et al. 2014).

More than 80% of all BrCa express MGB, and the level of MGB expression in BrCa is at least 10-fold higher compared to that in normal breast epithelium (Watson, Dintzis et al. 1999). Although previous studies surmised a possible link between MGB expression and tumor aggressiveness, recent studies by Picot *et al.* show that MGB may play a direct role in activating some malignant features of BrCa cells (Picot, Guerrette et al. 2015). Using gene knock-down experiments, they showed that loss of MGB expression leads to reduction in cell proliferation and migration. Also, cells were found to

have reduced ability to form spheroids in soft agar as well as reduced migration and invasion in response to MGB knock-down, *in vitro*. Furthermore, they showed that MGB regulates epithelial to mesenchymal transition and modulates the expression levels of mesenchymal-related genes *Snail*, *Twist* and *ZEB1*. Interestingly, they also found that over-expression of MGB sensitizes BrCa cells to chemotherapeutics (Picot, Guerrette et al. 2015).

Since MGB shows mammary tissue specific expression, with elevated levels in BrCa, it has been studied as a possible diagnostic marker for BrCa. Its expression has been detected in BrCa metastasized to lymph node and brain (Dono, Ferro et al. 2009; Verbanac, Min et al. 2010). Furthermore, since MGB is not expressed by cells in blood and lymph nodes, it is considered to have diagnostic value for disseminated and circulating BrCa cells (Zehentner and Carter 2004). MGB is one of the most promising markers for BrCa (Zehentner and Carter 2004), with over 100 studies published, according to the ISI Web of Science, on its utility in the diagnosis of BrCa.

In cancer gene therapy, tissue-specific promoters are useful for driving cytotoxic genes preferably at sites such as breast where loss of normal cells has minimal effect on viability or functioning of patients (Hart 1996). This makes the MGB promoter an excellent element for targeting a therapeutic gene to BrCa cells. In line with this, Dr. Hitt's group has previously shown that the MGB promoter can be used for BrCa specific expression of a reporter transgene (Shi, Long et al. 2004). The group also showed that elements controlling the BrCa specificity reside within 345 bp upstream of the coding sequence (Shi, Long et al. 2004). This minimal promoter (345 bp), although specific, is much weaker than the ubiquitously expressed immediate early promoter of

cytomegalovirus (CMV). They also identified a putative enhancer element several kilobases upstream of the minimal promoter and then inserted two tandem copies of this potential enhancer element upstream of the minimal MGB promoter (MPE2). This engineered MPE2 promoter was shown to have nearly comparable activity (within an order of magnitude) to the murine CMV promoter in BrCa cells *in vitro* and in tumors in mice (Shi, Graham et al. 2006). The activity of MPE2 was much lower compared to that of the CMV promoter in non-tumorigenic cell lines of human and murine origin *in vitro*, and MPE2 was found to be almost inactive in livers of mice (Shi, Graham et al. 2006).

1.5.4 Therapeutic Genes

A large number of genes ranging from pro-apoptotic genes such as Bax, TNF-alpha, TNF-alpha related apoptosis inducing ligand (TRAIL), Bik and p53 (see an excellent review on this topic by (Lo, Day et al. 2005)) to immune-stimulatory genes such as interleukin-2 (IL-2), IL-12, IL-15, IL-21 and GM-CSF (reviewed by (Hitt and Gauldie 2000; Qian, Liu et al. 2006)) have been studied in preclinical models as therapeutic genes in cancer gene therapy. This study focuses on IL-2 as a therapeutic gene for BrCa therapy.

1.5.4.1 IL-2 as Therapeutic Gene

IL-2 was discovered in the mid-1970s as T cell growth factor because of its proliferative effect on T lymphocytes (Morgan, Ruscetti et al. 1976). Later studies showed that IL-2 is a pleiotropic cytokine which helps in the growth and function of natural killer cells, macrophages, B cells, helper-T cells, cytotoxic T lymphocytes and other immune cells (Gaffen and Liu 2004). This cytokine is secreted by activated T cells and acts in both an autocrine and a paracrine manner (Hagiwara, Abbasi et al. 1995). IL-2

is a monomeric, secreted glycoprotein with molecular weight of ~15 kDa that exerts its function by binding to the high affinity receptor, IL-2R, expressed on the target cells (Nelson and Willerford 1998). Interestingly, IL-2 is not only required for the growth of T cells but it also plays a crucial role in maintaining immune cells homeostasis as evident from severe autoimmunity due to failure of eliminating activated T cells in mice lacking IL-2 (Kundig, Schorle et al. 1993). T cells homeostasis is maintained by a phenomenon called 'activation induced cell death', in which T cells undergo apoptosis after repeated antigenic stimulation and IL-2 is critically required for this phenomenon (Lenardo 1991).

During late 1970s, Kurnick *et al.* demonstrated that supernatants from *in vitro* cultures of stimulated T cells have the ability to mediate long-term survival and functioning of T cells in culture (Kurnick, Gronvik et al. 1979). Later, it was noted that exposure to IL-2 could generate cytotoxic cells, *in vitro*, from mouse splenocytes or human peripheral blood mononuclear cells (Grimm, Mazumder et al. 1982). These cells, called lymphokine activated killer (LAK) cells, were found to have the ability to selectively kill cancer cells which made investigators surmise that IL-2 treatment may potentially stimulate functional T cells *in vivo* and result in an anti-tumor effect (Grimm, Mazumder et al. 1982). However, initially the studies with IL-2 were very difficult because of the inability to obtain a large amount of IL-2 from cell culture. After the discovery of the IL-2 gene in 1983, and subsequent cloning of the IL-2 cDNA in *E. coli* it became feasible to produce and purify recombinant IL-2 (rIL-2) on a large scale (Devos, Plaetinck et al. 1983; Taniguchi, Matsui et al. 1983). In mice, rIL-2 showed an anti-tumor effect however only at doses that resulted in significant toxicities (Rosenberg, Mule et al. 1985). Soon after it became feasible to produce large scale rIL-2, several clinical trials

were performed. Unfortunately, no sign of anti-tumor response was seen in any of the studies (reviewed in (Rosenberg 2014)). Later, different doses of rIL-2 in combination with LAK cells were evaluated in clinical trials and objective responses were observed in a fraction of patients but only at maximum tolerable doses of IL-2. The addition of LAK cells did not increase anti-tumor activity of the IL-2, hence LAK cells were not used in further studies (reviewed in (Rosenberg 2014)). Later, several preclinical and clinical studies showed that IL-2 has appreciable anti-tumor activities; however it is not exactly clear how this cytokine exerts its anti-tumor effect. Histologic studies in animal models have found necrotic zones in IL-2 treated tumors and infiltration by cytotoxic lymphocytes such as macrophages, lymphokine activated killer cells, NK cells and neutrophils (Cavallo, Giovarelli et al. 1992; Bannerji, Arroyo et al. 1994; Cordier, Duffour et al. 1995). CD8+ T cells are thought to be important in IL-2 mediated tumor regression and long-term tumor-specific protection while CD4+ T cells are not important for the anti-tumor response induced by IL-2 (Fearon, Pardoll et al. 1990; Hock, Dorsch et al. 1993; Slos, De Meyer et al. 2001). Macrophages, LAK and NK cells probably have roles in non-antigen specific tumor cell killing (Rosenberg, Lotze et al. 1989; Cavallo, Giovarelli et al. 1992; Slos, De Meyer et al. 2001).

In 1992, IL-2 was approved by the FDA for the treatment of renal cancer and metastatic melanoma (Coventry and Ashdown 2012). In order to achieve therapeutic benefit, high dose IL-2 is systemically administered to patients: about 15-20% of patients show objective responses whereas 5-7% patients show durable complete responses (Fisher, Rosenberg et al. 2000; Atkins 2002; Atkins, Regan et al. 2004). Although meaningful treatment response is achieved in a subset of patients, high dose IL-2 can

result in life-threatening side effects such as vascular leak syndrome (VLS) (Baluna and Vitetta 1997). VLS is a particularly severe form of toxicity which is marked by accumulation of extravascular fluid in vital organs such lungs and liver (Nakagawa, Miller et al. 1996; Epstein, Mizokami et al. 2003). Currently there is no treatment for VLS and the only option is to discontinue IL-2 therapy (Laurent, Touvrey et al. 2013). Furthermore, preclinical and clinical studies have shown that systemic administration of IL-2 may cause anemia, cardiovascular damage, anorexia, seizures and coma (Siegel and Puri 1991). The side effects associated with systemic use of IL-2 severely limit its use in cancer therapy. Different approaches have been studied by different research groups to bypass IL-2 toxicities. One way to minimize the side effects of systemic IL-2 therapy could be to limit the high concentration of IL-2 to the tumor vicinity (Den Otter, Jacobs et al. 2008). One of the few approaches for targeting IL-2 to tumor is through the use of fusion protein in which IL-2 is fused with an antibody against a protein that is highly expressed by tumor cells (Becker, Varki et al. 1996; Penichet, Dela Cruz et al. 2001; Davis and Gillies 2003). Furthermore, restricting IL-2 to the tumor microenvironment should not only reduce toxicity but may also increase the therapeutic effect. This hypothesis is based on the finding by Lee *et al.* that IL-2-transfected cancer cells formed tumors in mice that were more vascularized and had higher numbers of infiltrating lymphocytes compared to tumors made by the parental cancer cells (Lee, Fenton et al. 1998).

Previous studies have shown that IL-2 has minimal anti-tumor effect in BrCa when used as a single agent. We, therefore, wished to study anti-tumor effect of IL-2 gene therapy in combination with a pro-apoptotic drug with the anticipation that the drug-

mediate apoptosis of cancer cells may release tumor antigens that might boost the overall anti-tumor activity of IL-2.

1.6 PAC1: a Pro-apoptotic Drug

Procaspase activating compound 1 (PAC1) is a novel molecule that was synthesized by Dr. Hergenrother's group at the University of Illinois, USA. This compound has been shown to induce apoptosis selectively in cancer cells of different origins both *in vitro* and in animal models (Putt, Chen et al. 2006). Apoptosis is a type of programmed cell death that can be initiated by two routes: the extrinsic pathway and the intrinsic pathway. A family of proteins called cysteine-aspartic proteases (caspases) play critical roles in both extrinsic and intrinsic pathways of apoptosis (Elmore 2007). In the extrinsic pathway, binding of a death ligand such as Fas ligand to its specific transmembrane receptor on the cell marks the initiation of apoptosis. A 'death domain' connected to the cytoplasmic portion of the receptor transmits the signal to the intracellular players of apoptosis. In response to the signal, initiator caspases (caspase-2, 8, 9, and -10) are activated followed by the activation of executioner caspases (caspase-3, 6, and -7) (Cohen 1997). The executioner caspases normally remain in their inactive zymogenic form, called procaspases, that need to be cleaved in order to become active (Chang and Yang 2000). Cleavage of executioner caspases is mediated by the active initiator caspases. Among the 3 executioner caspases, the most intensively studied and the most critical is caspase-3 (Elmore 2007). The intrinsic or mitochondrial apoptotic pathway begins with the release of cytochrome c from mitochondria to the cytoplasm (Wang 2001). Both the extrinsic and intrinsic apoptotic pathways rely on executioner

caspases, especially caspase-3, in order to induce apoptosis in the cell through the cleavage of various proteins, and DNA fragmentation (Elmore 2007).

Levels of intracellular zinc have been known to modulate the ability of a cell to undergo apoptosis (Truong-Tran, Ruffin et al. 2000). Studies have shown that addition of zinc could reduce the levels of apoptosis in cells, whereas depletion of zinc could induce apoptosis(Treves, Trentini et al. 1994). The effect of zinc on apoptosis is thought to be through direct binding of zinc to procaspase-3 preventing its cleavage and activation (Perry, Smyth et al. 1997; Truong-Tran, Ruffin et al. 2000). Interestingly, compared to normal cells, many types of cancers including BrCa, have been found to have higher levels of procaspase-3 (Fink, Schlagbauer-Wadl et al. 2001; O'Donovan, Crown et al. 2003; Krepela, Prochazka et al. 2004). This suggests that a drug that could activate procaspase-3 by relieving zinc-mediated inhibition may be able to selectively kill cancer cells. Furthermore, with the ability to directly activate procaspase-3, the drug could bypass defects in the apoptotic pathways upstream of executioner caspases, which are not uncommon in cancer cells. Interestingly, PAC1 has high affinity for zinc ions; it is thus able to chelate and sequester zinc ions that are loosely bound to procaspase-3 allowing the procaspase-3 to undergo auto-cleavage, ultimately leading to apoptosis (Peterson, Goode et al. 2009). Several studies have demonstrated the ability of PAC1 to induce apoptosis in a variety of cancer cell lines *in vitro* and in several animal tumor models (Putt, Chen et al. 2006; Peterson, Hsu et al. 2010). A phase I clinical trial has been recently started with PAC1 against multiple malignancies (NCT02355535).

1.7 Oncolytic Viruses

1.7.1 Overview and History

Oncolytic viruses represent a novel class of cancer bio-therapeutics that can selectively replicate in and kill cancer cells while leaving normal cells unharmed. The idea of using viruses for the treatment of cancer is not new; it dates back to the beginning of 20th century when spontaneous regressions of tumors were observed in cancer patients after vaccination with live rabies virus or during the course of a natural infection with other viruses (Dock 1904; Pelner, Fowler et al. 1958). At that time there was little faith in the contemporary therapeutic approaches; and the observation that viral infection could have some anti-tumor effect led researchers to propose that viruses may be used to treat cancer (Dock 1904; Kelly and Russell 2007). The advent of cell and tissue culture during the 1950s allowed researchers to propagate and study viruses with consistency, and it was during this period that the field of virotherapy rapidly progressed (Weller, Robbins et al. 1949; Gey 1952). This was the time when the oncolytic properties of several viruses were determined in human cancer cell lines *in vitro* and in rodent models as well as in human patients. Among the first viruses to be tested in humans were Hepatitis B virus, measles and West Nile viruses (Hoster, Zanes et al. 1949; Southam and Moore 1952; Georgiades, Zielinski et al. 1959). In these early studies, tissue or fluids harvested from patients with ongoing viral infection were injected in cancer patients through multiple routes. By current ethical standards, most of those earlier studies would be deemed inappropriate; however, the desperate need for effective therapy must have played a role in dismissing any safety concerns. In terms of efficacy, most of the human trials resulted in discouraging outcomes. Occasional tumor regressions, mostly in immune-suppressed

patients, were observed, although they suffered severe side effects or even died when the virus spread to normal tissues (reviewed in(Kelly and Russell 2007)). The lack of appreciable anti-tumor efficacy of oncolytic virotherapy and the severe side effects emanating thereof, together contributed to the loss of interest in the field during the 70s and 80s (Kelly and Russell 2007). In the century since viruses were first recognized to be beneficial for cancer patients, the field of cancer virotherapy has been a roller-coaster ride, reaching near-abandonment at one point followed by a vigorous resurgence of interest, culminating in the approval of an oncolytic adenovirus in China (2005) and approval of an oncolytic herpesvirus by the FDA (2016) (Garber 2006; Kelly and Russell 2007; Greig 2016).

1.7.2 Mechanism of Viral Onco-tropism

Although tumor progression is thought to be a stochastic process, during the process of tumorigenesis cancer cells acquire hallmark changes including resistance to apoptosis, growth independence, immune evasion, enhanced angiogenic capacity and metabolic deregulation (Hanahan and Weinberg 2011). Interestingly, these properties of cancer cells that allow them to thrive also make them better hosts for many types of viruses. For example, cancer cells often have non-functional innate immune defense mechanism as a consequence of their need to evade detection and destruction by immune system. Cancer cells with defective interferon pathways are highly permissive to vesicular stomatitis virus, myxoma virus and raccoonpox virus which are otherwise attenuated in normal cells (Stojdl, Lichty et al. 2000; Everts and van der Poel 2005; Evgin, Vaha-Koskela et al. 2010). Also, cancer cells tend to resist apoptosis and translational suppression, both of which are favorable for the growth of several types of

viruses (Russell, Peng et al. 2012). Furthermore, over-expression of certain virus-receptors by cancer cells allow higher uptake of viruses compared to normal cells. For example, some types of cancer cells over-express CAR (Martin, Watkins et al. 2005), laminin (Sanjuan, Fernandez et al. 1996), CD155 (Masson, Jarry et al. 2001) and CD46 (Anderson, Nakamura et al. 2004) which allow for higher uptake of adenovirus, sindbis virus, polio virus and measles virus, respectively.

In 1950s, viruses were found to be capable of adapting to replication in specific tissues (Moore 1952; Southam and Moore 1952). This property was then utilized for targeted evolution of viruses to make them more cancer specific. Moore et al. (1952) were able to increase cancer specificity of a virus by continuously propagating them in cancer cells (Moore 1952). The increased cancer specificity was thought to be the result of acquired mutations in some progeny viruses giving them a selective growth advantage (Moore 1952). Although, it was suggested that modulation in viral genome could increase cancer specificity, direct manipulation of viral genome was not possible at that time (Southam 1960).

By the early 1990s when recombinant technology became a standard tool, researchers focused on directly manipulating viral genomes to specifically target them to cancer cells. With the increasing knowledge about how viral gene products control the mammalian cell cycle and disable cellular defense mechanisms, it is becoming more feasible to augment or eliminate specific viral functions to enhance their antineoplastic efficacy (Mullen and Tanabe 2002). In general, three strategies are used to make a virus cancer specific: (i) deletion of viral genes whose function would be complemented in cancer cells but not in normal cells, (ii) transcriptional targeting of essential viral gene(s)

with cancer specific promoters, and (iii) transductional targeting by modification of viral surface proteins (reviewed in (Vaha-Koskela, Heikkila et al. 2007)). I will return to the first of these mechanisms after a discussion of anti-tumor modes of action that are common to numerous oncolytic viruses, regardless of their mechanism of targeting.

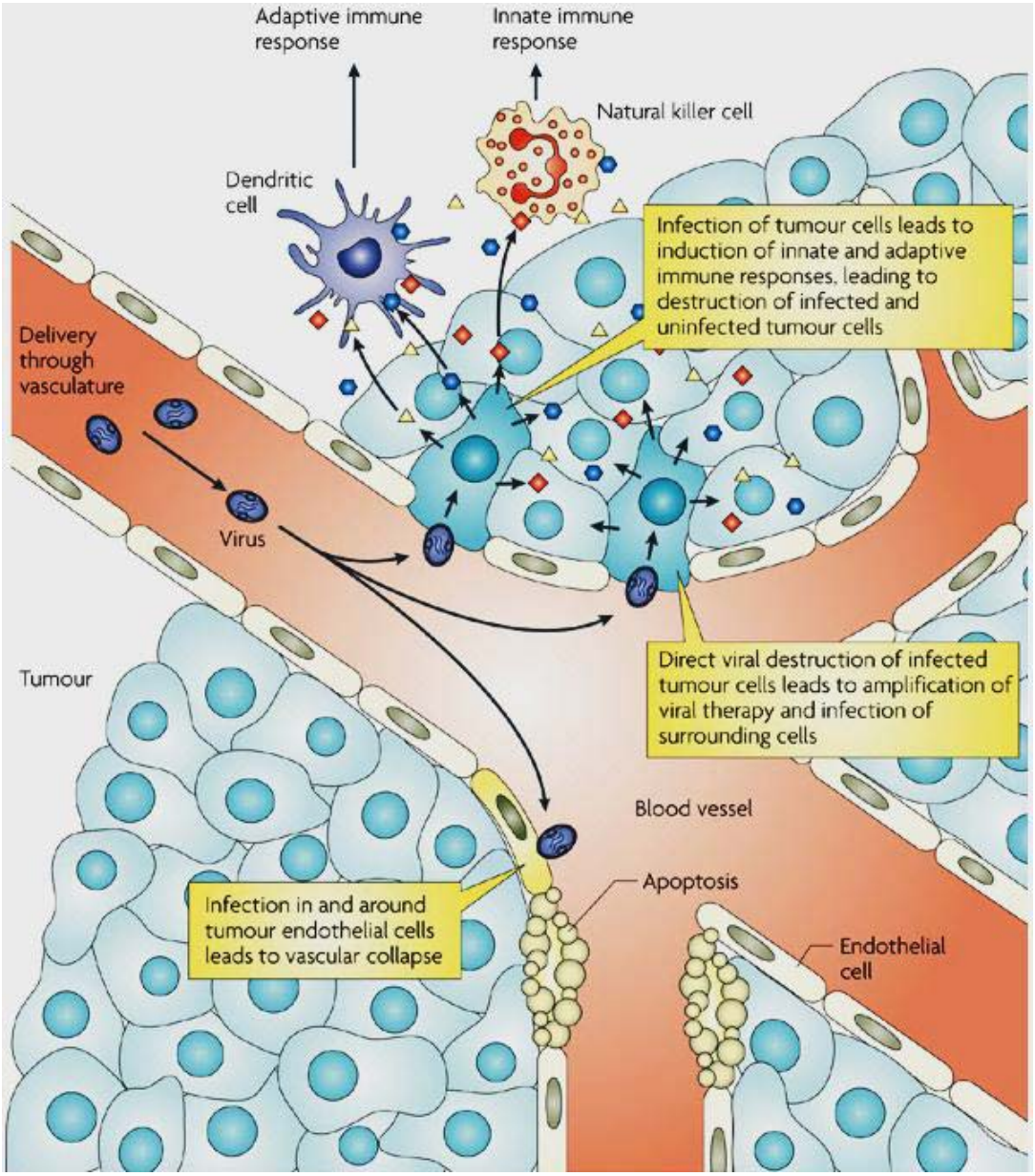
1.7.3 Oncolytic Viruses: Modes of Action that Directly Affect Cancer Cells

Oncolytic viruses are thought to exert their antineoplastic activities through a variety of ways. While the exact mechanism of oncolysis differs from virus to virus and even for the same virus depending on the structure and encoded transgene; there are some common mechanisms employed by most oncolytic viruses to achieve an antineoplastic effect (Figure 1.2). First, replication of many different viruses in a cancer cell can induce lysis of the cell (Mullen and Tanabe 2002). Cell death caused by direct replication of oncolytic viruses is complex and does not clearly fit into anyone of the traditional modes of cell death such as apoptosis, necrosis and autophagy (Russell, Peng et al. 2012). This is partly because oncolytic viruses are thought to hijack the cell death machinery, allowing death to occur only when cellular resources have been fully exploited for maximal production of progeny viruses (Russell, Peng et al. 2012). Oncolytic viruses are self-perpetuating and a single dose of the virus, in theory, could eliminate all the cancer cells unless the virus itself is cleared by the immune system before the cancer cells are eliminated (Liu, Thorne et al. 2008).

Second, some viral proteins are toxic to cancer cells which can directly kill the cell before replication-mediated lysis. For example, the 11.6 kDa E3 death protein and E4orf4 proteins encoded by adenovirus are toxic to cells (Tollefson, Ryerse et al. 1996; Shtrichman and Kleinberger 1998). However, this type of cell death by oncolytic viruses

is often undesired since premature cell death prevents optimal release of oncolytic viruses from the infected cells for subsequent rounds of infections.

Figure 1.2: Different mechanisms by which oncolytic viruses exert antineoplastic effect(Kirn and Thorne 2009). This figure is adapted with permission from Macmillan Publishers Ltd: Kirn DH and Thorne SH. ***Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer.*** Nat Rev Cancer 2009;9(1):64-71.



Vaccinia delivered to the tumour through the vascular system can produce an anti-tumour effect through multiple mechanisms, which include viral infection and tissue destruction. This leads to release of cytokines (blue symbols), danger signals (yellow symbols) and antigens (red symbols) that can stimulate the innate and adaptive immune responses. Viral infection of tumour cells leads to replication of the virus and viral spread through and between tumors. Viral infection in and around tumor endothelial cells leads to vascular collapse. Endothelial cells are destroyed either as a result of direct infection with virus, or subsequent to infection of surrounding tumour cells, which leads to infiltration of neutrophils into the tumour and thrombosis (Kirn and Thorne 2009).

1.7.4 Oncolytic Viruses: Modes of Action that Involve Anti-tumor Immunity

Oncolytic viruses could induce specific and non-specific anti-tumor immunity which can aid to the antineoplastic efficacy of the virus. Although, the role of immune system has been a matter of debate for a long time in oncolytic virotherapy, recent advancements suggest that immune system can play a favorable role (Kaufman, Kohlhapp et al. 2015).

Oncolytic viruses are often constructed to encode a therapeutic gene and the expression of such therapeutic genes can further exert anti-neoplastic activity. A variety of transgenes ranging from immune-stimulatory gene to pro-apoptotic genes have been inserted into different oncolytic viruses to enhance their anti-tumor efficacy. For example, the immune-stimulatory genes IL-4, IL-12 and GM-CSF as well as pro-apoptotic genes such as tumor necrosis factor alpha, p53 and tumor necrosis factor-related apoptosis inducing ligand have been studied as therapeutic genes in different oncolytic viruses (Andreansky, He et al. 1998; Parker, Gillespie et al. 2000; Kim, Oh et al. 2006; Han, Assenberg et al. 2007; Heiber and Barber 2011; Bai, Yu et al. 2014).

1.7.5 Oncolytic Viruses: Modes of Action that Affect the Tumor Vasculature

Recent studies have shown that oncolytic viruses can indirectly kill tumor cells by destroying tumor vasculature (Angarita, Acuna et al. 2013; Breitbach, Arulanandam et al. 2013). Adequate blood supply within the tumor is a critical requirement for tumor progression and metastasis. Therefore, inhibition of angiogenesis and/or disruption of established tumor vasculature could potentially result in tumor regression or cure. Several anti-angiogenic drugs have been approved for cancer therapy, but resistance develops when tumors re-vascularize through adaptive or intrinsic resistance mechanism (Angarita,

Acuna et al. 2013). Oncolytic viruses show promise in that they have been shown to kill both endothelial cells and cancer cells within a tumor (Angarita, Acuna et al. 2013; Breitbach, Arulanandam et al. 2013). While direct killing of endothelial cells within a tumor can be mediated by viruses, the main mechanism by which oncolytic viruses disrupt the vasculature in the tumor may be more indirect, through targeted inflammation (Breitbach, Paterson et al. 2007). It is believed that oncolysis by a virus causes the release of pro-inflammatory cytokines allowing for the recruitment of inflammatory cells to the tumor microenvironment (Breitbach, Paterson et al. 2007). A study by Breitbach *et al.* showed that neutrophils play a critically important role in disruption of blood supply to the tumor following treatment with oncolytic viruses (Breitbach, Paterson et al. 2007). Under normal conditions, neutrophils can distort their shape in order to pass through smaller capillaries; however they adopt a "rigid" shape in the inflammatory tumor microenvironment which may cause clogging of micro-vessels within the tumor ultimately blocking the blood supply and increasing tumor hypoxia (Burns, Smith et al. 2003; Breitbach, Paterson et al. 2007). The critical role of neutrophils in disruption of tumor vasculature is evident from the finding that the anti-vascular effect of an oncolytic VSV is completely abolished in mice depleted of neutrophils (Breitbach, Paterson et al. 2007; Breitbach, De Silva et al. 2011). While the disruption of the intra-tumoral blood supply allows killing of uninfected tumor cells it can also limit the spread and persistence of oncolytic viruses. Therefore, it may be desirable to design a therapeutic regimen in which neutrophil recruitment is reduced initially to allow optimal replication and dissemination of the virus within the tumor, followed by enhanced recruitment of

neutrophils to block intra-tumor blood supply, thus mediating killing of uninfected tumor cells (Breitbach, Paterson et al. 2007).

1.7.6 Oncolytic Virotherapy: Current Status

A brief search on Pubmed shows a plethora of studies published on oncolytic viruses targeting different types of cancer. A wide diversity of virus families have been studied for their oncolytic potential including rhabdoviridae (*e.g.*, VSV, Maraba virus), poxviridae (*e.g.*, vaccinia virus, myxoma virus), herpesviridae (*e.g.*, HSV-1), reoviridae (*e.g.*, reovirus type 3), adenoviridae (*e.g.*, adenovirus type 2 & 5), paramyxoviridae (*e.g.*, Newcastle disease virus, measles virus), picornaviride (*e.g.*, poliovirus, coxsackievirus), togaviridae (*e.g.*, sindbis virus) and parvoviridae (*e.g.*, H1-parvovirus) (reviewed by (Vaha-Koskela, Heikkila et al. 2007)). While most of them are still at a preclinical stage, several oncolytic viruses have entered clinical trials. Some examples of oncolytic viruses currently in various phases of clinical trials are shown in Table 1.2.

Oncolytic viruses tested so far have been well tolerated even in immunodeficient animals and immune-suppressed humans (Russell, Peng et al. 2012; Bell and McFadden 2014; Pol, Buque et al. 2016). Mild side effects are seen in patients treated with the currently defined highest feasible doses of oncolytic viruses. However, it is likely that even higher doses of viruses will be used in future trials as manufacturing yields improve (Knop and Harrell 2007; Russell, Peng et al. 2012). Thus, it would be too early to say how high, in terms of viral dose, one could go in order to achieve maximum anti-tumor activity without experiencing dose-limiting toxicities.

Talimogene laherparepvec (T-VEC), an oncolytic HSV encoding GM-CSF, has shown clinical efficacy against metastatic malignant melanoma in various phases of

clinical trials. This virus harbours a deletion of the ICP34.5 gene, a neuro-virulence factor, which makes it non-pathogenic and allows the virus to selectively replicate in rapidly dividing cancer cells (Liu, Robinson et al. 2003). In a phase II trial, 8 out of 50 melanoma patients achieved complete regression of injected and un-injected tumors after intra-tumoral administration of T-VEC (Senzer, Kaufman et al. 2009). This study is an excellent example of the potential of an intra-tumorally administered oncolytic virus to cross-prime and amplify anti-tumor immunity. In a phase III multicenter clinical trial, 16.3% patients showed objective response which lasted for a minimum of six months. This led to the approval of T-VEC by FDA in 2015 for the treatment of melanoma (Greig 2016). Another oncolytic virus in advanced clinical trials is Pexa-Vec, a vaccinia virus encoding GM-CSF and deleted of *J2R* (Anderson, Nakamura et al. 2004). This virus has been tested in multiple clinical trials in combination with chemotherapeutics (https://clinicaltrials.gov). So far Pexa-Vec has shown promising results in phase I and II trials for hepatocellular carcinoma and is currently in a phase III trial (NCT02562755).

In most of the clinical trials performed so far, oncolytic viruses have been administered intra-tumorally (Prestwich, Harrington et al. 2008; Russell, Peng et al. 2012). However, if there is no accessible tumor, systemic delivery of oncolytic viruses will be required. Unfortunately, when delivered systemically, a large fraction of viruses fail to reach the tumor as a result of virus sequestration in liver and spleen, neutralization by serum factors, and the virus's inability to cross the endothelial lining (Russell, Peng et al. 2012). In a phase I clinical trial, when Pexa-Vec was delivered intravenously, virus was recoverable from tumor biopsies only when the virus dose exceeded a threshold of 10^9 infectious units (Breitbach, Burke et al. 2011). Therefore, one current goal in the field

of oncolytic virus therapy is to develop methods to circumvent the barriers in efficient systemic delivery of viruses to the tumors (Russell, Peng et al. 2012).

Taken together, there have been tremendous advancements in the field of oncolytic virotherapy over the last 2 decades. Given the excellent safety profile and efficacy seen in preclinical and clinical studies, it would be logical to surmise that oncolytic viruses hold promise to be included in front-line treatment regimens in the future.

Table 1.2: Examples of Oncolytic Viruses Currently in Clinical Trials (as of April 27, 2016. Abstracted from http://clinicaltrials.gov)

Virus	Modification	Cancer	Phase	Sponsor	Reference
DNX-2401 (Adenovirus)	Δ24-RGD	glioblastoma, ovarian cancer	I	DNAtrix	NCT02197169
Oncos-102 (Adenovirus)	Δ24-RGD; encodes GM-CSF	solid tumors	I	OncosTherapeutics	NCT01598129
Colo-Ad1 (Adenovirus)	Chimeric A11/3 group B	colon cancer, NSCLC, renal cancer, ovarian cancer	I/II	PsiOxus Therapeutics	NCT02053220
CG0070 (Adenovirus)	E3 deleted; encodes GM-CSF	bladder cancer	III	Cold Genesys	NCT02365818
Pexa-Vec (Vaccinia virus)	ΔTK; GM-CSF insertion	hepatocellular carcinoma	III	SillaJen	NCT02562755
GL-ONC1 (Vaccinia virus)	ΔTKΔF14.5L ΔA56R	lung cancer	I/II	Genelux	NCT01766739
T-Vec (Herpes simplex virus)	ΔICP34.5, ΔICP47, insertion of US11 and GM-CSF insertion	breast cancer	II	Amgene	NCT02658812
HSV1716 (Herpes simplex virus)	ΔICP34.5	malignant pleural mesothelioma,	II	Virttu Biologics	NCT01721018
Reolysin (Reovirus)	none	metastatic breast cancer	III	Oncolytics Biotech	NCT01656538

1.8 Vaccinia Virus

1.8.1 Classification and Origin

Vaccinia virus (VACV) belongs to the family *Poxviridae*. Poxviruses are large, brick shaped particles measuring ~300 x 270 x 250 nm with single linear double-stranded DNA genome of ~130-300 kbs (Fenner 2001). The family is divided into two sub-families based on their host range. Members of the sub-family *Chordopoxviniae* infect vertebrates whereas members of the sub-family *Entomopoxvirinae* infect arthropods(Fenner 2001). The *Chordopoxviniae* consists of 8 distinct genera which lack immunologic cross-protection (Moyer RW 2000). Among the 8 genera of this sub-family, the genus *orthopoxvirus* has been most extensively studied. Members of the *Orthopoxvirus* genus vary greatly in their host range; for example, the cowpox virus shows greatest genetic diversity and a broad host-range while the variola virus shows relatively little diversity and it strictly infects humans (Qin, Favis et al. 2015). Members of same genus are morphologically similar and provide cross-protection against each other (Katsafanas and Moss 2007). Indeed, DNA sequencing studies have revealed that VACV and variola virus share greater than 90% amino acid sequence identity (Massung, Liu et al. 1994). Variola virus, the causative agent of smallpox disease, changed the course of history by killing millions of people prior to its eradication in 1980(Belongia and Naleway 2003). It is VACV that was used as a vaccine in the successful eradication of smallpox. VACV is the most intensively studied poxvirus, however, the biological origin of this virus is still uncertain and its natural host is unknown (Turner 1982; Qin, Favis et al. 2015). Some pox virologists believe that VACV may have originated from

horsepox-like virus even though a current strain of a horse-pox virus encodes more genes than VACV (Tulman, Delhon et al. 2006; Qin, Favis et al. 2015).

1.8.2 Life-Cycle

Poxviruses are unusual among the DNA viruses in that they complete their life cycle within the cytoplasm of infected cells (Figure 1.3). The only other DNA virus known to replicate in the cytoplasm is African swine fever virus (Moss 2007; Dixon, Chapman et al. 2013). Knowledge about poxvirus replication is derived mainly from studies of VACV. The genome of VACV is ~200 kb and encodes approximately 200 proteins that are involved in combating host defenses, enabling transcription and replication of the viral genome, and assembling virus particles (Katsafanas and Moss 2007). The central region of the genome, encoding proteins essential for virus production, is highly conserved among the members of *Orthopoxvirus*. However, the terminal regions of the genome, encoding proteins important for host range determination, are less conserved (Mackett and Archard 1979; Qin, Favis et al. 2015). The life cycle of VACV follows a temporal order in which expression of early genes precedes DNA replication which in turn is followed by expression of intermediate genes and finally late genes(Moss, Ahn et al. 1991; Kovacs, Vasilakis et al. 2001).

The life cycle of VACV begins with virus binding to the cell surface and entry into the cell. Although the cellular receptors for VACV remain largely unknown, it is generally thought that the virus binds to carbohydrate moieties such as glycosaminoglycans (Smith, Murphy et al. 2003), heparin sulfate (Chung, Hsiao et al. 1998) and chondroitin sulfate (Hsiao, Chung et al. 1999) that are ubiquitously found on cells (Smith, Murphy et al. 2003). Once the virus binds to the cell surface, fusion of the

viral envelope with the cell membrane allows penetration of the virus core into the cytoplasm through a low pH-dependent endosomal pathway (Smith, Murphy et al. 2003; Laliberte and Moss 2009).

After entering the cell, virus cores are transported to juxtanuclear locations where early genes are transcribed within the core with the help of encapsidated RNA polymerase and transcription factors. Transcription of early genes begins within 20 minutes and peaks at 1 hour (Boone and Moss 1978; Baldick and Moss 1993). The early mRNAs are extruded from the viral core and translated by host ribosomes (Smith, Murphy et al. 2003). Nearly half of viral genes are transcribed before DNA replication begins (Boone and Moss 1978). The early gene products include proteins involved in host interactions, viral DNA synthesis, and transcription of intermediate genes (Knipe 2001). Products of early genes together with some host proteins uncoat the viral core ultimately releasing the viral genome into the cytoplasm (Knipe 2001).

Viral DNA replication proceeds 1-2 hours post-infection in localized cytoplasmic domains called virus factories from which cellular organelles are largely excluded (Cairns 1960). Replication of the viral genome gives rise to concatemers, which are resolved into monomers before packaging by the help of late gene products (Merchlinsky and Moss 1989). In a typical infected cell, up to 10,000 genome copies are made, half or less of which are ultimately packaged into mature virions (Salzman 1960; Joklik and Becker 1964). Transcription of intermediate genes can begin only after initiation of DNA replication; it is thought that the viral genome within the infecting particle may be inaccessible to newly synthesized RNA polymerase and transcription factors which prevent the transcription of intermediate genes (Keck, Baldick et al. 1990). Following the

expression of intermediate and late genes and resolution of concatamers, virions are assembled within the viral factories. At the end, each cell produces 4 distinct forms of virions which differ in the number of membranes and their antigenicity as a result of different molecules bound to their surface. The 4 forms of virion are: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (Smith, Vanderplasschen et al. 2002; Smith and Law 2004) (Figure 1.3). The IMV form is the most abundant type of infectious progeny formed from a non-infectious crescent-shaped precursor that is wrapped in a single lipid bilayer envelope within the viral factory. IMV are released only after the lysis of cell (Smith and Law 2004). A fraction of IMV gets wrapped by a second membrane layer derived from endosomes or the trans-golgi network. These virions are called IEV (Sodeik, Doms et al. 1993). Some IEV particles move to the cell surface *via* microtubules where the outer membrane of the virus fuses with the plasma membrane of the cell exposing the virions at the outer cell surface. These particles are called CEV. Some CEV dissociate from the cell surface with the help of actin projectiles, thus becoming EEV (Sodeik, Doms et al. 1993; Smith, Vanderplasschen et al. 2002). CEV and EEV are physically indistinguishable from each other and both contain 2 membranes, whereas IMV contains 1 membrane and IEV contains 3 membranes (Sodeik, Doms et al. 1993; Smith and Law 2004) (Figure 1.3).

The membranes around the virions are thought to play a crucial role in dissemination of virus both *in vitro* and *in vivo* (Payne 1980; Smith, Vanderplasschen et al. 2002). IMV is physically robust and is efficiently transferred between hosts. However, it is sensitive to neutralization by complement, which makes it less efficient in

dissemination within a host (Payne 1980). In contrast to IMV, all enveloped viruses (IEV, CEV and EEV) are resistant to complement-mediated neutralization. Complement resistance of enveloped virions is thought to result from incorporation of a group of cellular proteins, called regulators of complement activation (RCA), into the viral envelope (Vanderplasschen, Mathew et al. 1998). However, RCA proteins fail to protect the virions from complement from a species different than the one used to grow the virus (Vanderplasschen, Mathew et al. 1998). The CEVs induce the formation of actin projectiles from beneath virions at the cell surface which allow efficient cell-to-cell spread of virus by CEV and also mediates the release of EEV(Smith, Vanderplasschen et al. 2002). EEVs which are less prone to inactivation by complement and antibodies are highly efficient in establishing distant infections within the host (Smith, Vanderplasschen et al. 2002).

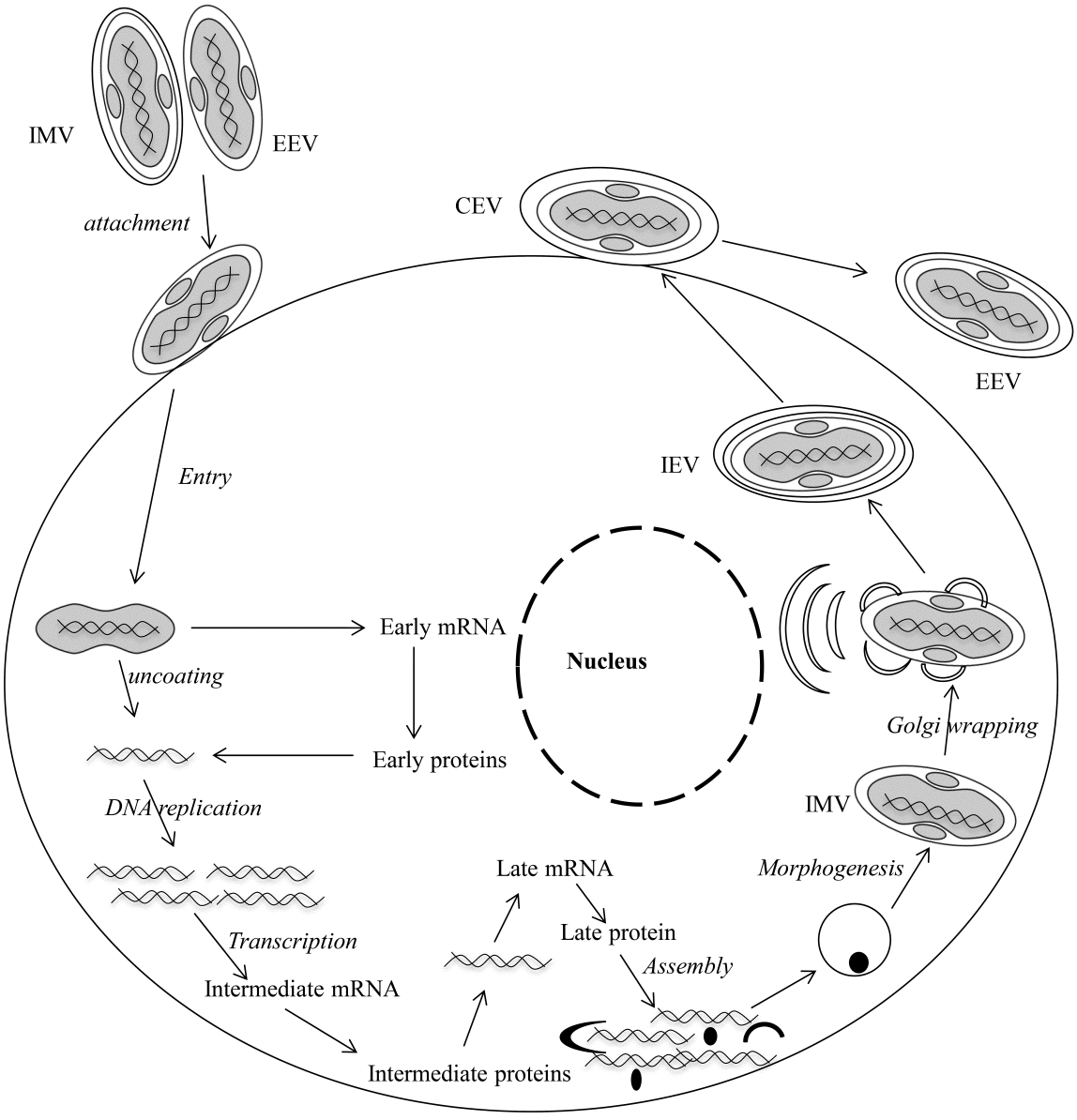


Figure 1.3: Life-cycle of vaccinia virus. Vaccinia virus completes its life cycle in the cytoplasm of infected cells. Shown in the figure are the different steps involved in the life-cycle of the virus (see section 1.8.2 for details). Figure adapted and modified from (Moss 2007). (IMV, intracellular mature virus; EEV, extracellular enveloped virus; IEV, intracellular enveloped virus; CEV, cell-associated enveloped virus)

1.8.3 Vaccinia Virus Encodes Genes Involved in Nucleotide Metabolism

Metabolism of deoxyribonucleotides (dNTPs), precursors of DNA, in mammalian cells is a complex process involving many enzymes. Interestingly, VACV encodes a suite of genes whose products are involved in the synthesis of dNTPs (Fenner 2001) (Figure 1.4). This property allows the virus to replicate even in non-dividing cells which usually have low levels of dNTP synthesis. VACV enzymes involved in dNTP synthesis include the homologs of cellular thymidine kinase (TK), thymidylate kinase (TMK) and ribonucleotide reductase (RR) (Bajszar, Wittek et al. 1983; Smith, de Carlos et al. 1989). The products of the viral *J2R* and *A48R* genes are homologs of cellular TK and TMK, respectively. Cellular RR is composed of two subunits: a large subunit (RRM1) and small subunit (RRM2) (Nordlund and Reichard 2006). VACV encodes *I4L* and *F4L* genes whose products are homologous to RRM1 and RRM2, respectively (Slabaugh, Roseman et al. 1988; Tengelsen, Slabaugh et al. 1988). In normal cells, expressions of TK, TMK and RR are highly regulated and are cell-cycle dependent. While TK and TMK are important only for the production of dTTP, RR is required for the production of all the four nucleotides i.e. dATP, dCTP, dGTP and dTTP (reviewed in (Mathews 2006)).

In a cell, dTTP is generated either *de novo* through reduction of uracil diphosphate or is salvaged from the deoxyribonucleoside thymidine (Sneider and Potter 1969). While TK is essential only in the salvage pathway, TMK is important in both *de novo* and salvage pathways for the synthesis of dTTP (Gordon, Bardos et al. 1968; Mathews 2006). TK exists in two isoforms TK1 and TK2 which are involved in salvage of dTTP in the cytoplasm and mitochondria, respectively (Berk and Clayton 1973; Anderson, Quintero et al. 2011). Unlike TK1, TK2 is not cell-cycle

dependent(Aufderklamm, Todenhofer et al. 2012). Studies have shown that levels and activity of TK are high in rapidly dividing cells and low in resting cells (Johnson, Rao et al. 1982; Aufderklamm, Todenhofer et al. 2012). Furthermore, TK has been found to be constitutively active in many types of cancer and levels of TK have been suggested to be of diagnostic and prognostic value (O'Neill, Buckwalter et al. 2001). Therefore, deletion of *J2R* from VACV should restrict growth of the virus to rapidly dividing cells such as cancer cells that can compensate for the deletion. Indeed, deletion of the viral TK gene *J2R*, alone or in combination with other genetic modifications is the most common approach for creating oncolytic vaccinia viruses. Multiple studies have demonstrated that *J2R*-deleted VACV shows cancer specific replication *in vitro* and *in vivo* (Puhlmann, Brown et al. 2000; Guse, Sloniecka et al. 2010; Guse, Cerullo et al. 2011; Lun, Ruan et al. 2013; Hughes, Wang et al. 2015). Furthermore, the most clinically advanced oncolytic VACV (Pexa-Vec) is deleted in the *J2R* gene (Liu, Hwang et al. 2008).

RR catalyzes the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, the most critical step in the synthesis of DNA precursors (Reichard and Estborn 1951; Thelander and Reichard 1979). Interestingly, the levels of the large subunit (RRM1) remains stable throughout the cell cycle whereas the small subunit (RRM2) is degraded at the end of S-phase (Engstrom, Eriksson et al. 1985). Hence, RRM2, with a short half-life of ~3 hours, is a rate limiting factor in dNTP synthesis (Engstrom, Eriksson et al. 1985; D'Angiolella, Donato et al. 2012). Studies have shown that many types of cancer including BrCa, express high levels of RRM2 in order to meet their high demand of dNTPs(Jensen, Page et al. 1994; Yun, Cho et al. 2008; Morikawa, Maeda et al. 2010; Wang, Lu et al. 2012). Furthermore, RRM2 has been

shown to actively contribute to cancer progression, and elevated levels of RRM2 correlate with poor prognosis as well as resistance to chemotherapeutics such as gemcitabine in many types of cancer (Fan, Villegas et al. 1996; Zhou, Tsai et al. 1998; Lee, Vassilakos et al. 2003; Duxbury, Ito et al. 2004; Itoi, Sofuni et al. 2007; Liu, Zhang et al. 2013). Given the high levels of RRM2 and continuous supply of dNTPs in cancer cells, it is logical to surmise that the viral F4 protein (homolog of RRM2) would be dispensable for the replication of the virus in cancer cells but not in normal cells. Accordingly, our group has previously shown that *F4L*-deleted VACV is highly attenuated while wild-type VACV is highly virulent in nude mice (Gammon, Gowrishankar et al. 2010). The potential oncolytic activities of VACV deleted of *F4L* alone or in combination with *J2R* (Figure 1.5), have been explored in this study.

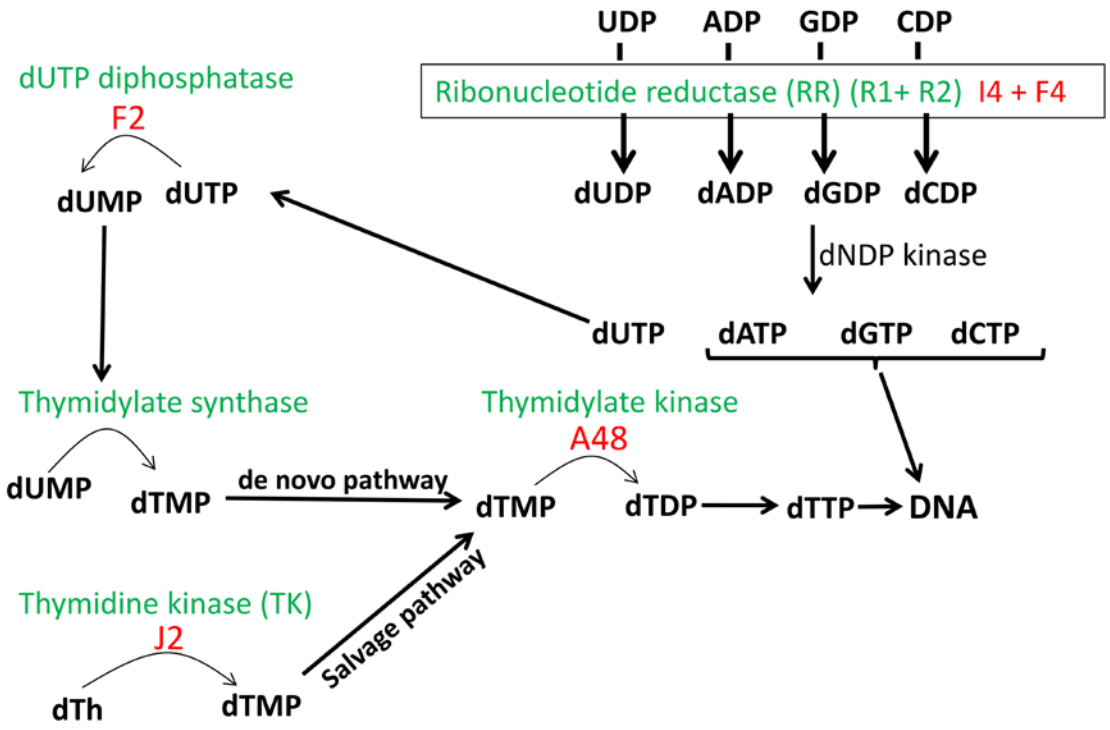


Figure 1.4: Cellular and viral enzymes involved in nucleotide metabolism. Nucleotide metabolism in eukaryotic cells is a highly complex process involving many enzymes. Vaccinia virus encodes many genes the products of which are homologs of cellular enzymes involved in nucleotide metabolism. Cellular enzymes involved in dNTPs metabolism are shown in green and the viral homologs are shown in red. (Figure modified from Dr. David Evans).

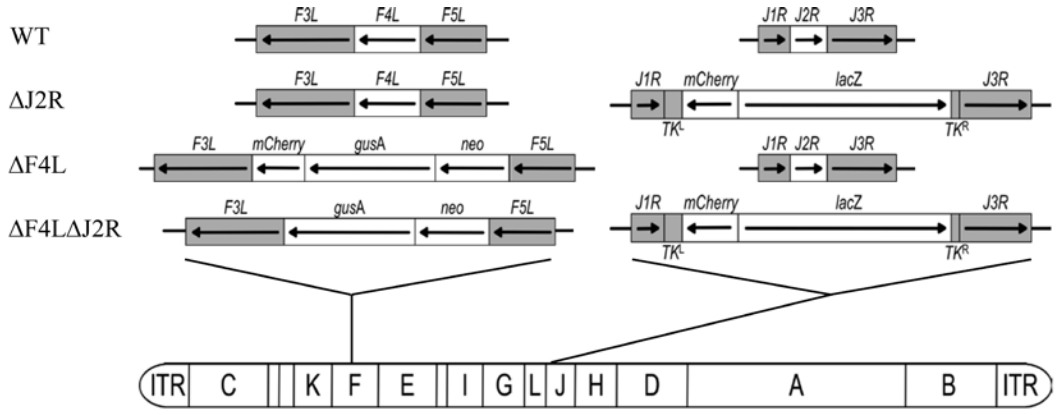


Figure 1.5: Cartoons depicting the map of wild-type and mutant VACVs used in this study. Viruses were generated from the Western Reserve VACV strain as described in section 3.5.2. (*neo*, neomycin; *gusA*, β-glucuronidase; *lacZ*, β-galactosidase; ITR, inverted terminal repeats; TK, thymidine kinase, TK^L, thymidine kinase left homology; TK^R, thymidine kinase right homology; WT, wild-type). (Figure courtesy: Kyle Potts).

1.8.4 VACV as Oncolytic Agent

VACV has several characteristics that make the virus an ideal candidate for an oncolytic virus. First, it has a proven safety record in humans as a result of its use in millions of people during smallpox eradication (Fenner 1982). The virus has been extensively studied and a wealth of knowledge is available about its molecular biology. Indeed, VACV was the first animal virus to be grown in cell culture and to be observed microscopically. This virus was among the first viruses to be purified, accurately titered, and chemically analyzed (Knipe 2001; Shen and Nemunaitis 2005). Second, VACV encodes more than 200 genes several of which make proteins that are necessary only for replication of the virus in slow-dividing normal cells but would be dispensable for its replication in rapidly dividing cancer cells (Knipe 2001). This provides an opportunity to make the virus cancer-selective by deleting one or more of those viral genes. Third, unlike adenovirus that does not replicate in mice (Jogler, Hoffmann et al. 2006), the most commonly used preclinical animal model, VACV replicates in a wide range of hosts including mice (McFadden 2005). Hence, the oncolytic properties of VACV can be studied in many syngeneic animal models that could facilitate translation into clinical trials (Guse, Cerullo et al. 2011). Furthermore, VACV replicates in almost all types of human cells, which implies that an oncolytic VACV can be used to target human malignancies of different tissue origin (McFadden 2005). Fourth, VACV completes its life cycle within the cytoplasm and its genome does not enter the nucleus, hence there is no risk of insertional mutagenesis (Moss 2007). Fifth, the virus particles are stable and it is relatively easy to prepare high titer stocks. Long term storage of VACV as a frozen suspension or dry powder is feasible with negligible loss of infectivity (Shen and

Nemunaitis 2005). Sixth, different structural forms of the virus allow for rapid spread of the virus in cell culture and in host animals (Smith and Vanderplasschen 1998; Smith, Murphy et al. 2003). For example, after intra-tumoral injection, IMV and CEV can efficiently spread within the primary tumor, and EEV, which is resistant to anti-viral antibodies (Ichihashi 1996) and complement-mediated neutralization (Vanderplasschen, Mathew et al. 1998), can travel through the bloodstream to reach distant tumors (Thirunavukarasu, Sathaiah et al. 2013). Seventh, the virus has a cloning capacity of ~25 kb which makes it possible to arm an oncolytic VACV with one or more potential therapeutic genes (Smith and Moss 1983). Furthermore, the timing and level of expression of a therapeutic gene could be controlled by using early, late, or synthetic viral promoters (Yang, Maruri-Avidal et al. 2013). Lastly, there are second-level of safety measures available against VACV, *i.e.* there are several anti-VACV agents available such as cidofovir (De Clercq 2002), ST-246 (Yang, Pevear et al. 2005) and vaccinia immunoglobulin (Wittek 2006) which could be used to limit toxicity in the unlikely event of uncontrolled virus replication (Guse, Cerullo et al. 2011).

During the smallpox vaccination campaign, VACV was propagated by different health agencies, pharmaceutical companies, and researchers, using a variety of methods which gave rise to different strains of the virus (Qin, Favis et al. 2015). Currently, several strains of VACV are available such as Western Reserve (WR), Lister, Copenhagen, Wyeth, Tian Tan, modified vaccinia virus Ankara (MVA), and New York City Board of Health (NYCBH) (Qin, Favis et al. 2015). These strains seem to differ widely in their oncolytic potential (Thorne, Hwang et al. 2007; Kirn and Thorne 2009). While the WR strain shows the strongest oncolytic effect, the MVA strain does not replicate in

mammalian cells and hence has no oncolytic effect at all (Thorne, Hwang et al. 2007). Wyeth, Lister and Copenhagen strains have shown appreciable oncolytic effect in preclinical as well as clinical trials (Guse, Cerullo et al. 2011). These strains were widely used in humans for vaccination and they are considered safe but pose difficulty in mass production (Guse, Cerullo et al. 2011). WR, a laboratory strain derived from the Wyeth, replicates robustly in a wide variety of mammalian cells and shows inherent selectivity for cancer cells (Thorne, Hwang et al. 2007). The WR strain was obtained by long-term repeated passaging of Wyeth in mouse brain and has not been used in humans for vaccination purposes (Artenstein 2008). Hence, there is lack of safety record for this strain in humans. Very recently, a phase I clinical trial was completed on a modified WR strain that is deleted of genes encoding viral TK and vaccinia growth factor. In this study, virus was injected intra-tumorally and no significant toxicity was observed even at the highest feasible dose of $3x10^9$ infectious units (Zeh, Downs-Canner et al. 2015). The study reported here in this dissertation has explored the potential oncolytic activities of WR VACV deleted in *F4L* alone or in combination with a *J2R* deletion.

1.9 Thesis Summary

There is an increasing interest in the use of viruses as cancer therapeutics. Viruses could be used either as vectors in cancer gene therapy or as oncolytic agents. We have studied adenovirus, the most commonly studied viral vector, as a vector for transcriptionally targeted IL-2 gene therapy for breast cancer. In our study we used the engineered mammaglobin (MPE2) promoter for transcriptional targeting of IL-2, one of the most commonly studied cytokines as a cancer therapeutic. Our study is, to the best of our knowledge, the first to evaluate a transcriptionally targeted IL-2 for its therapeutic

activity in breast cancer. We found that the MPE2 promoter is highly efficient in driving a reporter gene specifically in breast cancer cells of both human and murine origins. However, despite showing excellent cancer specificity, the MPE2 promoter was weak in driving the therapeutic gene (IL-2) *in vivo* in mice. Nonetheless, the MPE2 promoter-controlled IL-2, delivered by a non-replicating adenoviral vector, was found to be safer compared to an MCMV promoter-controlled IL-2 vector and exerted a significant anti-tumor effect in syngeneic mouse models. Aiming to further enhance the anti-tumor effect of our MPE2-controlled IL-2 vector, we studied the combination of this vector with a pro-apoptotic drug PAC1 with the hypothesis that the drug-mediated apoptosis of cancer cells will release tumor antigens which will boost the anti-tumor effect of exogenous IL-2. Although PAC1 showed appreciable killing of breast cancer cells *in vitro*, it induced little apoptosis in cancer cells in mouse models. However, PAC1, owing to its weak *in vivo* activity, did not enhance the anti-tumor effect of our IL-2 vector.

We also studied the safety and anti-tumor activities of *F4L*-mutant vaccinia viruses in the treatment of breast cancer. To our knowledge, this is the first study evaluating the oncolytic properties of the novel *F4L*-mutant vaccinia virus in breast cancer. Our data show that deletion of *F4L* alone or in combination with *J2R* highly attenuates the virus in slow-dividing normal cells both *in vitro* and in mice. However, as predicted, breast cancer cells of both human and murine origins compensate for the loss of *F4L* and/or *J2R* allowing the mutant viruses to replicate to high levels much like the wild-type virus. The mutant viruses were able to efficiently kill cancer cells *in vitro*, and completely controlled growth of a human breast tumor xenograft in nude mice. Furthermore, bio-distribution studies revealed that growth of the viruses was highly

attenuated in normal organs of both nude and immune-competent mice. Despite the ability of the viruses to completely halt the growth of human breast tumors in nude mice, their ability to slow tumor growth in immune-competent mice was found to be sub-optimal. Further studies are needed to determine whether the anti-tumor effect of the viruses could be enhanced in immune-competent mice by combining the viruses with chemotherapeutics or by arming them with immune-modulatory cytokines.

Chapter 2: Breast Cancer Gene Therapy Using an Adenovirus Encoding Human IL-2 under Control of Mammaglobin Promoter/Enhancer Sequences

Breast Cancer Gene Therapy Using an Adenovirus Encoding Human IL-2 under Control of Mammaglobin Promoter/Enhancer Sequences

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Note that section 2.5.5 has been added to the thesis (referred to as "data not shown" in the publication).

Contributions

Data presented in this paper were generated by Paggy Hew, a former M.Sc. student in Hitt lab, and myself. Paggy Hew generated the raw data for table 2.1 as well as figure number 2.1, 2.2 and 2.3. I used those raw data to create the final figures. All the remaining data in the paper were generated and the figures were created by myself. I wrote the manuscript and did the revision. Dr. Mary Hitt provided intellectual directions for this project and edited the manuscript.

2.1 Context and background information

Work done by Paggy Hew as part of her M.Sc. thesis in Dr. Hitt's laboratory led the foundation for this paper. Her project was to develop tissue-specific promoters for adenovirus-mediated gene therapy of breast cancer. It has long been realized that adenovirus mediated cancer gene therapy is limited by the associated toxicities which often result from unintended infection of normal tissues and expression of therapeutic genes therein. One approach to restrict transgene expression to the target tissue is through transcriptional targeting using tissue/tumor specific regulatory elements. In her M.Sc. research, Paggy investigated three promoters for their ability to direct breast cancer-specific expression: the human telomerase reverse transcriptase (hTERT) promoter, the human osteocalcin (hOC) promoter, and the mammaglobin (MGB) promoter. The specificity and efficacy of these promoters were compared in breast cancer cell lines of human and murine origin. Interestingly, Paggy found that, while the hTERT and MGB promoters both exhibited some levels of tissue specificity *in vitro*, the MGB promoter was uniquely able to induce breast cancer-specific expression in both human and murine cell lines. Furthermore, compared to hTERT or hOC promoter, the MGB promoter was found to be more efficacious in directing reporter gene expression. The transcriptional strength of the MGB promoter was further enhanced by adding 2 tandem copies of the putative mammaglobin enhancer element upstream of the MGB promoter (MPE2). This engineered MPE2 promoter was found to retain the breast cancer-specificity while enhancing the activity of the minimal MGB promoter. *In vivo*, a first generation (non-replicating) adenovirus vector encoding human IL-2 (hIL-2) under the control of the MPE2 promoter (Ad-MPE2-IL-2) was found to induce much lower levels of liver toxicity compared to that with an adenoviral vector encoding hIL-2 under the control of the

constitutively active, immediate early promoter derived from murine cytomegalovirus (MCMV) (Ad-MCMV-IL-2).

The observed superiority of MPE2 promoter over hOC and hTERT promoters in terms of safety and efficacy led us to select the MPE2 promoter for studying the feasibility of adenovirus-mediated, breast cancer targeted IL-2 gene therapy. Toward this objective, I compared adenovirus infectivity and MPE2 activity in 3 murine breast cancer cell lines (4T1, EMT6 and MTHJ) in order to select the best cell line to generate tumors in mice for studying the safety as well as anti-tumor efficacy of Ad-MPE2-IL-2. The polyoma-virus-middle-T-antigen-over-expressing MTHJ cell line was found to support the highest levels of virus infection (based on expression of GFP encoded by the vector) and MPE2 promoter activity (based on vector-encoded luciferase activity). Thus, I chose MTHJ cells to generate tumors in immune-competent mice. I found that Ad-MPE2-IL-2 or Ad-MCMV-IL-2 vector alone induced sub-optimal tumor regression which is in agreement with the preliminary data obtained by Paggy. I, therefore, decided to study the anti-tumor effect of the Ad-MPE2-IL-2 vector in combination with a pro-apoptotic drug, with the hypothesis that apoptosis induced by the drug would expose tumor antigens which would allow activation and expansion of tumor-antigen-specific T cells in the presence of high levels of IL-2 within the tumor microenvironment. The pro-apoptotic drug that I used in this study was procaspase activating compound 1 (PAC1) which is a small-molecule that has been shown to induce apoptosis by directly activating procaspae-3 in cells of different malignancies. Levels of procaspase-3 are elevated in many types of cancer including breast cancer (Fink, Schlagbauer-Wadl et al. 2001; O'Donovan, Crown et al. 2003; Krepela, Prochazka et al. 2004). In our study, I found that PAC1 efficiently

kills breast cancer cell lines *in vitro,* however, PAC1 fed to mice by oral gavage for up to 21 days, in combination with 3 intratumoral injections of IL-2 vectors, did not improve the anti-tumor activity of the IL-2 vectors. While Ad-MPE2-IL-2 or Ad-MCMV-IL-2 vector alone induced significant delay in growth of tumors compared to control treated tumors, PAC1 failed to induce any anti-tumor effect.

Breast Cancer Gene Therapy Using an Adenovirus Encoding Human IL-2 Under Control of Mammaglobin Promoter/Enhancer Sequences

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The authors have no conflicts of interest.

2.2 Abstract

Interleukin-2 (IL-2) has been used clinically for the treatment of some malignancies, however, toxicities associated with systemic IL-2 therapy is a major challenge. Here we have determined whether transcriptional targeting of IL-2 to breast cancer (BrCa) using an engineered human mammoglobin promoter/enhancer (MPE2) is a feasible option for reducing IL-2 associated toxicities while still achieving a meaningful anti-tumor effect. We have constructed non-replicating adenovirus vectors encoding either a reporter gene (luciferase) or human IL-2 cDNA under control of the MPE2 sequence, the murine cytomegalovirus immediate early (MCMV) promoter or the human telomerase reverse transcriptase (hTERT) promoter. Luciferase and hIL-2 cDNAs under the control of the MPE2 sequence in Ad vectors were expressed at high levels in BrCa cells and at lower levels in normal cells of human and murine origin. Cancer specificity of the hTERT promoter was found to be similar to that of the MPE2 promoter in cells of human origin, but reduced specificity in murine cells. The MPE2 regulatory sequence demonstrated excellent tissue specificity in a mouse tumor model. While the MCMV-promoter-controlled IL-2 vector generated high liver toxicity in mice, the MPE2-controlled IL-2 vector generated little or no liver toxicity. Both IL-2 vectors exerted significant tumor growth delay, however, attempts to further enhance anti-tumor activity of the IL-2 vectors by combining with the pro-apoptotic drug PAC1 were unsuccessful.

Keywords: Adenovirus, IL-2, Mammoglobin promoter, breast cancer, PAC1

2.3 Introduction

Breast cancer (BrCa) is the most common cancer and the leading cause of cancer-related death in females worldwide (Vogelzang, Benowitz et al. 2012). Although surgery and radiation therapies have high success rates for the treatment of primary BrCa, they are restricted to the treatment of local disease. For the management of metastatic BrCa, chemotherapy and hormone therapy are commonly used, but with limited success (Higgins and Wolff 2008). Although effective in prolonging the life of patients, these therapies are not sufficient to cure BrCa. Development of resistance to the therapeutics and recurrence of the disease after complete remission are the two major hurdles in the success of BrCa treatment (Jones 2008). Another challenge in the treatment of BrCa is the heterogeneity of the disease. Not all types of BrCa respond to the same chemotherapy or hormone therapy, and this has further complicated the treatment selection process (Polyak 2011). A drug that targets a pathway/molecule that is common to many types of BrCa would be desirable as it could potentially reduce the complexity of treatment stratification.

Gene therapy is a relatively recent approach investigated for its application to cancer treatment. The most common strategies of cancer gene therapy involves direct killing of cancer cells through the delivery of cytotoxic genes, suppression of oncogenes, delivery of tumor suppressor genes and activation of anti-tumor immunity (Roth and Cristiano 1997; Amer 2014). Recently, immunotherapies have been heavily studied either as single treatment modalities or in combination with traditional treatment modalities such as chemotherapy and radiation therapy. Cancer immunotherapies are broadly aimed at activation of anti-tumor immunity and/or relieving the immune-suppressive

environment found in many types of solid tumors. Regulatory T cells and/or cancer cells secrete transforming growth factor-β that plays a pro-tumor role by inhibiting the proliferation and cytolytic activities of natural killer and CD8+ T cells. Consequently, reducing the levels of transforming growth factor-β by various means such as gene knockdown using antisense oligonucleotides, neutralization using monoclonal antibodies and blocking transforming growth factor-β receptor kinase have all shown promising anti-tumor results in preclinical and early phases of clinical trials in different types of cancer (Smith, Robin et al. 2012; Alvarez, Bouchlaka et al. 2014). Similarly, use of monoclonal antibodies against immune check-point proteins, for example, cytotoxic T-lymphocyte-associated antigen 4, programmed death 1 and its ligand, have also been shown to relieve immune suppression, resulting in enhanced anti-tumor immune activity (Pardoll 2012). Direct activation of immune cells in the immune-suppressive tumor environment could also be achieved by exogenous supply of various cytokines. For example, interleukin-2 (IL-2), IL-12, IL-15, IL-21 and granulocyte-macrophage colony-stimulating factor have all been shown to exert anti-tumor activity through the activation of cytotoxic immune cells (Dranoff 2004).

Among the cytokines, IL-2 has been the most widely studied and has been shown to have potent ability to induce activation and proliferation of T cells and natural killer cells (Gaffen and Liu 2004). Because of its anti-tumor effect, IL-2 has been approved by the FDA (Food and Drug Administration) for the treatment of renal cancer and metastatic melanoma. In order to achieve therapeutic benefit, high-dose IL-2 is systemically administered to patients: ~15–20% of patients show objective responses, whereas 5–7% patients show durable complete responses (Fisher, Rosenberg et al. 2000; Atkins

2002; Atkins, Regan et al. 2004). Although meaningful treatment response is achieved in a subset of patients, high-dose IL-2 can result in life-threatening side effects such as vascular leak syndrome (Baluna and Vitetta 1997). The side effects associated with systemic use of IL-2 severely limit its use in cancer therapy. Several approaches have been studied by different research groups to target IL-2 to tumors. One way to minimize the side effects of systemic IL-2 therapy could be to limit the high concentration of IL-2 to the tumor vicinity (Den Otter, Jacobs et al. 2008). An early approach involves using IL-2 fusion proteins in which IL-2 is fused with an antibody against a protein that is highly expressed by tumor cells (Becker, Varki et al. 1996; Penichet, Dela Cruz et al. 2001; Davis and Gillies 2003). Alternatively, restricting high levels of IL-2 expression to the tumor may not only reduce toxicity but also result in better therapeutic effect. This notion is based on the findings by Lee *et al.* that IL-2-transfected cancer cells formed tumors in mice that were more vascularized, less hypoxic and had higher numbers of infiltrating lymphocytes compared with tumors made by the parental cancer cells (Lee, Fenton et al. 1998). Hypoxia, a common event in solid tumors, is thought to affect the ability of tumor cells to be recognized by immune cells (Lee, Fenton et al. 1998; Rao, Dyer et al. 2006). Thus, expression of IL-2 in the tumor environment could activate anti-tumor immunity not only by inducing the generation of cytotoxic immune cells but also by promoting increased tumor infiltration by activated immune cells. We have taken a different approach for targeting IL-2 to the tumor site, that is, adenovirus-based transcriptionally targeted gene therapy.

Adenovirus (Ad) is one of the most commonly used viral vectors in cancer gene therapy. Ads have many features that make them a suitable vector for gene therapy

including: (1) the viral genome is relatively easy to manipulate by recombinant DNA technology; (2) scaling up and purification of the recombinant virus for use in the clinic is relatively easy; (3) the virus infects both quiescent and dividing cells with high efficiency; (4) recombinant viruses are fairly stable as the viral genome does not undergo rearrangement at a high rate; (5) during propagation, the vector replicates to high levels producing up to 1000 plaque-forming units (PFUs) per infected cell; and (6) high levels of transgene expression are achieved in target cells (Sadeghi and Hitt 2005). Moreover, unlike retroviruses, the Ad genome is maintained as an episome in the infected cell, and therefore the risk of insertional mutagenesis is low.

Intratumoral injection of an Ad vector encoding IL-2 should, theoretically, limit the production of IL-2 to the tumor environment. However, studies have shown that even after intratumoral injection, a significant amount of Ad escapes the tumor environment and infects the liver (Bramson, Hitt et al. 1997). Therefore, to further restrict IL-2 expression to the tumor cells, the IL-2 gene can be transcriptionally targeted using tumor/tissue-specific promoters. One such promoter is derived from the mammaglobin gene that is almost exclusively expressed in mammary tissue (Goedegebuure, Watson et al. 2004). More than 80% of all breast cancer cells express mammaglobin, and the level of mammaglobin expression in BrCa cells is >10-fold higher than that in normal breast epithelial cells (Watson, Dintzis et al. 1999). We have previously reported the use of mammaglobin regulatory sequences for BrCa-specific expression of a reporter gene (Shi, Long et al. 2004). However, compared with viral promoters such as those from cytomegalovirus (CMV) and Simian virus 40 (SV40), most mammalian tumor/tissue-specific promoters are inferior in terms of expression intensity (Nettelbeck, Jerome et al.

1998). In order to enhance the expression strength of the mammaglobin promoter we have fused the promoter to a tandem duplication of upstream sequences from the mammaglobin gene (Shi, Graham et al. 2006). We have constructed nonreplicating Ad vectors encoding human IL-2 (hIL-2) under the control of this engineered mammaglobin promoter/enhancer (Ad-MPE2-IL-2) or the murine CMV immediate early promoter (Ad-MCMV-IL-2). Here we report the safety and anti-tumor efficacy of these hIL-2 encoding vectors in mouse models of breast cancer.

Aiming to enhance the efficacy of the IL-2 encoding vectors, we combined this IL-2 treatment with a recently discovered proapoptotic drug: procaspase activating compound 1 (PAC1). PAC1 is a small-molecule drug that has been shown to induce apoptosis in cancer cells by chelating zinc, thus activating procaspase-3 that is elevated in many types of cancer including BrCa (Putt, Chen et al. 2006; Peterson, Goode et al. 2009). We hypothesize that PAC1-mediated apoptosis could expose hidden tumor antigens that, in combination with IL-2 expression within the tumor environment, should induce rapid activation and expansion of tumor-antigen-specific T cells leading to further cytolysis of the tumor.

2.4 Material and Methods

2.4.1 Cell Culture

PyMidT antigen-expressing murine BrCa cell lines MT1A2 (Addison, Hitt et al. 1997)) and MTHJ (Desilva, Wuest et al. 2012) were maintained in Dulbecco's modified Eagle's medium containing high glucose. Human BrCa cell lines MDA-MB-231 (ATCC HTB-26, Manassas, VA, USA), MDA-MB-468 (ATCC HTB-132) and T47D (ATCC HTB-133) were maintained in RPMI-1640. Similarly, SKBR-3 (human BrCa, ATCC HTB-30), 516 (murine kidney cells)(Addison, Hitt et al. 1997) and MRC5 (human lung fibroblast, ATCC CCL-171) were maintained in McCoy's 5A, Dulbecco's modified Eagle's medium containing high glucose and minimal essential medium, respectively. All media were supplemented with 10%fetal bovine serum, 2 mM L-glutamine and 100 U ml^{-1} penicillin–streptomycin. The cells were maintained at 37 °C and 5% CO_2. All the cells used in this study were free of mycoplasma contamination as determined by 4',6-diamidino-2-phenylindole staining.

2.4.2 Adenovirus Vectors

Ad-BHG and Ad-dl70-3 are Ad5-based E1- and E3-deleted nonreplicating control viruses lacking any transgene insert (Bett, Haddara et al. 1994; Putzer, Bramson et al. 1998). Ad vectors were constructed using Cre-*loxP*-based site-directed recombination between the viral genomic plasmid pBHGloxΔE1,3Cre containing the entire viral genome with a deletion in E1 and E3 regions, and shuttle plasmids carrying the firefly luciferase gene or hIL-2 complementary DNA (Addison, Braciak et al. 1995) under the control of regulatory sequences of the MCMV immediate early gene(Addison, Hitt et al. 1997), the human telomerase reverse transcriptase gene (hTERT) (encompassing

nucleotides −285 to +55 relative to the transcription start site) (Cong, Wen et al. 1999) or the mammaglobin gene (the engineered MPE2 promoter) (Shi, Graham et al. 2006). Briefly, Ad E1-transformed human embryonic kidney cells (HEK-293) were co-transfected with the genomic plasmid, containing a *loxP* site and expressing a Cre recombinase, and a shuttle plasmid carrying a *loxP* site and the transgene under the control of MPE2, MCMV or hTERT promoters. Isolated plaques were selected and amplified. Restriction enzyme digestions were performed on DNA isolated from the amplification to confirm that the isolated viruses were the correct recombinants. Finally, viruses were expanded and then purified by cesium chloride banding. Stocks were tested for the presence of replication-competent adenovirus (Hitt, Addison et al. 1997).

2.4.3 PAC1 Formulation

For *in vitro* experiments, a 50 mM (19.6 mg ml^{-1}) stock solution of PAC1 (Putt, Chen et al. 2006) was made in dimethyl sulfoxide. For *in vivo* experiments, PAC1 was dissolved in 200 mg ml^{-1} hydroxypropyl-β-cyclodextrin (HPβCD; Cyclodextrin Technologies Development, Alachua, FL, USA) to obtain a final concentration of 15 mg ml^{-1}PAC1. Briefly, in order to make 20 ml PAC1, 4 g HPβCD was dissolved in 20 ml distilled water and 300 mg PAC1 was added to it. The pH was adjusted to 1.5 with 2 N HCl to obtain absolute solubility, and stirred for 45 min. The pH of the solution was then raised to 5.5 using 2 N NaOH and further stirred for 15 min. Finally, the solution was filtered through 0.22 μM polyvinylidene difluoride filter (Merck Millipore, Tullagreen, Ireland), aliquoted and stored at −20 °C. PAC1 solution prepared as described was stable for at least 1 week.

2.4.4 Cytotoxicity Assay

A total of 5000 cells per well were plated into 96-well plates and incubated at 37 °C overnight. The next day, medium in each well was replaced with fresh medium supplemented with or without the indicated concentrations of PAC1 in triplicate. At 3 days after treatment, Alamar Blue (Resazurin; Sigma, St Louis, MO, USA) was added to a final concentration of 44 μM, and plates were returned to the incubator. After 2 h, the fluorescence of each well was measured (excitation at 544 nm, emission at 590 nm) using a FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany). The percentage of cell survival in the treated wells was calculated relative to the control-treated wells.

2.4.5 Dual Apoptosis Assay

The Dual Apoptosis Kit (Biotium, Hayward, CA, USA) was used to simultaneously detect caspase-3 cleavage and exposed phosphatidylserine (Cen, Mao et al. 2008). The kit contains NucView 488 caspase-3 substrate and CF594 Annexin V (both from Biotium). Briefly, 5000 cells per well were plated into 96-well plate with clear bottom (Cat. No. M0562; Sigma-Aldrich, Oakville, ON, Canada). The next day, cells were treated with PAC1 or dimethyl sulfoxide and incubated for 48 h at 37 °C. Cells were stained with Annexin V and NucView following the manufacturer's instruction. Finally, cells were counterstained with Hoechst 33342 (Thermo Scientific Pierce, Burlington, ON, Canada) and imaged with a confocal microscope at × 10.

2.4.6 Western Blot Analysis

MTHJ cells were treated with PAC1 or dimethyl sulfoxide, and cell lysates collected 24 or 48 h later. RIPA buffer supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 × Protease Inhibitor Cocktail (Sigma) was used to lyse the cells. Protein concentrations of the cell lysates were determined by Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, IL, USA). Lysates containing equal amounts of protein (20 μg) were separated electrophoretically on 0.1%-SDS-12% polyacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, München, Germany). Membranes were blocked with Odessey blocking buffer (Li-COR Biosciences, Lincoln, NE, USA) for 1 h and then treated with rabbit anti-caspase-3 antibody (Cat. No. 9662, Cell Signaling Technology, Denver, MA, USA) and rabbit anti-α-tubulin antibody (Cat. No. ab4074, Abcam, Cambridge, MA, USA) overnight at 4 °C. The following day, membranes were washed 3 × with phosphate-buffered saline–Tween buffer and incubated for 1 h with donkey anti-rabbit IgG (H+L) antibody labeled with infrared IRDye 680RD (LI-COR Biosciences). Finally, the membranes were scanned using an Odyssey Infrared Imaging System (LI-COR Biosciences).

2.4.7 Luciferase Assay

Cultured cells or mice were infected with Ad vectors encoding the firefly luciferase gene. Lysates from the infected cells (48 and 72 h after infection) or organs (2 and 7 days after infection) of infected mice were prepared in Cell Culture Lysis Reagent (Promega, Madison, WI, USA). Protein concentrations in the lysates were measured by Detergent-Compatible Protein Assay Kit (Cat. No. 500-0112, Bio-Rad). Luciferase

activity in the lysates was determined using the Luciferase Assay System (Cat. No. E1501, Promega) according to the manufacturer's protocol.

2.4.8 IL-2 Assay

Cultured cells or mice were infected with Ad vectors encoding the hIL-2 complementary DNA. Cell supernatants were collected every 24 h and replaced with fresh medium. Supernatants were assayed for hIL-2 by enzyme-linked immunosorbent assay (Becton Dickinson OptEIA hIL-2 set, Mississauga, ON, Canada formerly Pharmingen OptEIA hIL-2 kit, Cat. No. 555190). Tumor and liver of infected mice were harvested on days 2 and 7 after infection and homogenized using a GentleMACS dissociator (Miltenyl Biotec, Bergisch Gladbach, Germany). The concentration of hIL-2 was determined by enzyme-linked immunosorbent assay (OptE1A hIL-2 kit).

2.4.9 Measurement of ALT and AST Levels

Serums from treated mice were sent to Pathology Service at McMaster University for the measurement of ALT and AST.

2.4.10 Tumor Models

All animal experiments were approved by the Animal Care Committees at the University of Alberta or McMaster University and were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Female FVB mice (Charles River Laboratories, Kingston, NY, USA) were 7–9 weeks old at the time of tumor cell injection. Subcutaneous tumors were established using 10^6 cells isolated from primary tumor explants of MMTV-PyMidT transgenic mice (Addison, Braciak et al. 1995). For the orthotopic tumor model, a cell line (MTHJ) derived from a mammary tumor from that transgenic mouse line was used (Desilva, Wuest et al. 2012). Two million MTHJ cells in

50% matrigel were injected in the mammary fat pad of each mouse. Tumors were palpable in both models in all mice by 2–3 weeks after injection. Before the start of treatment, mice were grouped so as to obtain a similar average tumor volume for each group. Replicates of tumor regression experiments were performed by different individuals.

2.4.11 Tumor Regression and Survival

Once tumors were palpable, mice were divided into different treatment groups such that the average tumor size of all groups was roughly the same. For the subcutaneous tumor model, each group (n=5 or 6) was given a single intratumoral injection of Ad-BHG control virus, Ad-MPE2-IL-2 or Ad-MCMV-IL-2 at the doses indicated. Tumors were measured twice weekly using digital calipers. Mice with tumor sizes >10 mm in any two dimensions were killed.

For the orthotopic tumor model, each group received 3 injections (5×10^8 pfu each) of Ad-dl70-3 control virus, Ad-MPE2-IL-2 or Ad-MCMV-IL-2 on days 1, 4 and 7 relative to the first virus injection. Mice were administered 100 mg kg^{-1} of PAC1 or the vehicle HPβCD by oral gavage daily for 21 days starting at day 0. Tumors were measured twice weekly using a digital caliper. Tumor volume was calculated as $1/24 \times 3.1416 \times \text{length} \times (\text{width}+\text{height})^2$.

2.4.12 Immunohistochemical Analysis

In some cases, orthotopic tumor-bearing mice that were treated with IL-2 vectors and/or PAC1 were killed 48 h after the last virus injection. Tumors and livers were harvested, fixed in formalin for 48 h, paraffin embedded and 5 μm thick sections were obtained from the tissues. The tumor sections were stained with TACS-XL *In*

Situ Apoptosis Detection Kit–DAB TUNEL (TdT-mediated dUTP nick end labeling) kit (Cat. No. 4828-30-DK; Trevigen, Gaithersburg, MD, USA) following the manufacturer's instructions to detect apoptotic cells. Tumor sections were also stained for T cells using antibodies against CD3. Briefly, tissue sections were deparafinized and rehydrated after which heat-mediated antigen retrieval was performed (Ramos-Vara 2005). Endogenous peroxidase was quenched by immersing the sections in H_2O_2 for 10 min. The sections were blocked with Dako Antibody diluent (Cat. No. S3022; Dako North America, Carpinteria, CA, USA) and treated with rabbit anti-CD3-antibody (Cat. No. ab16669; Abcam) overnight at 4 °C. The following day, tissue sections were washed and then treated with biotinylated donkey anti-rabbit IgG (H+L) (Cat. No. ab6801, Abcam) antibody for 1 h. Finally, the sections were stained with streptavidin-conjugated horseradish peroxidase (Cat. No. ab7403, Abcam) and diaminobenzidine (Sigma-Aldrich). Images of the tissue sections were taken with an Axioskope color camera (Carl Zeiss Microscopy, Thornwood, NY, USA).

2.4.13 Isolation of CD3+ Cells

Mice injected with Ad vectors were killed and spleens were harvested and homogenized to obtain single-cell suspension in 5 ml phosphate-buffered saline containing 2% fetal bovine serum using a GentleMACS dissociator. CD3+ cells were isolated by negative selection using EasySep (Cat. No. 19851, Stem Cell Technologies, Vacouver, BC, Canada) following the manufacturer's protocol.

2.4.14 Statistical Analyses

Statistical analyses were performed using Student's *t*-tests with 95%confidence interval using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). *P*-values of <0.05 were deemed significant.

2.5 Results

2.5.1 MPE2 Promoter-Driven Reporter Gene is Highly Expressed in BrCa Cells Compared with Normal Cells in vitro and in vivo

Previously, using helper-dependent Ad vectors, we have shown that the MPE2 promoter is highly active in BrCa cells compared with normal cells (Shi, Long et al. 2004; Shi, Graham et al. 2006). To determine whether BrCa specificity of the MPE2 promoter is maintained in a first-generation Ad vector backbone, we constructed E1-,E3-deleted Ad vectors encoding luciferase under the control of the MPE2 (Ad-MPE2-luc) or MCMV promoter (Ad-MCMV-luc). Luciferase expression, as a measure of promoter strength, directed by the two vectors was compared in normal and BrCa cell lines of human and murine origin (Figure 2.1A). Not surprisingly, the activity of MCMV promoter was almost the same in cancer and normal cell lines. Although the MPE2 promoter showed almost the same levels of activity as the MCMV promoter in both human and murine BrCa cell lines (MDA-MB-468 and T47D, and MT1A2, respectively), MPE2 activity was reduced 2–4 logs compared with the activity of MCMV promoter in normal cells (human MRC5 and murine 516). This high level of MPE2 activity in breast cancer cells *in vitro* prompted us to examine MPE2 activity in an *in vivo* breast cancer model.

BrCa specificity of the MPE2 promoter was examined *in vivo* in immune-competent mice bearing subcutaneous (s.c.) flank PyMidT tumors. A single dose of Ad-MPE2-luc or Ad-MCMV-luc was injected intratumorally into each mouse. Tumors and livers were harvested at day 2 or 7 after injection and the levels of luciferase expression induced by each vector were determined (Figure 2.1B). Ad-MCMV-luc showed no tumor specificity at day 2 or at day 7. In contrast, luciferase levels in livers of Ad-MPE2-luc-treated animals were about 4 logs lower than that in tumors at both the time points. Based on this reporter gene expression analysis, it appeared that the MPE2 promoter was capable of driving therapeutic levels of highly selective transgene expression in our syngeneic BrCa model.

2.5.2 MPE2 Controlled IL-2 Expression is Greater in BrCa Cell Lines than in Normal Cell Lines in vitro

We next examined specificity of expression of an immunomodulatory cytokine, hIL-2, under control of the MPE2 promoter, relative to hIL-2 controlled by the hTERT promoter that has been widely used for transcriptional targeting of therapeutic genes to cancer cells (Komata, Kondo et al. 2001; Wack, Rejiba et al. 2008). Non-replicating hIL-2 encoding Ad vectors were constructed for this purpose. We infected BrCa cell lines T47D and MT1A2 as well as normal cell lines MRC5 and 516 with Ad-MCMV-IL-2, Ad-MPE2-IL-2 or Ad-hTERT-IL-2 and then tested the supernatants at 48 h after infection for secreted hIL-2 (Figure 2.2). As expected, the MCMV promoter showed no cell type selectivity, whereas hTERT and MPE2 promoters showed a high degree of specificity for the human BrCa cell line T47D. However, the MPE2 promoter showed greater cancer specificity than the hTERT promoter in murine cell lines. Consequently, the MPE2-IL-2

vector, but not the hTERT-IL-2 vector, was selected for further studies in the syngeneic mouse BrCa model.

2.5.3 Ad-MPE2-IL-2 is Safe and is Well Tolerated in Mice

To determine the toxicity profile of the IL-2 vectors in mice, a single dose of Ad-BHG control, Ad-MPE2-IL-2 or Ad-MCMV-IL-2 was given intravenously to non-tumor-bearing mice. Livers and sera from mice killed at day 7 after injection were analyzed for toxicity markers. The serum levels of both alanine transaminase and aspartate transaminase liver enzymes were considerably higher in Ad-MCMV-IL-2-treated mice compared with Ad-MPE2-IL-2-treated mice (Figure 2.3A). Liver sections were stained with hematoxylin and eosin and then graded on a five-point scale based on increasing indication of liver inflammation. Liver sections from mice treated with Ad-BHG control or Ad-MPE2-IL-2 appeared normal (grade 1); however, liver sections from Ad-MCMV-IL-2-treated mice were highly inflamed.

Consistent with previous results using an Ad-HCMV-IL-2 in the s.c. flank PyMidT model (Addison, Braciak et al. 1995; Addison, Bramson et al. 1998), intratumoral injection of Ad-MCMV-IL-2 was poorly tolerated: 4 out of 5 mice injected intratumorally with 2×10^8 pfu Ad-MCMV-IL-2 were killed or died because of toxicity (Table 2.1). In contrast, no mortality was seen in any of the mice injected with Ad-MPE2-IL-2 at a dose 5 times higher.

To address the tissue specificity of Ad-MPE2-IL-2, we compared the levels of hIL-2 in s.c. PyMidT tumors and livers of mice after a single intratumoral injection of the Ad vectors encoding hIL-2 (Figure 2.3B). The levels of hIL-2 in tumors of Ad-MPE2-IL-2-treated mice were much lower than that in Ad-MCMV-IL-2-treated mice. Similarly,

low levels of hIL-2 were detected in the livers of Ad-MCMV-IL-2 treated mice, but hIL-2 was undetectable in livers of Ad-MPE2-IL-2 treated mice.

2.5.4 Ad-MPE2-IL-2 Shows Weak Anti-tumor Activity in a Subcutaneous Murine Model of BrCa

We compared the ability of Ad-MPE2-IL-2 and Ad-MCMV-IL-2 to regress s.c. PyMidT tumors using equivalent doses of the two viruses based on viral load, as well as equivalent doses of the two viruses based on induced IL-2 expression levels, as estimated from our *in vitro* experiments. Mice bearing s.c. PyMidT tumors were injected intratumorally with a single injection (2×10^8 pfu) of Ad-MPE2-IL-2 or Ad-MCMV-IL-2 for equivalent viral loads. Mice were injected intratumorally with either 1×10^9 pfu Ad-MPE2-IL-2 or 5×10^7 pfu Ad-MCMV-IL-2 to give comparable hIL-2 levels in the tumor. Ad-BHG (1×10^9 pfu) was used as control. Complete responses (tumors completely regressed and were undetectable for at least 60 days) were achieved in 2 out of 5 mice treated with the low dose of Ad-MCMV-IL-2, although extensive toxicity was observed at higher doses. No complete responses were observed in Ad-MPE2-IL-2-treated mice, although partial tumor responses (tumor volumes reduced to half the original size or smaller) were observed in 2 out of 12 mice treated with Ad-MPE-2-IL-2 (Table 2.1). Given the excellent safety profile and predicted specificity of the Ad-MPE2-IL-2 vector, an investigation of combination therapy was warranted using this vector with other drugs that potentially enhance the anti-tumor effect.

2.5.5 Development of the Orthotopic Murine MTHJ BrCa Model

Prior to initiating analysis of any combination therapy, we investigated whether an alternate murine BrCa model could be used to replace the PyMidT model described

above, as that model requires primary cells isolated from tumor explants of MMTV-PyMidT transgenic mice. One concern with the explant model was reproducibility due to tumor heterogeneity in terms of aggressiveness of the tumors cells as well as variability in stromal cells within each tumor explant sample. This heterogeneity may account for the variabilities observed in our study (Table 2.1). In addition, for ethical reasons, we wanted to avoid use of a model that requires maintenance of a transgenic mouse colony solely for the purpose of providing donor tumor tissue. To select a suitable model for our study, three murine BrCa cell lines with established tumorigenicity profiles, EMT6, 4T1 and MTHJ (a cell line derived from an explanted PyMidT tumor), were tested for infectivity by an Ad-GFP vector. Among the 3 cell lines, MTHJ was found to be most susceptible to adenovirus infection (Figure 2.4A). We then compared MPE2 activity with that of the MCMV promoter in MTHJ cells, using Ad-MPE2-luc and Ad-MCMV-luc. Both promoters were highly active, and the luciferase expression levels in cells infected with the two vectors were comparable (within an order of magnitude), as expected (Figure 2.4B). Next, we compared hIL-2 expression following infection of MTHJ cells by Ad-MPE2-IL-2 and Ad-MCMV-IL-2. We observed that hIL-2 expression in Ad-MPE2-IL-2 infected cells was approximately 1000-fold lower compared to that in Ad-MCMV-IL-2 infected cells (Figure 2.4C), similar to our observations using these viruses in other murine (MT1A2) and human (T47D) BrCa cell lines (Figures 2.1A and 2.2). This transgene-dependent difference in relative expression levels comparing the MPE2 promoter to the mCMV promoter led us to question whether hIL-2 could have an inhibitory effect on the MPE2 promoter. This could account for the unexpectedly low levels of IL-2 that were observed in AdMPE2-IL-2 infections. To address this question,

we examined luciferase expression following Ad-MPE2-luc infection in the presence or absence of recombinant hIL-2, or following co-infection of MTHJ cells with Ad-MPE2-luc and Ad-MCMV-IL-2 or the empty vector Ad-dl70-3. We found that the added recombinant IL-2 or the IL-2 expressed by AD-MCMV-IL-2 vector did not affect luciferase expression in Ad-MPE2-luc infections, ruling out the possibility that hIL-2 has an inhibitory effect on MPE2 activity (Figure 2.4D).

Because MTHJ cells showed the highest infectivity by adenovirus among the 3 cell lines compared, we proceeded with using MTHJ cells in our subsequent combination studies. We reasoned that even the low expression of hIL-2 by Ad-MPE2-IL-2 might exert appreciable anti-tumor effect in combination with other agents. PAC1, a drug shown to induce apoptosis in several types of cancer (Putt, Chen et al. 2006), was chosen for the combination study, with the rationale that PAC1 would induce apoptosis in tumor cells, potentially releasing tumor antigens that could be recognized by cytotoxic T cells activated by vector encoded hIL-2.

2.5.6 PAC1 Induces Apoptosis in BrCa Cells in vitro

Previous studies have shown that PAC1 potently kills different types of cancer cells by directly activating procaspase-3 to caspase-3 (Putt, Chen et al. 2006). To test activity in BrCa, we examined PAC1-mediated cytotoxicity in a panel of BrCa cell lines. Subconfluent cells were treated with different concentrations of PAC1 and cell survival was measured. PAC1 was found to kill all the cell types more or less to the same extent (Figure 2.5A). Procaspase-3 cleavage following PAC1 treatment was confirmed in MTHJ (murine BrCa) cells by western blot analysis (Figure 2.5B). The cytotoxic effect of PAC1 was significantly attenuated in presence of Z-VAD that specifically blocks caspase-3

cleavage (Figure 2.5C). Further evidence that killing is mediated by activation of procaspase-3 was provided by simultaneously detecting exposed phosphatidylserine (a marker of apoptosis) and cleaved caspase-3 activity using the Dual Apoptosis Kit. High proportions of cells were found to be positive for exposed phosphatidylserine and caspase-3 activity (Figure 2.5D & E).

2.5.7 Ad-MPE2-IL-2 Significantly Retards Tumor Growth in the Orthotopic MTHJ BrCa Model but Addition of PAC1 does not Boost Anti-tumor Effect of the Vector

The ability of PAC1 to enhance the anti-tumor effect of Ad-MPE2-IL-2 was examined in an orthotopic BrCa model established by injecting MTHJ cells in the mammary fat pad of FVB mice. Tumor-bearing mice were injected intratumorally over a period of 1 week with 3 doses of 5×10^8 pfu Ad-MPE2-IL-2, Ad-MCMV-IL-2 or Ad-dl70-3 control vector, in combination with either PAC1 or the vehicle control delivered by oral gavage for 21 days starting 1 day before the first virus injection. No overt signs of toxicity were seen in any of the treatment groups. Compared with the control group, tumor growth was significantly retarded in a fraction of animals treated with Ad-MCMV-IL-2 or Ad-MPE2-IL-2 either alone or in combination with PAC1. However, PAC1 alone was not sufficient to delay tumor growth (Figure 2.6A & B).

We used TUNEL staining to visualize apoptotic cells in tumor sections on day 9 following the initiation of treatment. The control (Ad-dl70-3+HPβCD) or Ad-dl70-3 plus PAC1-treated mice had undetectable apoptotic cells. Mice treated with Ad-IL-2 vectors alone showed some apoptotic cells, whereas mice treated with the combination of Ad-IL-2 vectors and PAC1 showed higher levels of apoptosis in their tumor sections (Figure 2.6C).

Tumor sections were stained with anti-CD3 antibody to estimate the amount of T cells infiltrated into the tumor on day 9 from the initiation of treatment. Whereas T cells were undetectable in the tumors of mice treated with control vector, high levels of T cells were detected in tumors of Ad-MCMV-IL-2-treated mice. T cells in the tumors of Ad-MPE2-IL-2-treated mice were comparable to that in Ad-MCMV-IL-2-treated mice (Figure 2.6D). Furthermore, spleens of IL-2-treated mice were visibly enlarged by day 9, and there were increased numbers of CD3+ spleen cells in Ad-MPE2-IL-2- and Ad-MCMV-IL-2-treated mice compared with those in Ad control-treated mice (Figure 2.6E).

Furthermore, we also analyzed liver sections from the treated mice on day 9, in order to determine liver injuries. Liver sections of mice from all treatment groups were stained with hematoxylin and eosin (H & E) and imaged (Figure 2.6F). The H & E stained sections were sent to a veterinary pathologist for detailed analysis. According to the pathologist's report, livers from mice treated with Ad-MCMV-IL-2 alone or in combination with PAC1 had numerous clustered infiltrates of small lymphocytes and neutrophils in the parenchyma and around the portal triads. The infiltrates were associated with necrotic hepatocytes and the overall damages to the liver were severe. However, livers from mice treated with Ad-MPE2-IL-2 alone or in combination with PAC1 appeared normal much like those from the Ad-dl70 control treated mice.

2.6 Discussion

IL-2 has been used in clinics for over two decades for the treatment of metastatic melanoma and metastatic renal cell carcinoma but with limited success (Coventry and Ashdown 2012). The therapeutic benefit of IL-2 is often limited by the severe toxicity associated with a high systemic dose. Vascular leak syndrome is a particularly severe form of toxicity that may result from systemic IL-2 therapy that is marked by accumulation of extravascular fluid in vital organs such as lungs and liver (Nakagawa, Miller et al. 1996; Epstein, Mizokami et al. 2003). There is no treatment for vascular leak syndrome and the only option is to discontinue IL-2 therapy (Laurent, Touvrey et al. 2013). In order to minimize IL-2-associated toxicity, several different approaches have been proposed, including targeting IL-2 specifically to the tumor. IL-2 fused to an antibody recognizing a glycolipid (GD2), expressed on the surface of melanoma and neuroblastoma cells, has been shown to accumulate in the tumor microenvironment in animal models. This fusion protein was found to inhibit growth of established metastases of human melanoma and neuroblastoma in mice (Sabzevari, Gillies et al. 1994; Becker, Pancook et al. 1996; Pancook, Becker et al. 1996). IL-2 fused with an antibody recognizing the human epithelial cell adhesion molecule EpCAM that is overexpressed in many different cancers has been shown to be effective against some murine cancer models (Xiang, Lode et al. 1997; Lode, Xiang et al. 1998; Balzar, Winter et al. 1999). In addition, fusion of IL-2 with antibodies against carcinoembryonic antigen, epidermal growth factor receptor or HER2/neu have also been studied for targeting IL-2 to different types of tumors (Davis and Gillies 2003).

We studied the safety and anti-tumor efficacy of IL-2 transcriptionally targeted to BrCa. Previously, transcriptionally targeted IL-2 has been studied for other types of cancer. Vile *et al.* used the murine tyrosinase promoter to direct expression of exogenous murine IL-2 to melanoma cells (Vile, Miller et al. 1994). Similarly, He *et al.* used the liver-specific albumin promoter together with a tumor-specific α-fetoprotein enhancer to achieve hepatocellular carcinoma-specific expression of an IL-2/interferon α2b fusion protein (He, Tang et al. 1999). Similar to our results with MPE2 luciferase constructs, our data show that mammaglobin promoter (MPE2)-controlled IL-2 is preferentially expressed in mammary tumor cells *in vitro* at levels comparable to that driven by the MCMV immediate early promoter. However, MPE2-driven IL-2 expression appeared to be much lower *in vivo* compared with MCMV promoter-driven IL-2 expression. Whereas hIL-2 protein was detected in the tumor and liver of mice injected intratumorally with Ad-MCMV-IL-2, it was detected only in the tumor, although at low levels, of Ad-MPE2-IL-2-injected mice. Most importantly, based on liver histology and levels of liver enzymes (alanine transaminase and aspartate transaminase) in sera of treated mice, Ad-MPE2-IL-2 vector appears to have little, if any, liver toxicity.

Although the transcriptionally targeted IL-2 treatment appears safe in our hands and treatment with Ad-MPE2-IL-2 significantly delayed tumor growth compared with control-treated mice, no complete tumor regressions were achieved in any of the treated mice. There are several possible nonmutually exclusive explanations for this. First, Ad-IL-2 treatment is known to have a narrow therapeutic window (Toloza, Hunt et al. 1996; Putzer, Bramson et al. 1998) and the low levels of IL-2 expression by Ad-MPE2-IL-2 may be outside that window. Second, PyMidT overexpressing cells form rapidly growing

tumors, and a study by Vaage (Vaage 1988) has shown that IL-2 therapy is more effective with slow-growing tumors. Third, breast tumors in general have been found to be less responsive to IL-2 monotherapy, both in animal models and early-phase clinical trials (Rao, Dyer et al. 2006). However, IL-2 in combination with other agents has been shown to be effective for breast cancer in animal models and early-phase clinical trials. For example, in a study by Putzer *et al.*,(Putzer, Bramson et al. 1998) a single dose of 1.8×10^8 pfu Ad vector expressing hIL-2 (human CMV promoter controlled) in combination with a single dose of 10^9 pfu of Ad-p53 resulted in complete tumor regression in 50% of mice (n=20) bearing PyMidT tumors. Single agents did not cause complete tumor regression in any animals in that study, although each agent was capable of causing significant delay in tumor growth, in accordance with our result where we see delayed tumor growth in a fraction of mice treated with Ad-MPE2-IL-2 or Ad-MCMV-IL-2 vector. One problem with delivering exogenous p53 by an Ad vector is that only a fraction of tumor cells (as little as 20%) are transduced with the virus even with intratumoral injection of as high as $\sim 10^9$ pfu of virus(Putzer, Bramson et al. 1998). Unlike IL-2, p53 is not a secreted protein and therefore its effect would be limited to the transduced cells. In contrast, a small-molecule drug such as PAC1 should be accessible to almost all the tumor cells. Furthermore, our approach for combining Ad-IL-2 with PAC1 was based on the rationale that the proapoptotic PAC1 would induce apoptosis in tumor cells revealing potential tumor antigens that would be recognized by cytotoxic T cells, after which IL-2 in the tumor milieu could help in amplification of these T cells.

PAC1 has been shown to be highly toxic to many types of cancer cell lines *in vitro* and causes significant tumor regression in animal models for several types of tumors (Putt, Chen et al. 2006). The dose of PAC1 used in this study (100 $mg\,kg^{-1}$) was selected because higher doses were reported to induce transient neurotoxicity in mice (Peterson, Hsu et al. 2010). Although we observed PAC1-induced apoptosis *in vitro*, few if any apoptotic cells were observed in the tumor section of mice treated with PAC1 alone. Whereas Ad-MPE2-IL-2 and Ad-MCMV-IL-2 vectors significantly delayed tumor growth *in vivo* compared with control, PAC1 alone failed to do so. Unfortunately, the addition of PAC1 did not boost the anti-tumor effect of Ad-IL-2 vectors, suggesting that the concentration of PAC1 used might not have been sufficient to release enough tumor antigens to stimulate immunity.

Taken together, our data show that the MPE2 promoter can be used for transcriptional targeting of IL-2 or other genes to BrCa cells using nonreplicating Ad vectors. However, despite showing transcriptional strength nearly comparable to the MCMV promoter in BrCa cells *in vitro* and in mice when using luciferase encoding vectors, the MPE2 regulatory sequence was inefficient in driving IL-2 gene expression in PyMidT tumor models in mice. Nonetheless, even low levels of IL-2 expression by Ad-MCMV-IL-2 resulted in significant delay of tumor growth, as did the Ad-MCMV-IL-2 vector. Importantly, the Ad-MPE2-IL-2 vector showed much lower liver toxicity compared with the Ad-MCMV-IL-2 vector. However, our effort to enhance the anti-tumor activity of Ad-IL-2 vectors by the addition of PAC1 was not successful. Combination of the Ad-MPE2-IL-2 vector with other potent apoptotic drugs may result in enhanced anti-tumor activity of the vector.

Table and Figures

Table 2.1: Tumor Regression by Ad-IL-2 Vectors in Subcutaneous Breast Cancer Model

Virus	Dose (pfu)	n =	Morta-lity	Complete Regression	Partial[a] Regression	No Response	Protec-tion[b]
Ad-BHG	1x10e9	5				5 (100%)	
Ad-MPE2-IL-2	1x10e9	6			1 (17%)	5 (83%)	
Ad-MPE2-IL-2	2x10e8	6			1 (17%)	5 (83%)	
Ad-MCMV-IL-2	2x10e8	5	4 (80%)			1 (20%)	
Ad-MCMV-IL-2	5x10e7	5		2 (40%)		3 (60%)	2 (40%)

[a] Partial regression is defined as reduction to half of initial tumor volume or smaller

[b] Mice that were tumor free for 30 days were re-injected with tumor cells. Mice that did not form tumors within 60 days were considered protected.

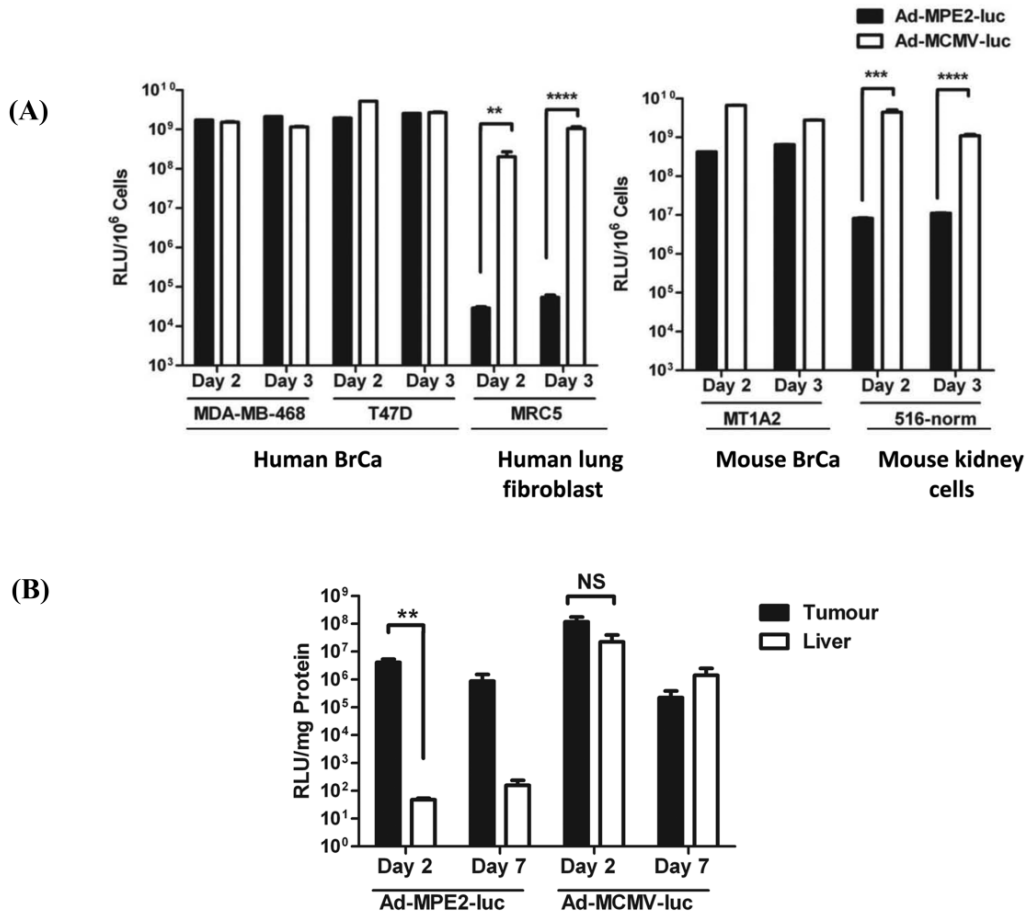


Figure 2.1: MPE2 promoter shows breast cancer specificity in driving reporter gene. (A) Normal human lung fibroblast (MRC5) and mouse kidney (516) cells as well as human BrCa (MDA-MB-468 and T47D) and mouse BrCa (MT1A2) cells were infected with Ad-MPE2-luc or Ad-MCMV-luc at a multiplicity of infection (MOI) of 50 pfu/cell. Cell lysates were collected 48 and 72 hours post-infection and assayed for luciferase activity. Data are shown as mean ± s.d. of three independent experiments. **(B)** FVB mice bearing subcutaneous PyMidT tumors were injected intra-tumorally with a single dose of $5x10^8$ pfu Ad-MCMV-luc or Ad-MPE2-luc. At day 2 or 7 post-injection, tumor and liver were harvested and lysates assayed for luciferase expression. Luciferase activity was normalized to protein concentration. Each data point represents mean ± s.d. (n=3). Student's *t*-test $^{**}P<0.01$, $^{***}P<0.001$, ****<0.0001, NS = not significant.

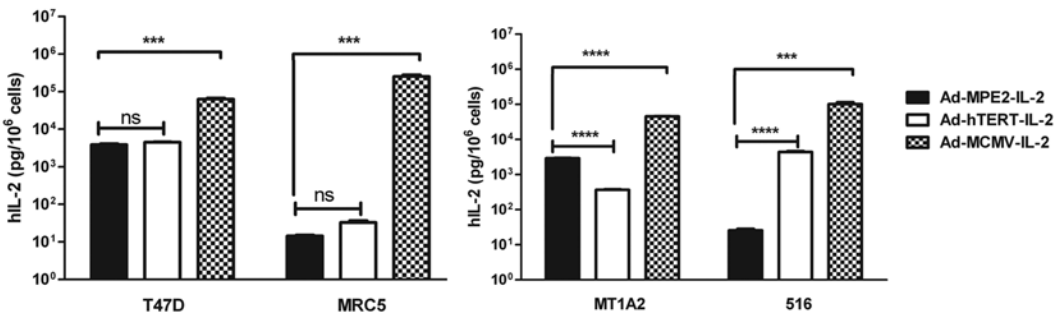


Figure 2.2: MPE2 promoter shows breast cancer specificity in driving therapeutic (hIL-2) gene. MPE2, MCMV and hTERT promoters were compared for strength and BrCa specificity in driving hIL-2 expression. Normal (MRC5 and 516) as well as BrCa (T47D and MT1A2) cells were infected with Ad-MCMV-IL-2, Ad-MPE2-IL-2 or Ad-hTERT-IL-2 at an MOI of 50 pfu/cell. Cell supernatants collected at 48 hours post-infection were assayed for hIL-2 by ELISA. Results are shown as mean ± s.d. of triplicate infections. Student's *t*-test $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, ns = not significant. The experiment was repeated at least once.

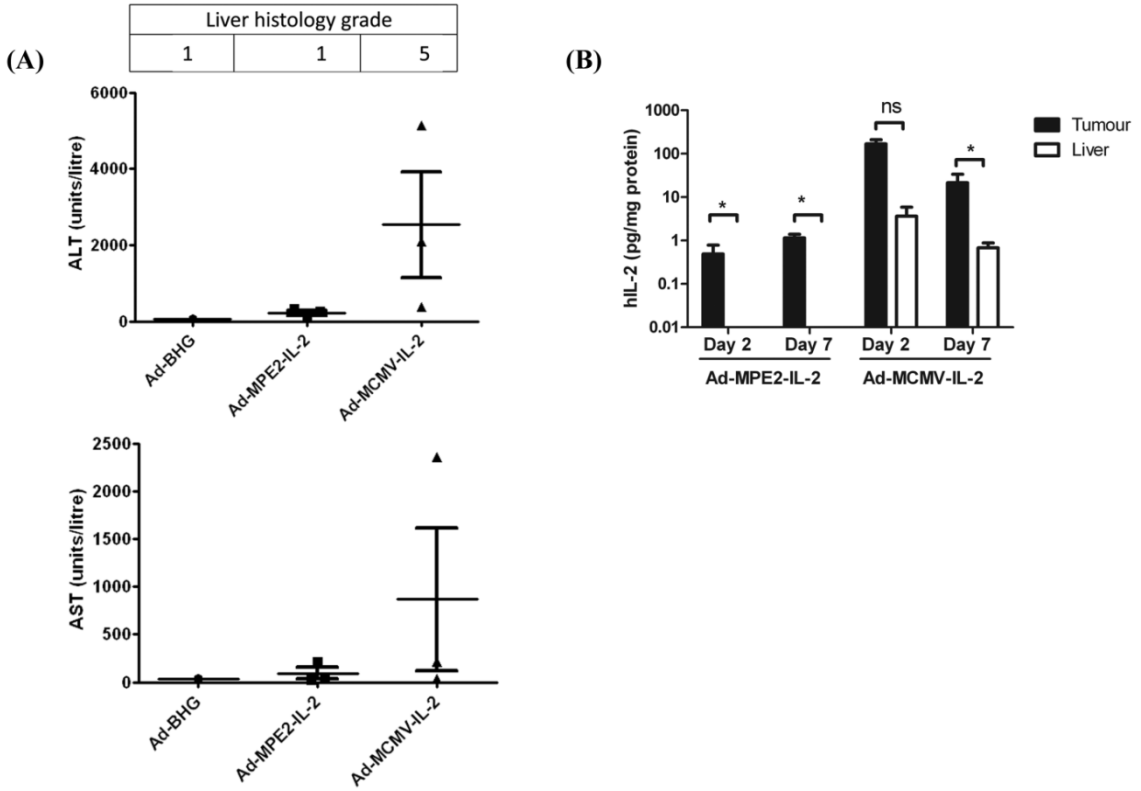
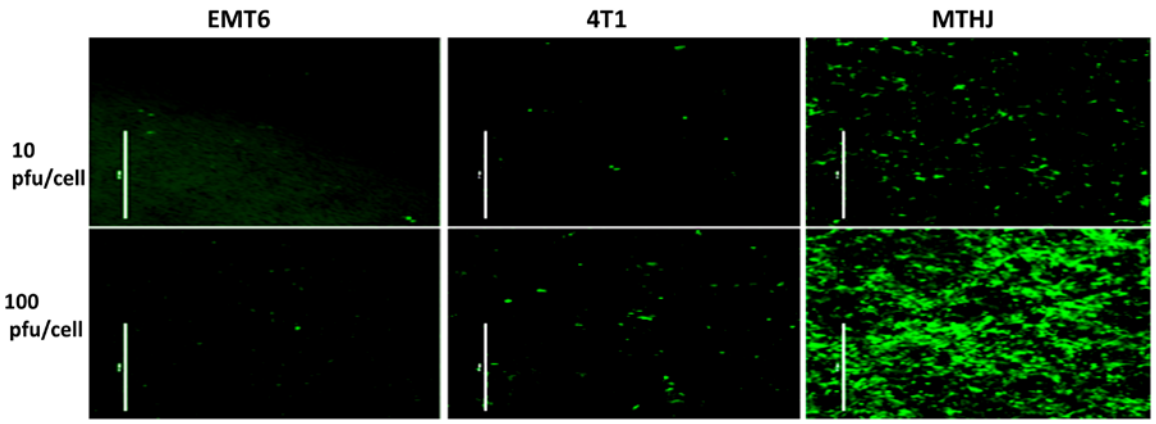
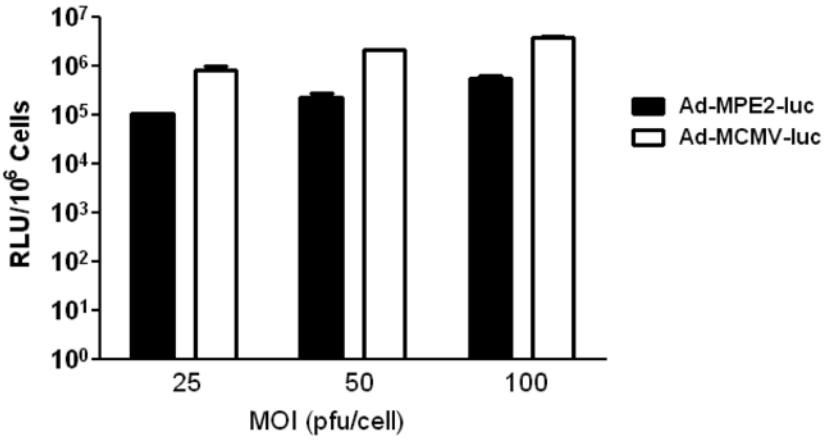


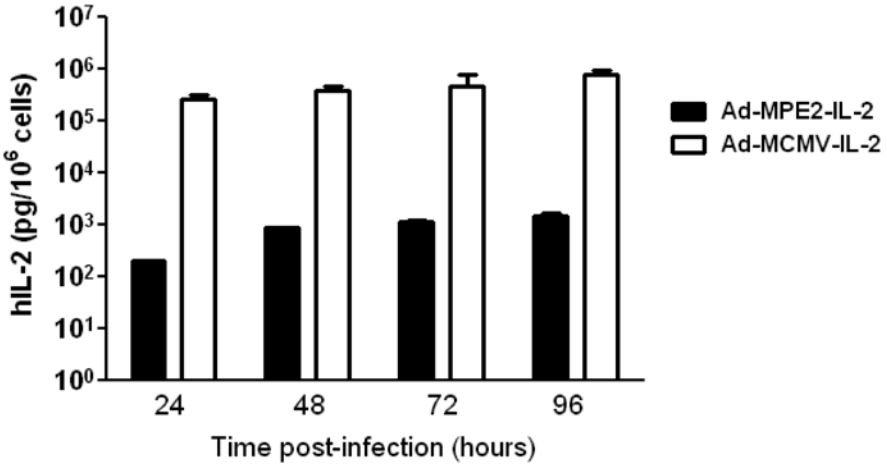
Figure 2.3: Ad-MPE2-IL-2 is less toxic compared to Ad-MCMV-IL-2 in mice. Toxicity markers and hIL-2 expression in mice treated with Ad vectors encoding hIL-2 were compared. **(A)** Non-tumour bearing FVB mice were injected via tail vein with $5x10^8$ pfu of AdBHG, Ad-MPE2-IL-2 or Ad-MCMV-IL-2. At day 7, animals were sacrificed and livers were harvested for histology and H & E staining. Also, serum was collected for analysis of serum liver enzymes (ALT and AST, markers of liver injury). Histology grades of stained liver sections based on the levels of inflammation are denoted in the table above the graphs. Data are given for each individual animal in a group (n=3). **(B)** hIL-2 levels in the tumor and liver of mice injected intra-tumorally with a single dose of $5x10^8$ pfu of the Ad vectors. Results are shown as mean ± s.d. (n=2), Student's *t*-test *$P<0.05$, ns = not significant.


EMT6
4T1
MTHJ
10 pfu/cell
100 pfu/cell

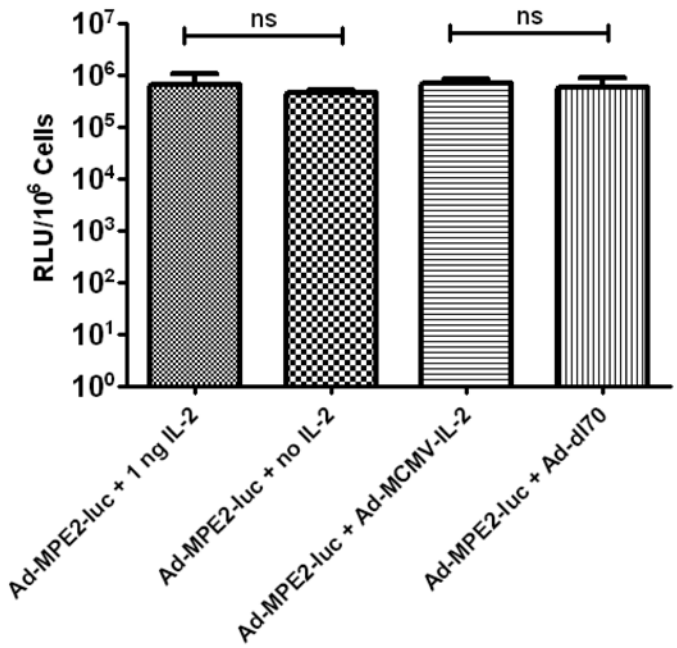
(A)


RLU/10^6 Cells
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
25
50
100
MOI (pfu/cell)
Ad-MPE2-luc
Ad-MCMV-luc

(B)

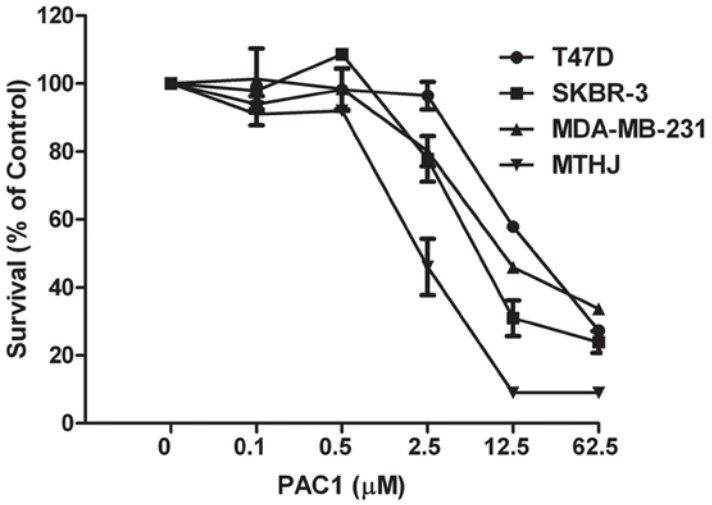

hIL-2 (pg/10^6 cells)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
24
48
72
96
Time post-infection (hours)
Ad-MPE2-IL-2
Ad-MCMV-IL-2

(C)

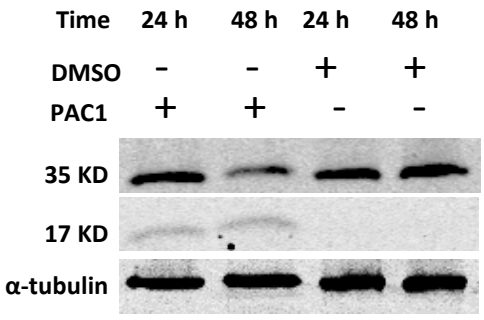

RLU/10^6 Cells
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
ns
ns
Ad-MPE2-luc + 1 ng IL-2
Ad-MPE2-luc + no IL-2
Ad-MPE2-luc + Ad-MCMV-IL-2
Ad-MPE2-luc + Ad-dl70

(D)

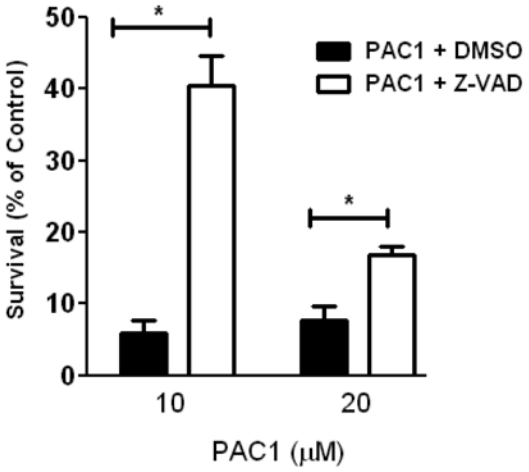
Figure 2.4: MTHJ cells support high levels of adenovirus infection and reporter gene expression by MPE2 promoter. (A) Three murine BrCa cell lines (EMT6, 4T1 and MTHJ) were infected with an adenovirus vector encoding GFP (Ad-MCMV-GFP) at an MOI of 10 or 100 pfu/cell. Cells were imaged for GFP expression using an EVOS fluorescent microscope, 48 hours post-infection. **(B)** MTHJ cells were infected with Ad-MPE2-luc or Ad-MCMV-luc at the indicated MOI. Cell lysates were collected 48 hours after infection and assayed for luciferase activity. Data are shown as mean ± s.d of three independent experiments. **(C)** MTHJ cells were infected with Ad-MPE2-IL-2 or Ad-MCMV-IL-2 at an MOI of 50 pfu/cell. Cell supernatants collected at the indicated time points were assayed for hIL-2 by ELISA. Data are shown as mean ± s.d of two independent experiments. (D) MTHJ cells were infected with Ad-MPE2-luc, and a final contration of 1 ng of recombinant IL-2 or vehicle was added to the infected cells 4 hours post-infection. Also, MTHJ cells were co-infected with Ad-MPE2-luc and Ad-MCMV-IL-2 or Ad-MPE2-luc and Ad-dl70 at MOIs of 50 pfu/cell for each of the viruses. Cell lysates were collected 48 hours after infection and assayed for luciferase activity. Data are shown as mean ± s.d of quadruplicate infections.


Survival (% of Control)
120
100
80
60
40
20
0
0
0.1
0.5
2.5
12.5
62.5
PAC1 (μM)
T47D
SKBR-3
MDA-MB-231
MTHJ

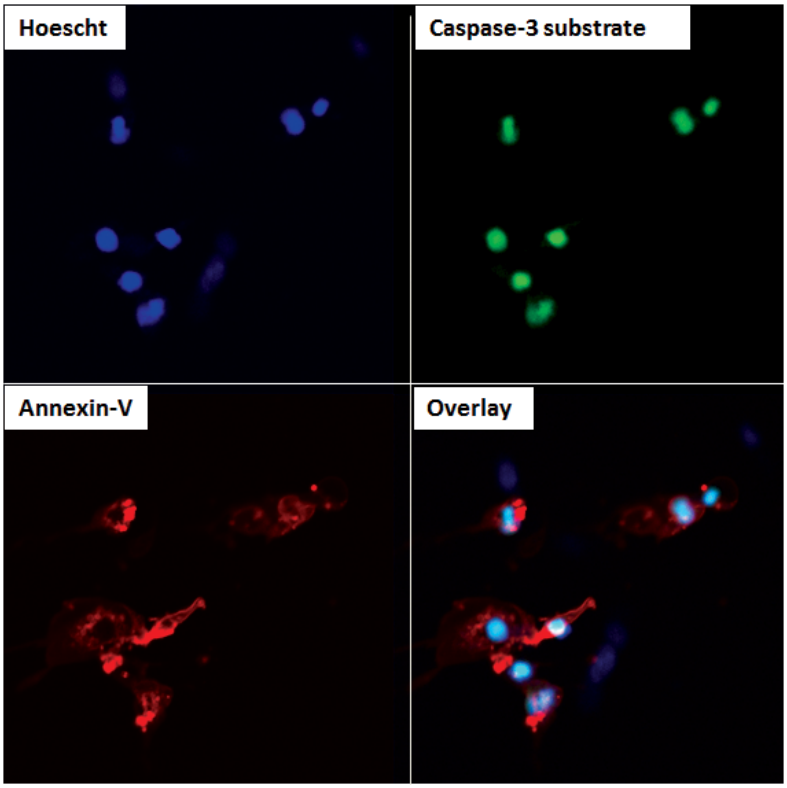
(A)


Time
24 h
48 h
24 h
48 h
DMSO
-
-
+
+
PAC1
+
+
-
-
35 KD
17 KD
α-tubulin

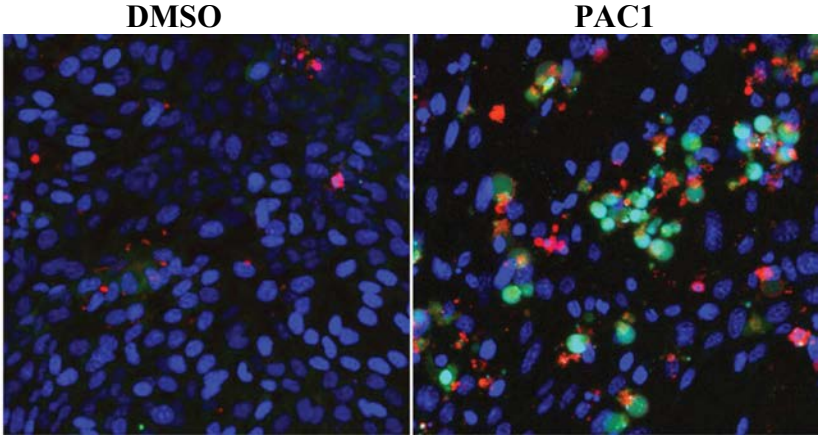
(B)


Survival (% of Control)
50
40
30
20
10
0
*
*
10
20
PAC1 (μM)
PAC1 + DMSO
PAC1 + Z-VAD

(C)

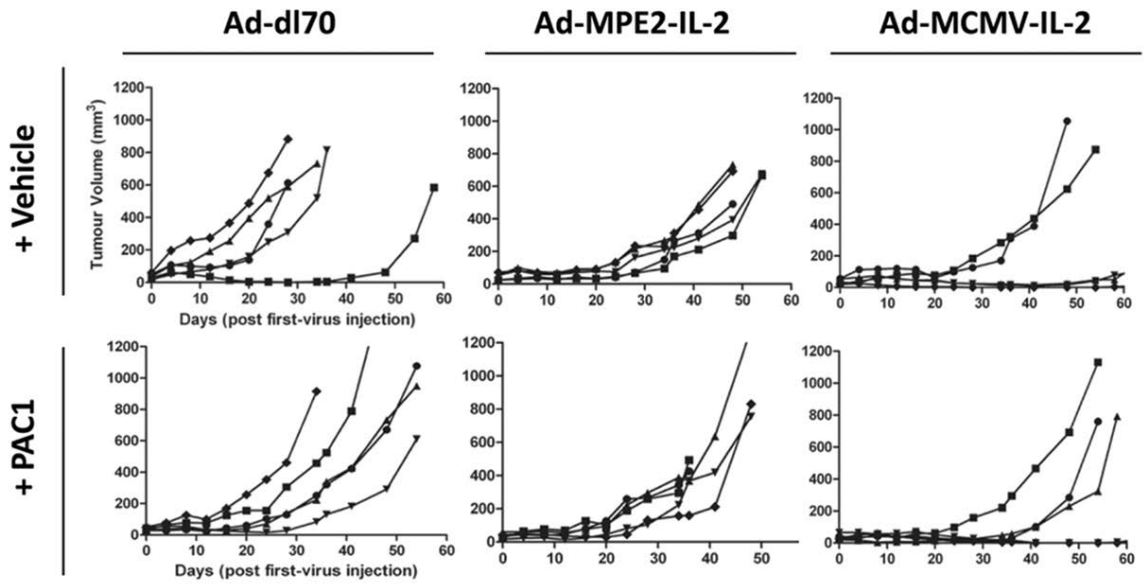


(D)

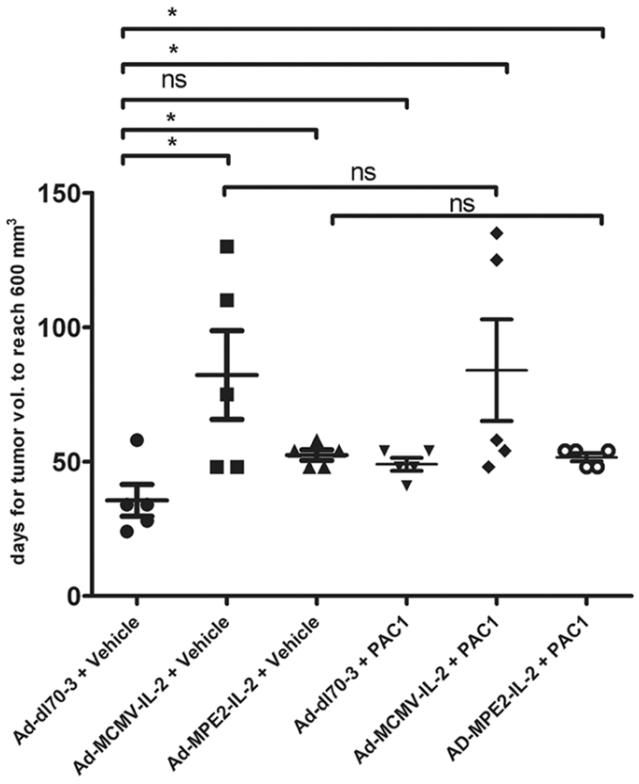


(E)

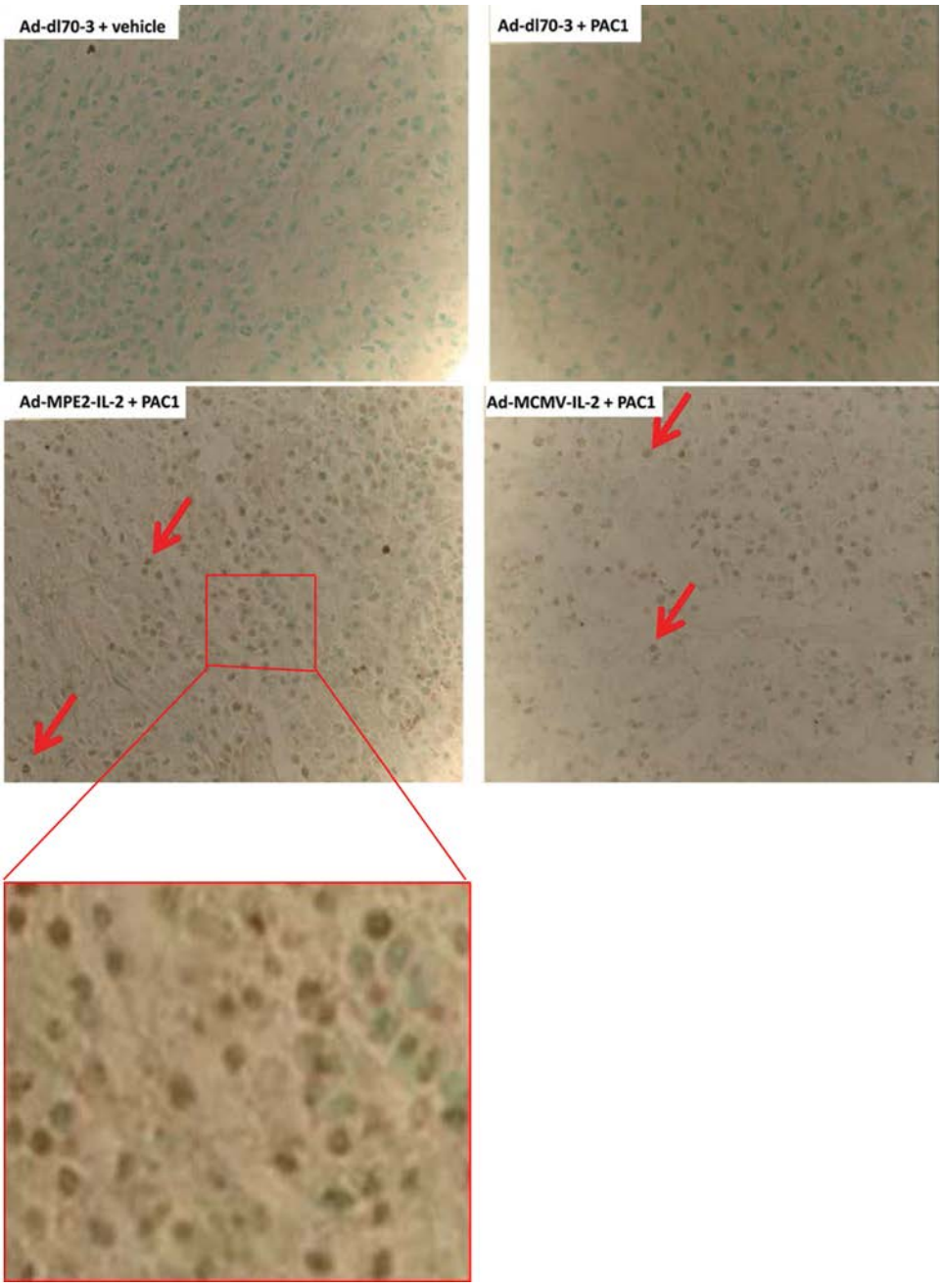
Figure 2.5: PAC1 induces apoptosis in breast cancer cells *in vitro*. (A) BrCa cells were treated with increasing concentrations of PAC1 as indicated. Cell viability was determined 72 hours post-treatment using the Alamar Blue viability assay. Results are shown as mean ± s.d. of three independent experiments. **(B)** MTHJ murine BrCa cells were treated with 5 μM PAC1 or DMSO (as control) and cell lysates were collected 24 or 48 hours later. Caspase-3 cleavage was determined by Western blot analysis. **(C)** MTHJ cells cells were treated with 100 μM Z-VAD or DMSO and 4 hour later PAC1 was added to the cells at the indicated concentration. Cell viability was determined 72 hours later by the Alamar Blue viability assay. The bars represent mean ± s.d of 2 independent experiments. **(D)** MTHJ cells were treated with 5 μM PAC1 and assayed for apoptosis 48 hours later using the Dual Apoptosis Kit (Biotium) that detects exposed PS by Annexin V binding (red) and caspase-3 activity by increased fluorescence of a caspase-3 substrate (green). **(E)** Representative pictures of MTJH cells stained with the reagents of the dual apoptosis kits 48 hours after treatment with 5 μM PAC1 or DMSO. Images were taken by confocal microscopy at 10X magnification. **(B), (D)** and **(E)** have been repeated at least once.


Ad-dl70
Ad-MPE2-IL-2
Ad-MCMV-IL-2
+ Vehicle
+ PAC1
Tumour Volume (mm3)
Days (post first-virus injection)

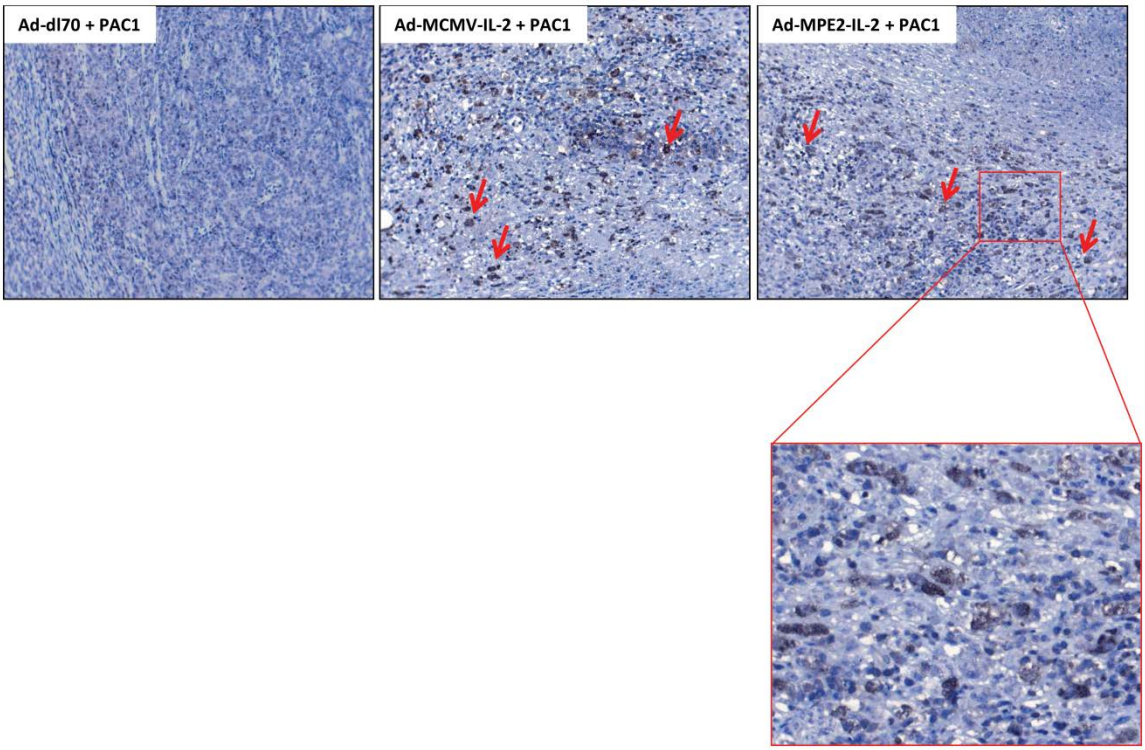
(A)


*
*
ns
*
*
ns
ns
days for tumor vol. to reach 600 mm3
Ad-dl70-3 + Vehicle
Ad-MCMV-IL-2 + Vehicle
Ad-MPE2-IL-2 + Vehicle
Ad-dl70-3 + PAC1
Ad-MCMV-IL-2 + PAC1
AD-MPE2-IL-2 + PAC1

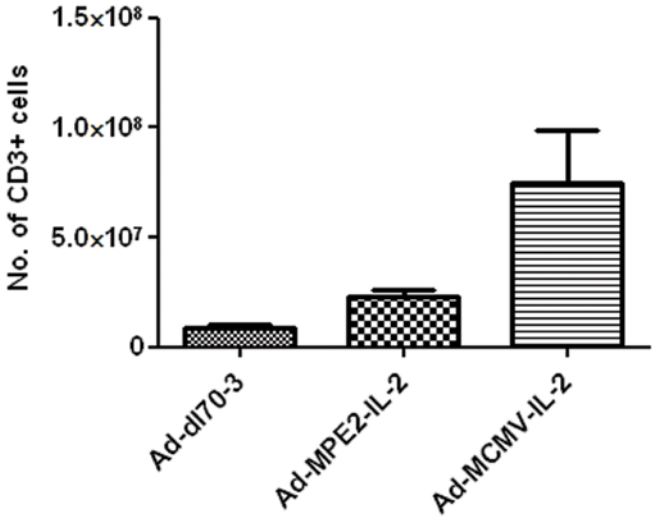
(B)


Ad-dl70-3 + vehicle
Ad-dl70-3 + PAC1
Ad-MPE2-IL-2 + PAC1
Ad-MCMV-IL-2 + PAC1

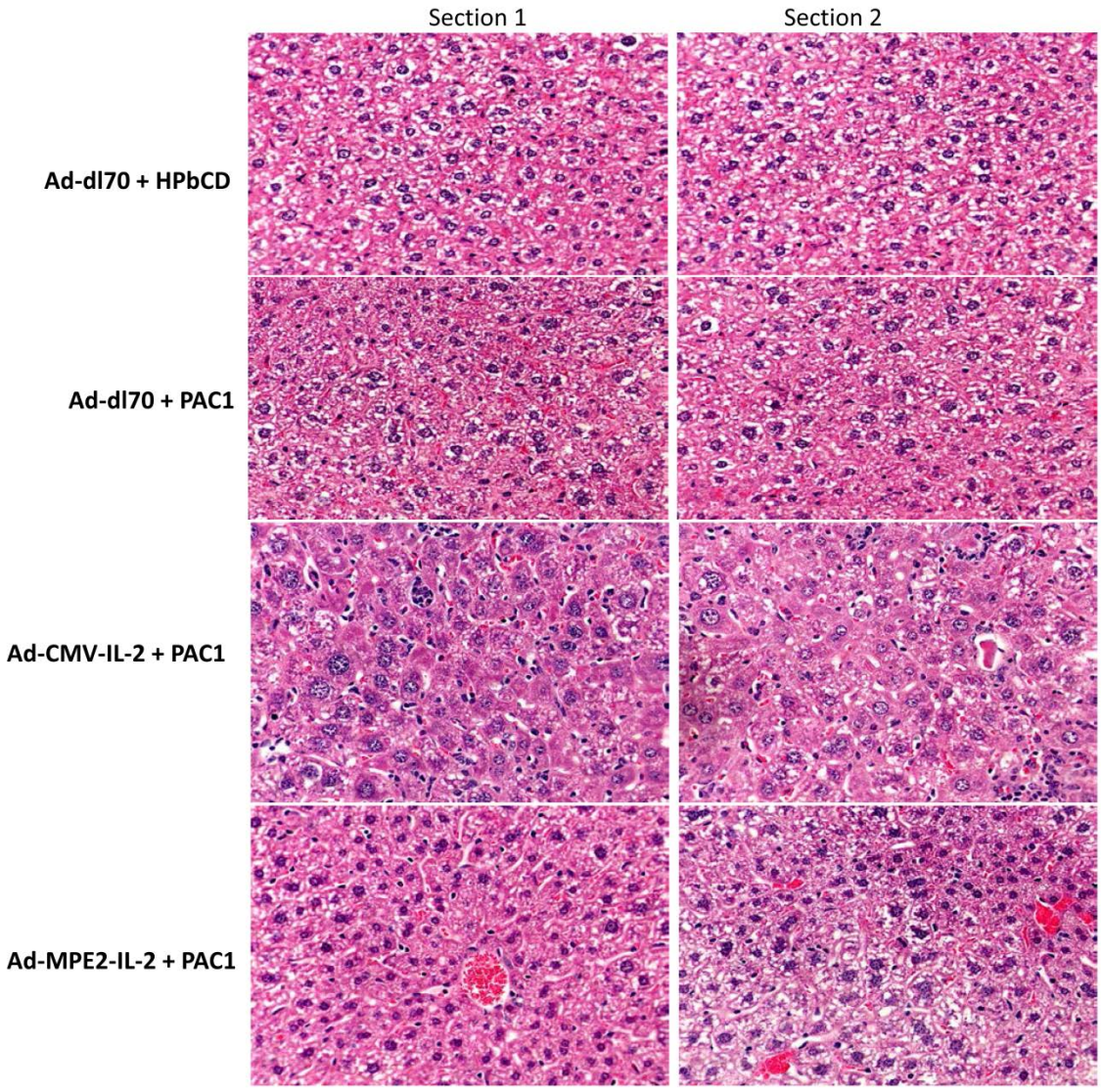
(C)


Ad-dl70 + PAC1
Ad-MCMV-IL-2 + PAC1
Ad-MPE2-IL-2 + PAC1

(D)


No. of CD3+ cells
1.5×10⁸
1.0×10⁸
5.0×10⁷
0
Ad-dl70-3
Ad-MPE2-IL-2
Ad-MCMV-IL-2

(E)


Section 1
Section 2
Ad-dl70 + HPbCD
Ad-dl70 + PAC1
Ad-CMV-IL-2 + PAC1
Ad-MPE2-IL-2 + PAC1

(F)

Figure 2.6: Ad vectors encoding hIL-2 gene have significant anti-tumor effects. (A) PyMIdT tumor-bearing mice (n=7) were given 3 injections of $5x10^8$ pfu of Ad-dl70-3, Ad-MPE2-IL-2 or Ad-MCMV-IL-2 on days 1, 3 and 7. Also, mice were administered 100 mg/kg PAC1 or HPβCD by oral gavage every day for 21 days starting on day 0. Tumors were measured twice-weekly with digital calipers. Each line in the graph represents tumor volume from a single mouse. **(B)** Comparison of time for tumor volumes to reach 600 mm^3 among the treatment groups. Days for tumors to reach 600 mm^3 were plotted and compared. Data are shown for individual animals in a group. Student's *t*-test $*P<0.05$, ns = not significant. Shown is a representative result out of two independent experiments. **(C)** Two mice from each group in (A) were euthanized 48 hours after the last dose of virus (*i.e.* on day 9 from the start of treatment); tumor, spleen and liver were harvested from each mouse. Tumors sections were assessed for DNA fragmentation as a marker of apoptotic cells using TUNEL kit (representative positive cells are indicated by arrows). **(D)** Sections of tumors were also stained with anti-CD3 antibody to determine the levels of T cells (representatives shown by arrow) in the tumor**.** **(E)** CD3+ cells were isolated from the spleen of each mouse using negative selection and the absolute numbers of CD3+ cells were determined by hemocytometric counting. Results are shown as mean ± s.d. (n=2). **(F)** H & E staining was performed on the liver sections from two mice from each treatment group to determine liver damage. According to a veterinary pathologist, the liver sections from mice treated with Ad-MCMV-IL-2 alone or in combination with PAC1 have numerous clustered infiltrates of small lymphocytes and neutrophils in the parenchyma and around the portal triads. The infiltrates are associated with necrotic hepatocytes and the overall damage to the liver is

severe. However, livers from mice treated with Ad-MPE2-IL-2 alone or in combination with PAC1 seem normal much like that from Ad-dl70 treated mice (See the pathologist's report in the APPEDIX section of this dissertation).

Chapter 3: Vaccinia Virus Lacking Genes for Nucleotides Synthesis Shows Oncolytic Activity in Breast Cancer

Vaccinia Virus Lacking Genes for Nucleotides Synthesis Shows Oncolytic Activity in Breast Cancer

Shyambabu Chaurasiya, Nicole Favis, Kyle Potts, Chad Irwin, Ryan Noyce, Kate Agopsowicz, David H. Evans, Mary M. Hitt

This chapter consists of an author-generated version of a manuscript soon to be submitted to the journal: ***Molecular Therapy-Oncolytics***

Contributions

Mutant viruses used in this study were rescued and purified by Dr. Chad Irwin and Kyle Potts in the laboratory of Dr. David Evans. Nicole Favis generated the raw data for tumor regression and survival studies in the xenograft model. Kate Agopsowicz helped with spheroid culture and did tumor cell injections in one of the animal experiments. Dr. Ryan Noyce helped design animal experiments. Dr. David Evans and Dr. Mary Hitt provided intellectual directions to this project and Dr. Hitt also edited the manuscript.

I did all the cytotoxicity assays, western blot analysis, virus growth curves, RRM2 knock-down experiments, cancer-specificity of virus in spheroid culture, and tested oncolysis by the viruses in cancer stem-like and differentiated populations isolated from the SUM-149 breast cancer cell line. Also, I performed the bio-distribution study and determined the effect of the viruses on tumor vasculature in nude and immune-competent mice. Nicole Favis assisted me with the tumor regression/survival study in the syngeneic mouse model. Finally, I wrote the manuscript and Dr. Mary Hitt edited it.

3.1 Abstract

We have generated mutants of vaccinia virus that lack either the *F4L* gene (ΔF4L VACV) or lack *F4L* together with *J2R* (ΔF4LΔJ2R VACV) and investigated their oncolytic activities. We hypothesize that the deletion of these genes, crucial for deoxyribonucleoside triphosphate (dNTP) synthesis, will attenuate growth of the virus in normal cells with minimal effect on its growth in cancer cells. We tested the cancer specificity, safety and anti-tumor efficacy of *F4L*-mutant viruses in breast cancer. Here we have shown that growth of *F4L*-deleted virus is dependent on levels of the small subunit (RRM2) of ribonucleotide reductase, the cellular homolog of the viral F4 protein, and a rate limiting factor in dNTP synthesis. The *F4L*-mutant viruses were found to replicate efficiently in and kill different types of breast cancer cell lines, *in vitro*, much like the wild type virus. Interestingly, the viruses were at least as cytotoxic to "triple-negative" (lacking estrogen and progesterone receptors and HER2) breast cancer cells, the most aggressive and difficult to treat, as to luminal breast cancer cells. Using 3-dimensional co-spheroid cultures of breast cancer and normal cells, we found that the *F4L*-mutant viruses have high selectivity for cancer cells. Also, growth and cytotoxicity of ΔF4L VACV were found to be similar in stem-like ($CD44^{+}CD24^{-/low}$) and differentiated cell populations following sorting of SUM-149, a triple-negative human breast cancer cell line. Importantly, the *F4L*-mutant viruses showed significant anti-tumor activity both in xenograft and syngeneic mouse models of breast cancer. The safety and anti-tumor efficacy of the *F4L*-deleted viruses were found to be comparable to the *J2R*-mutant virus. Taken together, our data show that *F4L*-mutant vaccinia virus could be a

promising oncolytic agent for the treatment of aggressive types of breast cancer but would likely require additional intervention to completely clear tumors.

3.2 Introduction

Breast cancer (BrCa) is the most common cancer and leading cause of cancer-related death in women worldwide (Jemal, Siegel et al. 2010). Surgery and radiation therapy are commonly used for the treatment of primary BrCa while chemo- and hormone therapies are used for the management of metastatic BrCa (Higgins and Wolff 2008). One challenge in the treatment of BrCa stems from the fact that it is a highly heterogeneous disease and not all types of BrCa respond to the same chemo- or hormone therapies (Polyak 2011). Although modern chemo-, hormone and targeted therapies have improved overall and relapse-free survival of BrCa patients, long-term survival remains low for metastatic BrCa, therefore alternative therapeutics with better efficacy are urgently needed (Hartkopf, Fehm et al. 2011; Liedtke and Rody 2015).

Many different viruses have been studied for their potential use as oncolytic agents. While some viruses such as human reovirus (Hashiro, Loh et al. 1977; Coffey, Strong et al. 1998) and vesicular stomatitis virus (Stojdl, Lichty et al. 2000) have natural selectivity for cancer cells, other viruses are genetically engineered to make them cancer-selective. Vaccinia virus (VACV) is one of the most intensively studied viruses for use in oncolytic therapy. VACV was used as a vaccine for the successful eradication of smallpox throughout the world and thus has a long-established safety profile in humans. Furthermore, characteristics of VACV such as rapid life cycle, stability, efficient cell-to-cell spread, and productive lysis of host cells make it an ideal candidate oncolytic virus (McCart, Ward et al. 2001). VACV encodes more than 200 genes (Simonian, Antonova

et al. 1979) some of which are homologs of cellular genes, and it replicates in the cytoplasm quasi-independently of cellular proteins (Buller and Palumbo 1991). Interestingly, the cellular homologs of some of the essential viral proteins are over-expressed in cancer cells. This provides an opportunity to make the virus selective for cancer cells by deletion of viral gene(s) whose function would be complemented in cancer cells but not in normal cells. Many different strategies have been studied for limiting VACV replication to cancer cells, the most common strategy being the deletion of the viral gene *J2R*, whose product is homologous to cellular thymidine kinase (TK). TK is an important enzyme in the salvage pathway of dTTP synthesis which is cell-cycle-regulated in normal cells and constitutively elevated in cancer cells (Hengstschlager, Knofler et al. 1994). Deletion of *J2R* has been shown to cause cancer-selective replication of VACV (Puhlmann, Brown et al. 2000). The *J2R* mutation in VACV is generally combined with other modifications such as deletion of the viral *C11L/R* gene encoding a homolog of epidermal growth factor (EGF) (Buller and Palumbo 1991; Puhlmann, Brown et al. 2000; McCart, Ward et al. 2001) or insertion of a gene encoding the cytokine GM-CSF. It should be noted that among the various strains of VACV, the Western Reserve (WR) strain has been shown to have the strongest anti-cancer effect (Thorne, Hwang et al. 2007). The WR strain was derived from the 'New York City Board of Health' strain by serial passaging in mouse brain (Parker, Bronson et al. 1941), but the WR strain itself was not used as a vaccine. For the last few decades this strain has been widely passaged in cell culture (Qin, Upton et al. 2011). Although previous studies have shown the WR strain to be neuro-virulent in mice (Parker, Bronson et al. 1941; Williamson, Reith et al. 1990), a more recent study by Zeh *et al.* found that WR-based

oncolytic VACV is well tolerated in humans following intra-tumoral injection to treat different types of cancers including BrCa (Zeh, Downs-Canner et al. 2015).

Like many viruses, VACV has evolved to replicate even in non-dividing cells which usually have very low levels of dNTP production. To achieve the high rates of synthesis of dNTPs required for robust replication of VACV, the virus carries a suite of genes whose products are involved in the dNTP metabolism (Fenner 2001). In addition to the TK homolog *J2R*, these viral genes include *I4L* and *F4L* that encode viral homologs of the large (RRM1) and small (RRM2) subunits, respectively, of the tetrameric ribonucleotide diphosphate reductase (RR). RR is a key enzyme involved in the biogenesis and maintenance of a balanced pool of dNTPs in a cell (Nordlund and Reichard 2006). Interestingly, in normal cells, the level of RRM1 remains stable throughout the cell cycle while RRM2 is targeted by the anaphase-promoting complex for its degradation during mitosis (Engstrom, Eriksson et al. 1985). Hence, RRM2 with a half-life ~3 h is more rate limiting for dNTP synthesis than RRM1 is (Engstrom, Eriksson et al. 1985). It has been shown that RRM2 actively contributes to cancer progression, and elevated levels of RRM2 correlate with poor prognosis of many types of cancer (Souglakos, Boukovinas et al. 2008; Zhang, Jones et al. 2009; Furuta, Okuda et al. 2010; Morikawa, Maeda et al. 2010). Moreover, a high level of RRM2 is associated with resistance to chemotherapeutics such as gemcitabine and DNA damaging agents (Duxbury, Ito et al. 2004; Lin, Belcourt et al. 2004). Viral I4 and F4 can combine with each other or with cellular RRM2 and RRM1, respectively, to form a functional RR complex (Chimploy and Mathews 2001). In accordance with the importance of RRM2 (F4), we have previously shown that an *F4L*-deleted (ΔF4L) WR VACV has highly

attenuated growth in normal cells, consistent with reduced pathogenicity in mice compared to *I4L*-deleted or wild-type WR virus (Gammon, Gowrishankar et al. 2010). In order to increase cancer selectivity by further attenuating virus replication in normal cells, we have constructed a double mutant WR virus that is disrupted in both the *F4L* and *J2R* genes (ΔF4LΔJ2R VACV) making it dependent on both cellular RR and TK activities.

Here we report our investigation of the oncolytic potential of *F4L*-deleted VACVs, with and without *J2R* deletion, for the treatment of BrCa. We examined the replication efficiency and cytotoxic potential of the VACV mutants *in vitro* in different types of BrCa and normal cell lines in monolayer and 3-dimensional spheroid culture systems. In addition, we analysed virus replication in and related cytotoxicity to differentiated and stem-like cell populations isolated from a human triple-negative BrCa cell line by fluorescence-activated cell sorting (FACS). Finally, we tested the safety and anti-tumor activities of the mutant VACVs in both xenograft and syngeneic mouse models of BrCa.

3.3 Results

3.3.1 Levels of RRM2 are Elevated in Rapidly Dividing Cells

The level of RRM2 is reported to be significantly higher in clinical specimens of breast tumors compared to that in the normal breast tissue (Jensen, Page et al. 1994; Kretschmer, Sterner-Kock et al. 2011). Initially using exponentially growing cultures, we found levels of RRM2 to be similar in both BrCa lines and normal cell lines (Figure 3.1A). It is important to note that *in vivo*, normal mammary cells are non-cycling in the absence of hormones (Brisken and O'Malley 2010). Therefore to mimic *in vivo*

proliferative capacity, cells were cultured in media supplemented with low levels of FBS (0.1%) which maintained viability yet reduced growth of normal cells in comparison to that of cancer cells. Under these conditions, the levels of RRM2 in normal human hTERT-HME and MCF10a cells and normal murine NIH3T3 and 516 cells were found to be much lower than those in cancer cells (Figure 3.1A, B & C). We also compared the level of RRM2 in different organs of mice with that in uninfected syngeneic tumors (MTHJ) isolated from the same mice. The levels of RRM2 were high in tumors and low in other organs as expected, except in brain (Figure 3.1D). Therefore, although there was not a strict correlation, there was a general trend for higher RRM2 levels in tumor cells than in normal non-proliferating cells *in vitro* and *in vivo*.

3.3.2 F4L-deleted VACVs Robustly Replicate in BrCa Cells and Induce Cell Death in vitro

We compared the growth of *F4L*-mutant VACVs with those of wild-type (WT) and *J2R*-mutant VACVs in a panel of BrCa and normal cell lines. All mutant VACVs grew to a similar extent as WT, increasing as much as 10,000-fold relative to input pfu, especially at late infection, in all cell lines tested regardless of the types of breast cancer from which the cells originated (Figure 3.2A). Consistent with their ability to replicate robustly in cancer cells, the mutant VACVs were found to efficiently kill cancer cells at a level comparable with that of WT as determined by the AlamarBlue assay for metabolically active cells (Figure 3.2B).

Normal cells grown under conditions that sustain proliferation (*e.g.*, 10% serum-containing medium) supported similar levels of virus growth as cancer cells. However, under low serum conditions (0.1%), replication of *F4L*-mutant VACVs in normal cells

was greatly reduced compared to that of the WT and *J2R*-mutant viruses, and compared to virus replication in cancer cells (Figure 3.2C).

3.3.3 Replication of F4L-mutant VACV is Dependent on Cellular RRM2

To determine whether the growth of *F4L*-mutant VACVs in BrCa cells depends on cellular RRM2 levels, we tested virus replication in MDA-MB-231 cells following treatment with RRM2-specific siRNA. Greater than 60% knockdown of RRM2 was obtained at 48 and 72-hours after siRNA transfection (Figure 3.3A). Growth curves for ΔF4L VACV and WT VACV were performed 24 hours after siRNA transfection of MDA-MB-231 cells (Figure 3.3B). Significantly lower titers of ΔF4L VACV were obtained in the RRM2-knocked-down cells compared to that in the control cells, however growth of WT VACV was not affected by RRM2 knock-down, suggesting that RRM2 expression is important for growth of ΔF4L VACV in BrCa cells.

3.3.4 Replication of F4L-mutants and Resulting Cytotoxicity are Similar in Differentiated and 'Stem-like' Populations of the SUM-149 BrCa Cell Line

As in tumors *in vivo,* a subset of cells in cancer cell lines *in vitro* appear capable of self-renewal and differentiation (Fillmore and Kuperwasser 2008; Charafe-Jauffret, Ginestier et al. 2009). These cells have been given the term "cancer stem cells" (CSC). Several studies have shown that CSCs are resistant to chemo- and radiation-therapies (Creighton, Li et al. 2009; Diehn, Cho et al. 2009). Also, CSCs are thought to play a crucial role in disease relapse after initial remission (Yu, Ramena et al. 2012; Smith, Roth et al. 2014). Several studies have pointed out the importance of CSCs in BrCa tumor initiation, metastasis and resistance to treatment (Creighton, Li et al. 2009; McDermott and Wicha 2010; Ahmad 2013). CSC markers commonly used for BrCa are

$CD44^{+ve/high}/CD24^{-ve/low}$ (Fillmore and Kuperwasser 2008) although several other markers have also been used by investigators to distinguish CSCs from the differentiated cell population (Charafe-Jauffret, Ginestier et al. 2009). We used SUM-149 cells to test whether our *F4L*-mutant VACV can replicate in and kill the CSC-enriched population. We chose the SUM-149 cell line because it is derived from a triple-negative inflammatory BrCa, the most lethal type of BrCa (Levine, Steinhorn et al. 1985; Hoffmeyer, Wall et al. 2005). CSCs derived from the SUM-149 cell line have been shown to be highly resistant to chemotherapeutic drugs such as paclitaxel and 5-fluorouracil *in vitro* (Gupta, Fillmore et al. 2011), therefore we were interested in determining whether oncolytic VACV might prove to be a viable alternative therapeutic.

$CD44^{+ve/high}/CD24^{-ve/low}$ and $CD44^{+ve/high}/CD24^{+ve/high}$ populations were isolated from uninfected SUM-149 (Figure 3.4A) using fluorescence activated cell sorting (FACS), and tested for their ability to support growth of ΔF4L VACV. Growth of the virus was very similar in the two populations (Figure 3.4B). Viability assays show that both populations were efficiently killed by the virus in a dose-dependent manner (Figure 3.4C).

In a parallel approach to test susceptibility of CSC to ΔF4L VACV, we isolated a CSC-enriched population from the main population of SUM-149 cells based on exclusion of Hoechst 33342 dye by ABC transporters expressed by CSCs (Britton, Kirby et al. 2011; Christgen, Ballmaier et al. 2012) and then compared growth of ΔF4L and virus-mediated cytotoxicity in those two sorted populations. Virus replication and virus-mediated cytotoxicity were found to be similar in both populations (Figure 3.4D & E), suggesting that BrCa stem cells may respond to VACV treatment.

3.3.5 F4L-mutant VACV Shows Cancer Cell-selectivity in a Spheroid Culture Model

Spheroid culture models are of intermediate complexity between simplistic monolayer cultures and animal models, and have been used previously by many investigators to test efficacy and specificity of anti-tumor drugs (Herrmann, Fayad et al. 2008; Dufau, Frongia et al. 2012) as they allow more accurate predictions of *in vivo* activity than monolayer culture models (Hirschhaeuser, Menne et al. 2010; Huh, Hamilton et al. 2011; Mehta, Hsiao et al. 2012). We used mono-spheroid and co-culture spheroid models to test the cancer cell selectivity of *F4L*-mutant VACV. Interestingly, MRC5 normal human fibroblast cells consistently assembled in co-cultures to form the inner sphere, with T47D (human BrCa) forming the outer sphere. Co-spheroids made from T47D and MRC5 as well as mono-spheroids of T47D and mono-spheroids of MRC5 cells were infected with ΔF4L VACV, then 2 days later were imaged by confocal microscopy to visualize the virally encoded mCherry signal in infected cells. We found that ΔF4L VACV spread throughout the mono-spheroid culture of T47D, including the core, but in the co-spheroid, virus infection was predominantly detected in the cancer cell layer (Figure 3.5A & B). Also, infection with ΔF4L VACV of mono-spheroids made of the normal cells MRC5 was found to be much lower compared to the T47D mono-spheroid (3.5C).

Our data from monolayer cultures are consistent with a requirement of RRM2 for ΔF4L VACV replication, therefore RRM2 levels in the spheroid cultures were of interest. Western blot analysis showed that while there was a small reduction in the RRM2 level in T47D spheroid cultures compared to monolayer cultures, there was a large reduction (>90%) in RRM2 in MRC5 spheroids compared to monolayer culture (Figure 3.5D). We

suggest that the RRM2 levels observed in the spheroid cultures may be more representative of RRM2 levels *in vivo,* as previous studies have shown that genetic expression profiles of cells in 3D cultures, compared to monolayer cultures, more closely represent those of cells from human tumor samples (Hirschhaeuser, Menne et al. 2010)

3.3.6 F4L-mutant VACVs Show Significant Anti-tumor Activities in Xenograft and Syngeneic Mice Models of BrCa

We have previously shown that wild-type WR VACV is highly virulent in nude mice while *F4L*-mutant VACVs are avirulent (Gammon, Gowrishankar et al. 2010). We expanded this study to test the specificity and anti-tumor efficacy of the mutant VACVs in orthotopic BrCa models in both nude and immune-competent mice. Orthotopic xenografts of the human triple-negative BrCa cell line MDA-MB-231 were treated with three intra-tumoral injections of live ΔJ2R, ΔF4L, ΔF4LΔJ2R VACVs or UV-inactivated VACV (10^6 pfu per injection). No overt toxicities or weight loss were seen in mice treated with any of the viruses. Complete tumor control was achieved in all groups treated with live virus (Figure 3.6A). All three mutant viruses significantly increased survival of mice compared to UV-inactivated virus (Figure 3.6B). In a separate experiment, mice treated with the viruses as described above were euthanized 2 days after the last virus injection to study the bio-distribution of ΔJ2R, ΔF4L, and ΔF4LΔJ2R VACV. Tumors and normal organs (brain, ovaries, spleen, liver and lungs) were excised, homogenized and assayed for the presence of virus. Although viruses were detected in some of the normal organs, the titers of viruses in normal organs were ~ 10^5 folds less than that in tumors. Titers were similar with all 3 mutant viruses in the tumors of mice (~10^8 pfu per gram of tissue) (Figure 3.6C). Also, staining for a late viral protein (A27L)

showed that all the mutant viruses were able to robustly replicate in the tumors of treated mice (Figure 3.6D).

MTHJ, a mouse breast cancer cell line derived from an explanted polyomavirus-middle-T-antigen-expressing murine tumor (Desilva, Wuest et al. 2012) was used to generate orthotopic tumors in immune-competent mice. ΔJ2R, ΔF4L, or ΔF4LΔJ2R VACVs were injected intra-tumorally with 10^8 pfu on each of days 1, 3 and 5. Tumor regression, survival and bio-distribution studies were performed as described above for the xenograft model. All live viruses delayed tumor growth and significantly increased survival of mice in this model (Figure 3.7A & B, respectively). High titers of ΔJ2R and ΔF4L VACV were recovered from tumors, but viruses were undetectable in the normal organs of the animals (Figure 3.7C). We were unable to recover virus from tumors or normal organs of mice injected with ΔF4LΔJ2R VACV, even though this virus induced significant anti-tumor activity.

3.3.7 F4L-mutant VACVs Robustly Replicate in Endothelial Cells in vitro and Increase Hypoxia in Tumors

Oncolytic viruses have been proposed to exert their anti-tumor effect through a wide variety of mechanisms including direct lysis of tumor cells, disruption of tumor vasculature and induction of anti-tumor immunity (Breitbach, Paterson et al. 2007; Kirn and Thorne 2009; Guse, Cerullo et al. 2011). Disruption of tumor vasculature causes increased hypoxia within the tumor leading to death of tumor cells. Hypoxia can have either negative or positive effects on growth of different types of viruses. For example, hypoxia has been shown to reduce replication efficiency of adenovirus by negatively affecting translation of crucial viral proteins such as E1A (Pipiya, Sauthoff et al. 2005;

Shen and Hermiston 2005). In contrast, hypoxia has been shown to increase replication efficiency of herpes simplex virus which is thought to result from increased MEK activity in hypoxic cells (Fasullo, Burch et al. 2009). In the case of VACV, hypoxia has been reported to enhance cytotoxicity in cancer cells, *in vitro*, without affecting virus replication (Hay 2005; Hiley, Yuan et al. 2010). Different oncolytic VACVs have been found to differentially affect tumor vasculature. For instance, the Dryvax strain JX-594 potently disrupts tumor vasculature (Breitbach, Arulanandam et al. 2013) whereas the Lister strain GLV-1h68 does not replicate in endothelial cells *in vitro* and has no disruptive effect on tumor vasculature *in vivo* in mouse models (Weibel, Raab et al. 2011).

In order to study the effect of our mutant viruses on tumor vasculature, we first compared the growth of the viruses with that of WT VACV in human (HUVEC & HMEC) and mouse (MCEC) endothelial cells *in vitro*. Our mutant viruses were found to replicate to levels similar to WT VACV in all three endothelial cell lines (Figure 3.8A). We then tested ΔJ2R, ΔF4L, and ΔF4LΔJ2R VACV for their ability to increase hypoxia in tumors *in vivo*. Mice bearing MDA-MB-231 tumors were given 3 intra-tumoral injections of the mutant VACVs or UV-inactivated VACV, two days later mice were injected with Hypoxyprobe 30 minutes before they were killed. *In vivo* staining for virus and endothelial cells in the tumor sections showed that the mutant viruses can infect tumor vasculature (Figure 3.8B). Analysis of corresponding tumor sections showed a significant increase in hypoxic regions in the tumors of all live-virus-treated groups compared to the UV-inactivated treatment group (Figures 3.8C & 8D). A similar observation was made with the MTHJ tumor model (Figure 3.8E).

3.4 Discussion

In this study we demonstrate that deleting the *F4L* gene from VACV made the virus dependent on cellular RRM2 which is often upregulated in many types of cancer including breast cancer(Ma, Salunga et al. 2003; Liu, Zhang et al. 2013; Zhang, Liu et al. 2014). Although we expected that deletions of *F4L* and *J2R* together would make the virus more cancer-selective compared to the viruses with only single deletions, we did not see much difference between ΔF4L and ΔF4LΔJ2R VACV in cancer-selectivity. Failure of the *J2R*-deletion to provide additional cancer-specificity to the *F4L*-mutant VACV may be due to the fact that there are other pathways for dTTP synthesis that are independent of cellular thymidine kinase (homolog of J2 protein) (Sneider and Potter 1969).

The *F4L*-mutant VACVs grew to high titers in BrCa cells *in vitro*, much like the WT virus. All mutant and WT viruses grew to high titers in proliferating non-tumorigenic cells as well. However, when proliferation of the non-tumorigenic cells was slowed down by culturing in low-serum-containing medium, growth of *F4L*-mutant viruses was clearly reduced, whereas growth of the WT and *J2R*-deleted VACVs was minimally affected. In contrast, when cancer cells were grown in low serum-containing medium, there was minimal reduction in cell proliferation, and the growth of WT as well as the mutant VACVs remained high. Cytotoxicity induced by the mutant VACVs was somewhat variable among the different breast cancer and non-tumorigenic cell lines, but it is interesting to note that the human TNBC lines were all susceptible to infection by the *F4L*-mutant viruses. The mouse breast cancer cell lines 4T1 and MTHJ were relatively

less sensitive to VACVs compared to the human cell lines, but the reasons for this are not known at this time.

Cancer stem cells are thought to be the initiators of tumors and probably play a critical role in metastasis and recurrence of cancer after initial remission (Nguyen, Vanner et al. 2012). In many types of cancer including BrCa, CSCs have been found to be resistant to radiation- and chemo-therapies. In addition to the ability of self-renewal and differentiation, CSCs share many other properties with normal stem cells such as relative quiescence, up-regulation of drug efflux pumps, and over-expression of anti-apoptotic proteins. These features allow the CSCs to resist the effect of conventional cancer therapies (Raguz and Yague 2008; Kanai, Wakimoto et al. 2011; Smith, Roth et al. 2014). Oncolytic viruses represent a completely different class of therapeutics employing a wide variety of mechanisms to kill cancer cells; hence CSCs that are resistant to conventional therapies may still be susceptible to oncolytic virotherapy. In line with this, some studies have shown that chemo- and radiation-resistant CSCs are efficiently killed by oncolytic viruses (Marcato, Dean et al. 2009; Smith, Roth et al. 2014). Not surprisingly, we found that our ΔF4L VACV efficiently killed both the CSC-enriched population ($CD44^{+}CD24^{-/low}$) and the non-CSC population *in vitro*.

Tumor vasculature is important for tumors to grow beyond a certain size, hence preventing the formation of neo-vasculature or disrupting the existing tumor vasculature can have anti-tumor effects. To harness the therapeutic benefits by inhibiting tumor vasculature, some anti-angiogenic drugs such as bevacizumab (Cohen, Shen et al. 2009) and sorafenib (Kane, Farrell et al. 2009) have been approved, while many others are in clinical trials for different malignancies (Al-Husein, Abdalla et al. 2012). However, the

benefit from anti-angiogenic drugs in the clinic has been modest, at best (Ma and Waxman 2008). One drawback of anti-angiogenic drugs is that they do not directly kill cancer cells. Unlike anti-angiogenic drugs, oncolytic viruses could infect and kill both the endothelial cells and cancer cells within the tumors (Angarita, Acuna et al. 2013). Oncolytic adenovirus, vesicular stomatitis virus and herpesvirus have been shown to disrupt tumor vasculature and increase hypoxia in different tumor models in animals (Benencia, Courreges et al. 2005; Saito, Sunamura et al. 2006; Ye, Wang et al. 2006; Breitbach, Paterson et al. 2007). While several studies have shown that vaccinia virus disrupts tumor vasculature, a study by Weibel *et al.* showed that their oncolytic derivative of the Lister strain of vaccinia virus does not replicate in endothelial cells *in vitro* and does not have a disruptive effect on tumor vasculature *in vivo* in mouse models(Breitbach, Paterson et al. 2007; Kirn, Wang et al. 2007; Weibel, Raab et al. 2011; Breitbach, Arulanandam et al. 2013). In contrast, our *F4L*-mutant WR viruses replicated to high levels in both human and murine endothelial cells *in vitro*, and they increased hypoxia following direct injection of human BrCa xenografts.

Like the *J2R*-deleted virus, our *F4L*-mutant viruses completely controlled the growth of xenograft tumors in nude mice for the duration of the study, in contrast to control virus-treated animals that all required euthanasia weeks earlier. Although no animal in the live virus treatment groups was euthanized due to tumor growth; there were a few mortalities during the late phase of study due to conditions unrelated to cancer growth or virus spread.

Our second *in vivo* model used the murine BrCa MTHJ cell line to generate tumors in immune-competent mice. Although the mutant VACVs delayed tumor growth

and increased survival of treated mice, the anti-tumor effect of the viruses was far less profound in this model than in the immune-defective model. This could be because killing of MTHJ cells by the VACVs was less efficient than killing of MDA-MB-231 cells as evident from the *in vitro* cytotoxicity assay. Nonetheless, we expected that even low levels of cell death induced by the virus might release tumor antigens promoting an anti-tumor immune response contributing to overall anti-tumor activity. Although we did not determine the contribution of the immune system to anti-tumor activity in this study, any anti-tumor immunity generated in the MTHJ model was not strong enough to result in tumor control, in contrast to our observations with the xenograft model. We propose that further modifications in the *F4L*-mutant viruses such as arming with GM-CSF or other cytokines to boost the anti-tumor immunity might enhance the therapeutic effect of the virus.

Taken together, we have shown that deletion of *F4L* alone or in combination with *J2R* makes VACV oncolytic. The oncolytic *F4L*-deleted VACVs killed CSCs and non-CSCs populations alike, at least *in vitro*. Like other oncolytic viruses, our viruses were able to grow in endothelial cells *in vitro* and increased tumor hypoxia. Most importantly, they were able to completely control tumor growth in MDA-MB-231 xenografts and significantly delayed tumor growth in a syngeneic mouse model of breast cancer.

3.5 Materials and Methods

3.5.1 Cell Culture

Cell lines and growth media used for this study are summarized in Table 3.1. All media and supplements were purchased from Gibco through Sigma, Life Technologies or ThermoFisher Scientific. All media were supplemented with 2 mM L-glutamine and 100 U/ml penicillin-streptomycin. The cells were maintained at 37^{o}C and 5% CO_2.

3.5.2 Construction of Recombinant Viruses

All recombinant VACVs used in this study were derived from a clonal isolate of the WR strain, originally obtained from the American Type Culture Collection. All recombinant viruses encode the fluorescent marker mCherry to allow both *in vitro* and *in vivo* imaging. Briefly, plasmid DNA encoding the mCherry cDNA under control of a synthetic early/late poxvirus promoter was sub-cloned from plasmid pE/L-mCherry-Topo2 into either pSC66 (to target the *J2R* locus) or R2-pZippyNeoGusA (to target *F4L*) (Gammon, Gowrishankar et al. 2010). Vero cells (ATCC CCL-81) were infected with wild-type VACV at an MOI of 2 pfu/cell for 1 hour, and then transfected with the appropriate plasmids using lipofectamine 2000 (Invitrogen). Two hours later, the medium was replaced and cells were incubated for 48 hours. At this point the cells together with the medium were collected then frozen and thawed 3 times to ensure the complete release of viruses from the cells. The harvested viruses were used to infect Vero cells and isolated mCherry-positive plaques selected for further purification (at least 3 rounds of plaque purification) and expansion. The ΔJ2R VACV has LacZ and mCherry cDNA disrupting the *J2R* gene, and the ΔF4L VACV has NeoGusA and mCherry cDNAs disrupting the *F4L* gene (see Figure 1.5) The double-deleted VACV (ΔF4LΔJ2R) was

constructed the same way except that the R2-pZippyNeoGusA sequence was rescued into the ΔJ2R VACV backbone instead of the wild-type VACV backbone. The resulting ΔF4LΔJ2R has the NeoGusA cDNA disrupting the *F4L* gene and LacZ/mCherry cDNA disrupting the *J2R* gene. PCR was used to confirm disruption of the respective genes, using the primers 5'-TGACGTAAATGTGTGCGAAAGT-3' and 5'-TCAGCACCCA-TGAATGTCGAT-3' to amplify *F4L,* and primers 5'-TATTCAGTTGAT-AATCGGCCCCATGTTT-3' and 5'-GAGTCGATGTAACACTTTCTACACACCG-3' to amplify *J2R*.

3.5.3 Western Blot Analysis

Cells or tissues were lysed using a modified Radioimmunoprecipitation Assay (RIPA) buffer supplemented with protease inhibitor cocktail (Sigma) and 1mM PMSF (Sigma), then briefly sonicated. Protein concentrations of the lysates were determined by BCA assay (ThermoFisher Scientific). 20 μg protein was separated by 0.1%-SDS-12% polyacrylamide-gel electrophoresis then transferred to a nitrocellulose membrane (Biorad, Mississauga, ON, CA). Membranes were blocked with Odyssey blocking® buffer (LI-COR Biosciences) for 1 hour and treated over-night at 4 ^{o}C with polyclonal goat anti-RRM2 (Cat# sc-10846, Santa Cruz Biotechnology, CA, USA), rabbit anti- β-tubulin (Cat# 2146S, Cell Signaling Technology Inc., USA) or rabbit anti-β-actin (Cat# 926-42210, LI-COR Biosciences) antibody. Finally membranes were treated for one hour with infrared IRDye®-labeled (680 nm or 800 nm) donkey anti-goat (LI-COR Biosciences) or donkey anti-rabbit (LI-COR Biosciences) secondary antibodies. The washed membranes were scanned using an Odyssey scanner (Li-COR Biosciences) and the images analyzed using *Li-CoR* Odyssey software (Li-COR Biosciences).

3.5.4 Virus Growth Curves in Cultured Cells

Growth kinetics of the viruses were determined by the classical plaque assay. Briefly, cells were plated in 6-well plates and were infected the following day (~70-80% confluency) at the multiplicity of infection (MOI) of 0.03 plaque-forming unit per cell (pfu/cell) in a total volume of 500 µl PBS for 1 hour. Following infection, fresh medium was added in each well and the plates were returned to incubator. Infected cells were harvested at indicated time points and frozen thawed 3 times to ensure complete lysis of cells and release of viruses. Serial dilutions of the lysates were used to infect a monolayer of BSC-40 (monkey kidney cell line) (Hruby, Lynn et al. 1979) in six-well plates. Forty-eight hours post-infection, medium was aspirated, the cells were stained with 0.5% (w/v) crystal violet, and the titers of each virus were determined by visually counting the plaques and multiplying by the dilution factors. Increase in pfu/cell at different time points was calculated relative to the input virus.

3.5.5 Cell Survival Assay

An assay for metabolically active cells was used to determine cell survival after virus treatment. Briefly, 5000 cells/well were plated in 96-well plates. The following day medium was removed and cells infected with virus as indicated. One hour later, fresh medium was added and the plates returned to the incubator. Seventy-two hours post-infection, alamarBlue (Resazurin; Sigma, St Louis, MO, USA) was added to each well to a final concentration of 44 µM, and cells were incubated again. Three hours later, fluorescence (excitation at 544 nm; emission at 590 nm) was measured using a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany**).**

3.5.6 RRM2 Knockdown

MDA-MB-231-luc-D3H2LN cells were seeded in 6-well plates in medium without antibiotics to achieve 70-80% confluency in 24 hours. The day after seeding, cells were transfected with RRM2-siRNA (Cat# SI02653441, Qiagen) or non-specific control siRNA (Cat# 1027281, Qiagen) using 5 µl/well lipofectamine 2000 (Cat# 11668-027, Invitrogen) and plates were returned to the incubator. Medium was replaced with fresh medium containing no antibiotics 4 hours after transfection, and then incubated for 40 hours before cell lysates were collected.

3.5.7 Flow Cytometry and Cell Sorting

For separating CSC and the non-CSC populations of SUM-149 cells, uninfected cells were stained with antibodies against human CD24 and CD44 conjugated with FITC (Cat# 555427, BD Pharmingen^{TM}) and Allophycocyanin (Cat# 559942, BD Pharmingen^{TM}), respectively. Gates were set based on a maximum of 4% positive cells in the isotype control population. $CD44^{+ve/high}/CD24^{-ve/low}$ and $CD44^{+ve/high}/CD24^{+ve/high}$ cells were sorted using the BD Influx cell sorter (BD Biosciences).

In order to separate the side population from the non-side population, SUM-149 cells were trypsinized, filtered through the cap of the FACS tube (Cat# 352235, Corning Science, Mexico), then collected by centrifuging at 300 x g for 5 minutes. Cells were then resuspended in pre-warmed DMEM supplemented with 5% FBS, 5 µg/ml Hoechst 33342 (Cat# 62249, ThermoFisher Scientific). Cells were incubated at 37 ^{o}C for 90 minutes with intermittent shaking every 20 minutes. Cells were pelleted at 300 x g for 5 minutes at 4 ^{o}C and resuspended in ice-cold PBS. Finally 2 µg/ml 7-AAD (Cat# 420404, Biolegend, San Diego, USA) was added 5 minutes prior to start of sorting. Live cells (7-

AAD negative) with low Hoechst signal (side-population) and high Hoechst signal (non-side-population) were sorted using BD influx cell sorter (BD Biosciences).

3.5.8 Spheroid Establishment and Culture

MRC5 cells were first labeled with CellTracker™ Green (5-chloromethylfluorescein diacetate) (Cat# C2925, ThermoFisher Scientific). To generate co-spheroids, 5000 labeled normal MRC5 cells and 5000 T47D BrCa cells, in 200 µl medium (RPMI: MEM, 1:1) with FBS and standard supplements, were added to each well of a 96-well plate pre-coated with 50 µl of 1% agarose. For mono-spheroid cultures, either 10,000 T47D cells or 10,000 labeled MRC5 cells were plated on agarose-coated plates. Plates were incubated for 5 days at 37 ^{o}C and 5% CO_2. On day 5, the spheroids were infected in triplicate with ΔF4L VACV at an estimated MOI of ~20 pfu/cell. Forty-eight hours post-infection, spheroids were fixed in formalin then mounted with glycerol containing 1 µg/ml DAPI. The spheroids were imaged through the Z-axis for mCherry, Cell-Tracker Green and DAPI at intervals of 25 µM using a Zeiss LSM710 confocal microscope.

3.5.9 Tumor Models

6-8 weeks old female immune-competent FVB mice and NIH-III nude mice were used for syngeneic and xenograft experiments, respectively. MTHJ, a line derived from polyoma middle T antigen over-expressing mammary tumor explants, was used to generate orthotopic tumors in immune-competent mice (Guy, Cardiff et al. 1992; Desilva, Wuest et al. 2012). The MDA-MB-231-luc-D3H2LN human triple-negative BrCa cell line was used to generate orthotopic tumors in nude mice. In both cases, two million cells in 50% matrigel (50 µl) were injected in the abdominal mammary fat pad. Tumors were

palpable in about 3 weeks. Each mouse received a total of 3 intra-tumoral injections (10^8 pfu/injection in immune-competent mice and 10^6 pfu/injection in nude mice, on each of days 1, 3 and 5). Mice were weighed and tumors sizes measured with calipers twice weekly.

To assess bio-distribution of viruses, mice were given 3 intra-tumoral injections of viruses over a period of 1 week, then were euthanized 2 weeks after first injection of virus for the MTHJ model, and 7 days after the first injection for the xenograft model. Tumors and indicated organs were harvested, chopped into small pieces and homogenized in 10 ml PBS using the GentleMACS tissue dissociator (Miltenyl Biotec). The viral titers in the tissue homogenates were determined by plaque assay on BSC-40 cells(Gammon, Gowrishankar et al. 2010).

All the animal protocols were approved by Health Sciences Animal Care and Use Committee at University of Alberta in compliance with Canadian Council on Animal Care.

3.5.10 Immunohistochemical Analysis

On day 7 after the first virus injection, hypoxy probe (Cat# HP2-100, Hypoxyprobe, Burlington, MA, USA) was injected intra-peritoneally and mice were killed 30 minutes later. Tumors were harvested, formalin-fixed for 48 hours and paraffin-embedded. For staining, tumor sections were deparafinized and rehydrated after which heat-mediated antigen retrieval was performed (Ramos-Vara 2005). The sections were blocked with Dako Antibody diluent (Cat. No. S3022; Dako North America, Carpinteria, CA, USA) and treated with hamster anti-CD31-antibody (Cat. No. MA3105, ThermoScientific) and rabbit anti-A27 (ab117453, Abcam) over-night. Sections were

washed and treated for 1 hour at room temperature with FITC-conjugated anti-rabbit (Cat No. Ab150077, Abcam) and cy5 conjugated anti-hamster (Cat. No. SC006, Applied Biological Materials Inc.). Slides were imaged using a fluorescence microscope.

3.5.11 Growth of VACVs in Endothelial Cells in vitro and Measurement of Hypoxia in Tumors

Growth kinetics of the viruses in HUVEC, HMEC and MCEC cells, *in vitro*, was determined as described in section 3.5.4. For the *in vivo* study, nude mice bearing MDA-MB-231-luc-D3H2LN orthothopic xenografts or immune-competent mice bearing MTHJ tumors were injected intra-tumorally on each of days 1, 3 and 5 with virus as indicated. Hypoxia in tumors was detected using the Hypoxyprobe Plus Kit (Cat# HP2-100, Hypoxyprobe, Burlington, MA, USA). Two days after the final virus treatment, mice were injected with hydroxyprobe intra-peritoneally and 30 minutes later mice were euthanized. Then tumors were harvested, formalin fixed, paraffin embedded, and sectioned into 5 μM thick slices. The tumor sections were stained for hypoxia following manufacturer's protocol. Images of the tumor sections were taken with an Axioskope color camera (Carl Zeiss Microscopy, LLC, USA). The areas of the hypoxic regions were quantified using 12 images from each group (4 images per tumor section) by ImageJ software.

3.5.12 Statistical analysis

All Statistical analyses for comparing 2 columns at a time were performed using Student's *t*-test with 95% confidence interval. While comparing more than 2 columns at a time, one way ANOVA was used with 95% confidence interval. *P*-values of <0.05 were deemed significant. Survival studies were analyzed for statistical significance using the

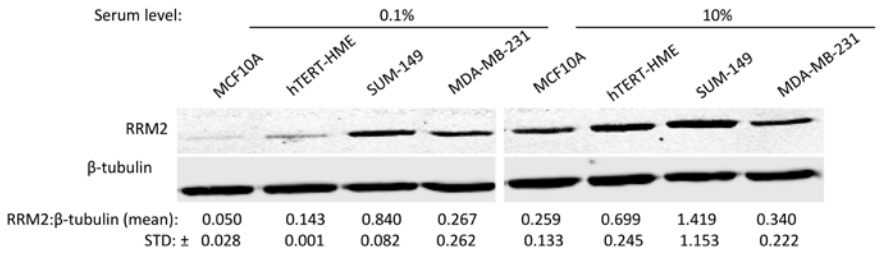
log-rank Mantel-Cox test. GraphPad Prism 5 Software (GraphPad Software, La Jolla, CA, USA) was used to calculate statistical values.

Table and Figures

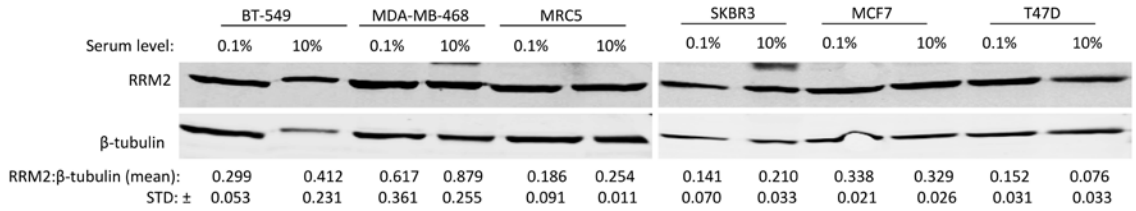
Table 3.1: Cell Lines Used in This Study

Cell line	Cell type	Source	Media
4T1	Mouse mammary adenocarcinoma	D. Evans[1]	RPMI + 10% FBS
516[a]	Mouse kidney fibroblast	F. Graham[2]	DMEM + 10% FBS
BSC-40	Monkey kidney fibroblast	D. Evans	MEM + 5% FBS
BT-549	Human triple-negative BrCa	ATCC	RPMI + 10% FBS
HMEC	Immortalized human endothelial cells	Y. Fu[1]	MCDB131 + 10% FBS
hTERT-HME	Human mammary epithelial	ATCC	DMEM/F12 + 5% horse serum + 20 ng/ml EGF +10 µg/ml insulin +500 ng/ml hydrocortisone +100 ng/ml cholera toxin
HUVEC[b]	Primary human endothelial cells	A. Murray[1]	M199 + 20 % FBS + 100 µg/ml ECGS
MCEC	Mouse cardiac endothelial cells	A. Murray	DMEM + 10% FBS
MCF7	Human luminal BrCa	ATCC	DMEM + 10% FBS
MCF10A	Human mammary epithelial	ATCC	Same as hTERT-HME
MDA-MB-231 D3H2LN	Human triple-negative BrCa	Caliper Life Sciences	RPMI + 10% FBS
MDA-MB-468	Human triple-negative BrCa	ATCC	RPMI + 10% FBS
MRC5	Human lung fibroblast	ATCC	MEM + 10% FBS
MTHJ[c]	Mouse mammary adenocarcinoma	K. Mossman[2]	DMEM + 10% FBS
NIH3T3	Mouse embryonic fibroblast	ATCC	DMEM + 10% FBS
SKBR3	Human HER2-over-expressing BrCa	ATCC	McCoy's5A+ 10 FBS
SUM-149	Human inflammatory triple-negative BrCa	L. Postovit[1]	Ham's F12 + 5% FBS + 5 µg/ml insulin +1µg/ml hydrocortisone
T47D	Human luminal BrCa	ATCC	RPMI + 10% FBS
Vero	Monkey kidney epithelial cells	ATCC	DMEM + 10% FBS

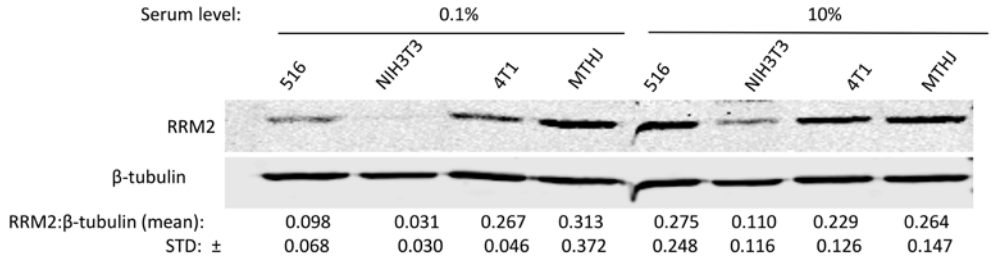
[1]University of Alberta, Canada; [2]McMaster University, Canada; [a] (Addison, Bramson et al. 1998); [b](Zhang, Nakhaei-Nejad et al. 2011); [c](Desilva, Wuest et al. 2012)


Serum level:
0.1%
10%
MCF10A
hTERT-HME
SUM-149
MDA-MB-231
MCF10A
hTERT-HME
SUM-149
MDA-MB-231
RRM2
β-tubulin
RRM2:β-tubulin (mean): 0.050 0.143 0.840 0.267 0.259 0.699 1.419 0.340
STD: ± 0.028 0.001 0.082 0.262 0.133 0.245 1.153 0.222

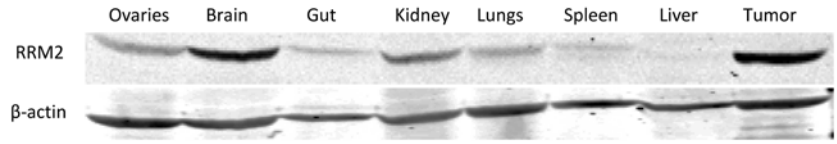
(A)


BT-549
MDA-MB-468
MRC5
SKBR3
MCF7
T47D
Serum level:
0.1% 10% 0.1% 10% 0.1% 10% 0.1% 10% 0.1% 10% 0.1% 10%
RRM2
β-tubulin
RRM2:β-tubulin (mean): 0.299 0.412 0.617 0.879 0.186 0.254 0.141 0.210 0.338 0.329 0.152 0.076
STD: ± 0.053 0.231 0.361 0.255 0.091 0.011 0.070 0.033 0.021 0.026 0.031 0.033

(B)

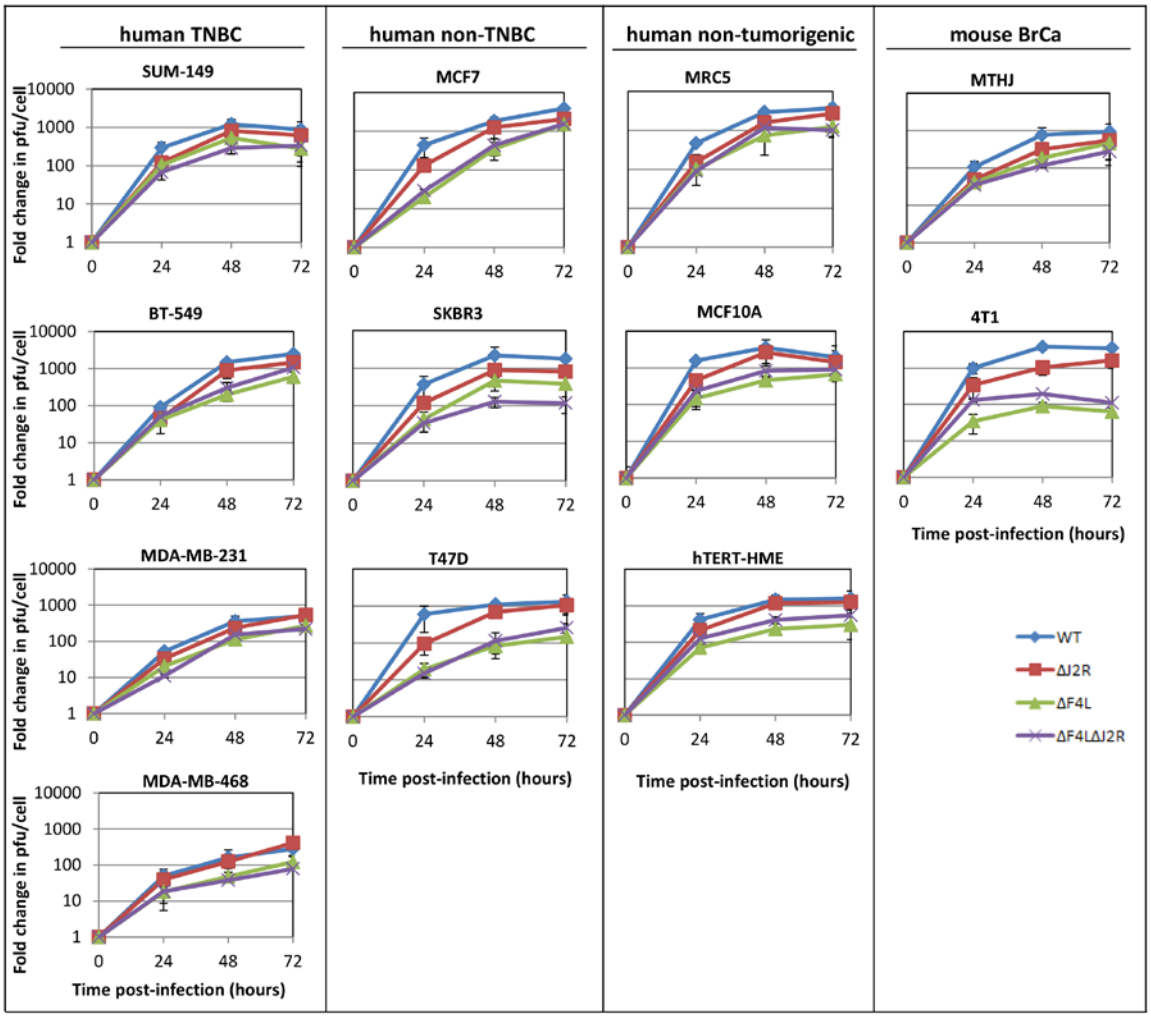

Serum level:
0.1%
10%
516
NIH3T3
4T1
MTHJ
516
NIH3T3
4T1
MTHJ
RRM2
β-tubulin
RRM2:β-tubulin (mean): 0.098 0.031 0.267 0.313 0.275 0.110 0.229 0.264
STD: ± 0.068 0.030 0.046 0.372 0.248 0.116 0.126 0.147

(C)

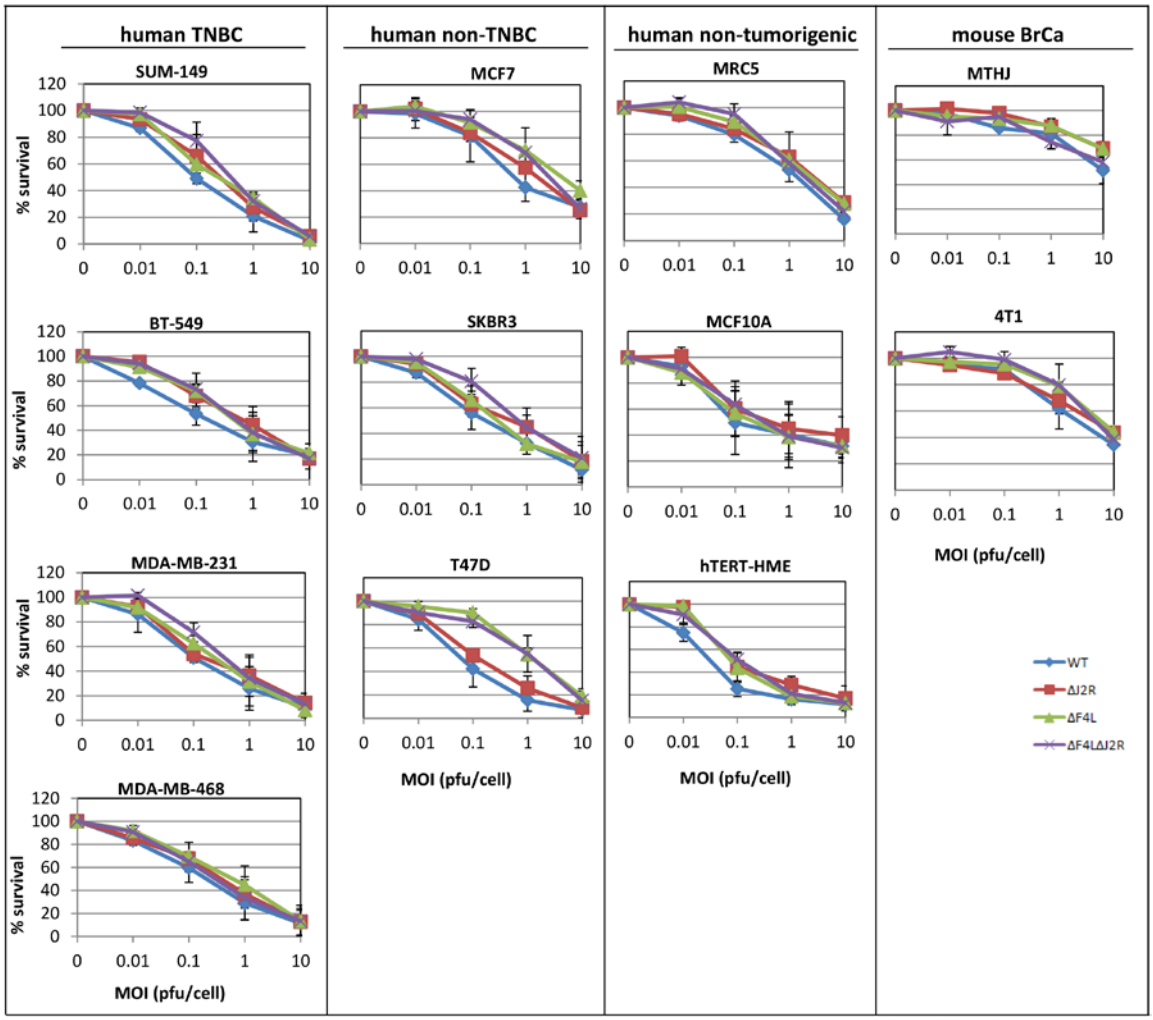

Ovaries
Brain
Gut
Kidney
Lungs
Spleen
Liver
Tumor
RRM2
β-actin

(D)

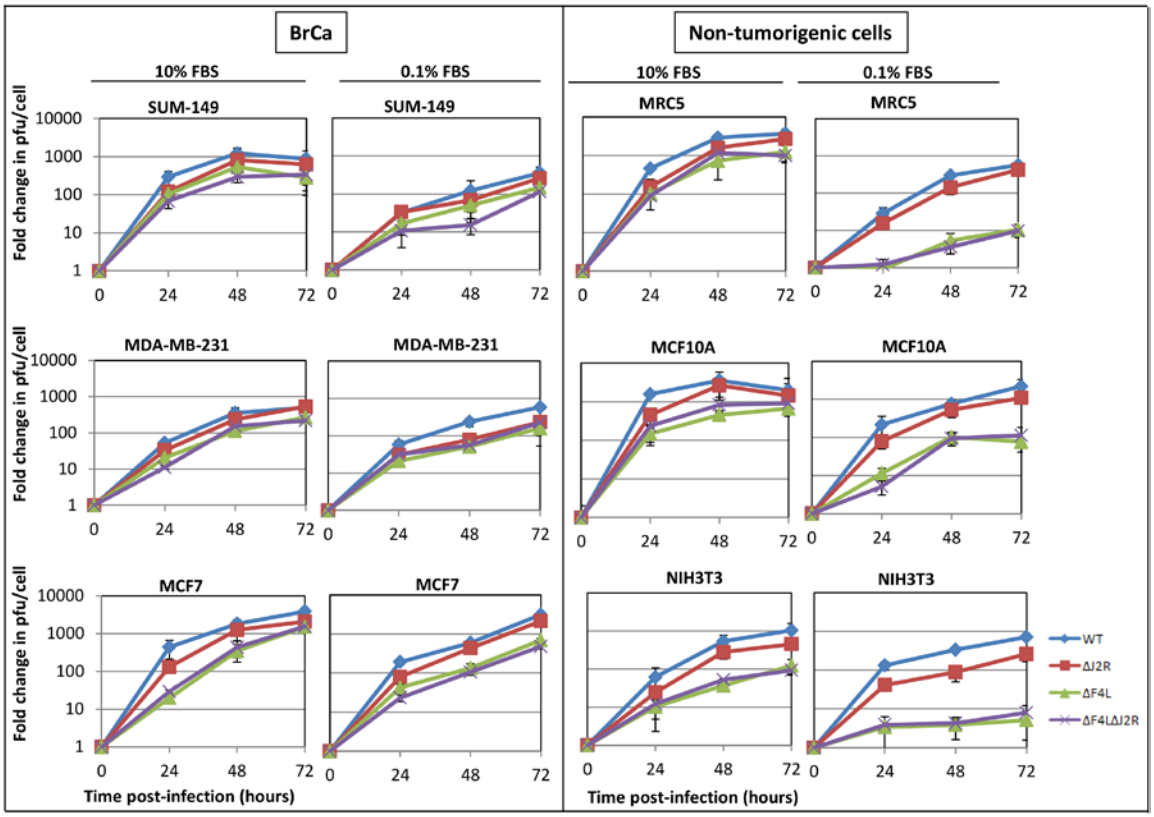
Figure 3.1. Levels of RRM2 are elevated in BrCa cells compared to non-tumorigenic cells *in vitro* and *in vivo*. (**A** & **B**) human BrCa cells (SUM-149, MDA-MB-231, BT-549, MDA-MB-468, SKBR3 MCF7, and T47D) and non-tumorigenic cells (MCF10A, hTERT-HME and MRC5) were grown for 72 hours in media supplemented with 0.1% or 10% fetal bovine serum (FBS). Cell lysates were analyzed for RRM2 (45 kDa) and β-tubulin (51 kDa) as described in the materials and methods section. The blots were quantified using the Odessey software. Mean values of RRM2:β-tubulin from 3 independent experiments ± STD from 3 independent experiments are shown. **(C)** Mouse BrCa (4T1 and MTHJ) and non-tumorigenic (516 and NIH3T3) cells were grown in media supplemented with 0.1% or 10% FBS for 72 hours, then RRM2 was determined as above. Mean values of RRM2:β-tubulin from 3 independent experiments ± STD from 3 independent experiments are shown. **(D)** MTHJ tumors and normal organs were harvested from FVB mice, lysates were prepared by homogenization, and RRM2 levels analyzed. β-tubulin or β-actin was used as loading control in these experiments.


human TNBC
human non-TNBC
human non-tumorigenic
mouse BrCa
SUM-149
MCF7
MRC5
MTHJ
BT-549
SKBR3
MCF10A
4T1
MDA-MB-231
T47D
hTERT-HME
MDA-MB-468
Fold change in pfu/cell
10000
1000
100
10
1
0
24
48
72
Time post-infection (hours)
WT
ΔJ2R
ΔF4L
ΔF4LΔJ2R

(A)


human TNBC
human non-TNBC
human non-tumorigenic
mouse BrCa
SUM-149
MCF7
MRC5
MTHJ
BT-549
SKBR3
MCF10A
4T1
MDA-MB-231
T47D
hTERT-HME
MDA-MB-468
% survival
MOI (pfu/cell)
WT
ΔJ2R
ΔF4L
ΔF4LΔJ2R

(B)


BrCa
Non-tumorigenic cells
10% FBS
0.1% FBS
SUM-149
MRC5
MDA-MB-231
MCF10A
MCF7
NIH3T3
Fold change in pfu/cell
Time post-infection (hours)
WT
ΔJ2R
ΔF4L
ΔF4LΔJ2R

(C)

Figure 3.2. ΔF4L and ΔF4LΔJ2R VACV replicate proficiently in and are cytotoxic to BrCa cells *in vitro*. (A) Subconfluent monolayers of human triple-negative BrCa (TNBC), non-TNBC, non-tumorigenic and mouse BrCa cell lines were infected with WT or mutant VACVs at an MOI of 0.03 pfu/cell. Infected cells were harvested at the indicated time points and viral titers were determined by plaque assay on BSC-40 cells. **(B)** Cells grown in 10% serum-containing medium were infected with WT or mutant VACVs at the indicated doses and cell viability was determined by the Alamar blue assay 72 hours post-infection. Survival of infected cells was normalized to that of mock-infected cells. **(C)** Replication of VACVs was assessed in BrCa (SUM-149, MDA-MB-231 and MCF7) and non-tumorigenic cells (MRC5, MCF10a and NIH3T3) grown in 10% or 0.1% FBS-containing media. Each data point represents the mean of 3 independent experiments ± STD.

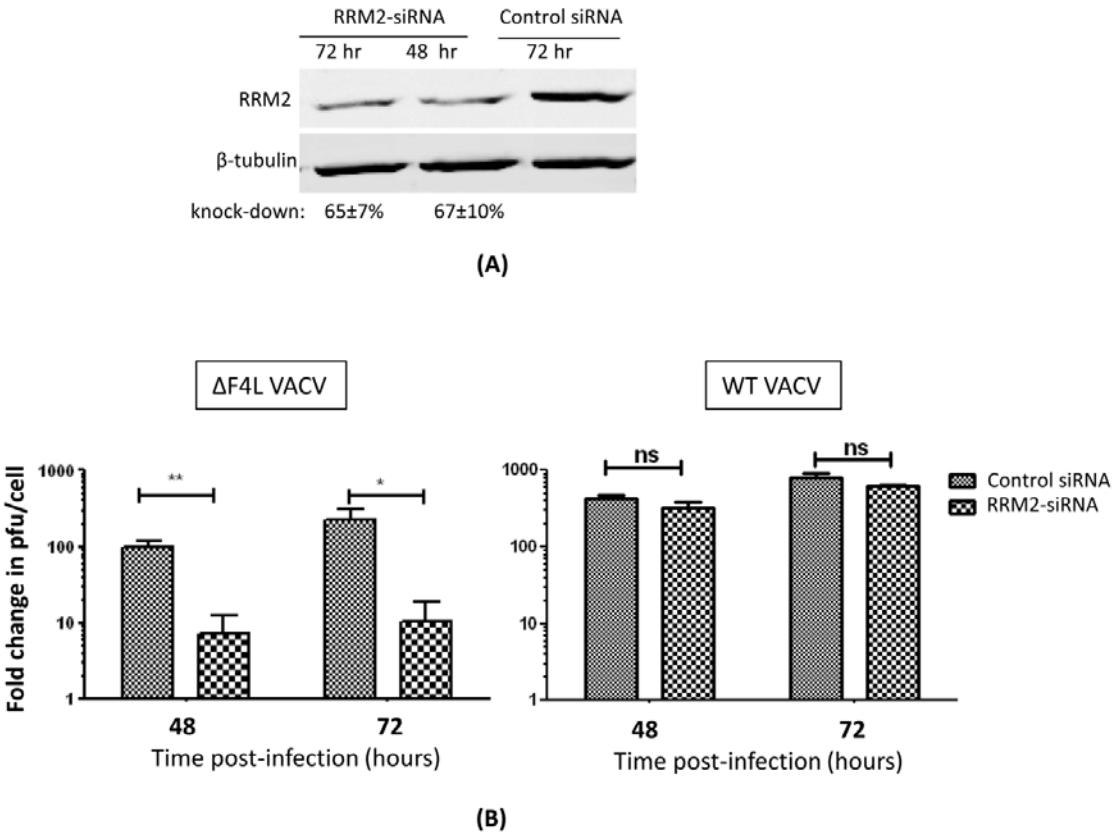
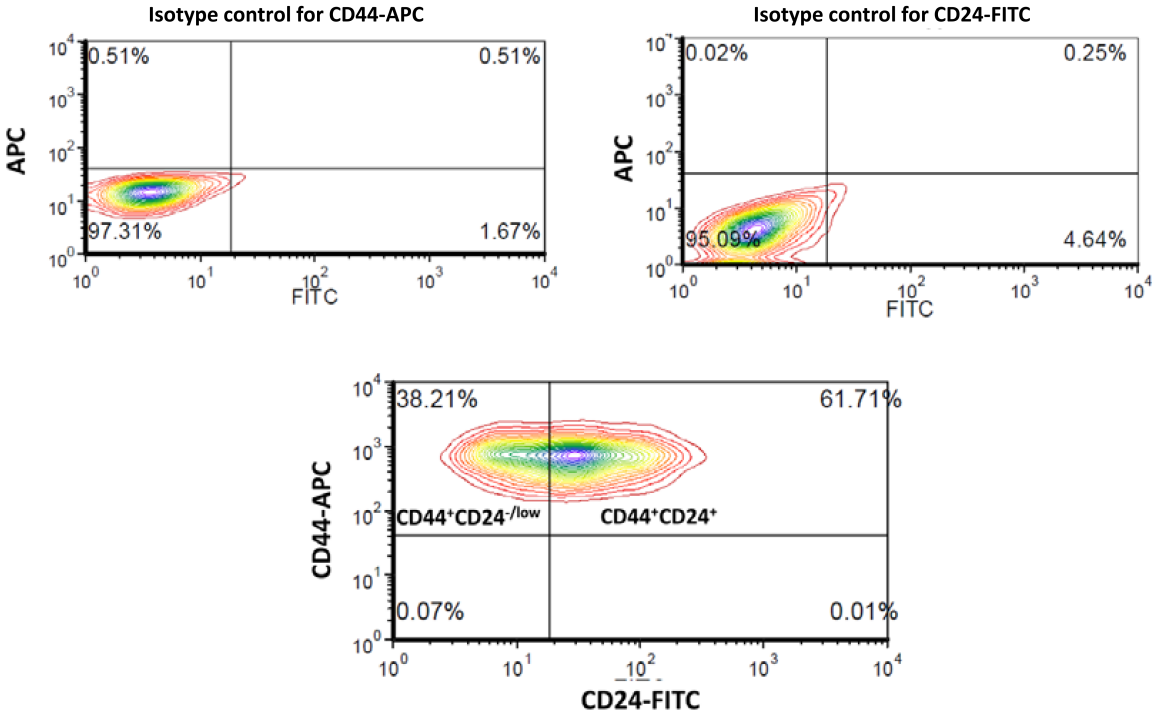
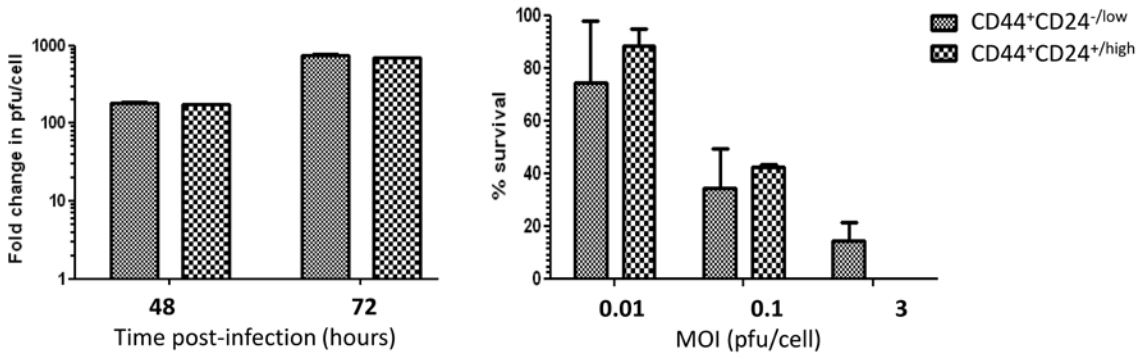


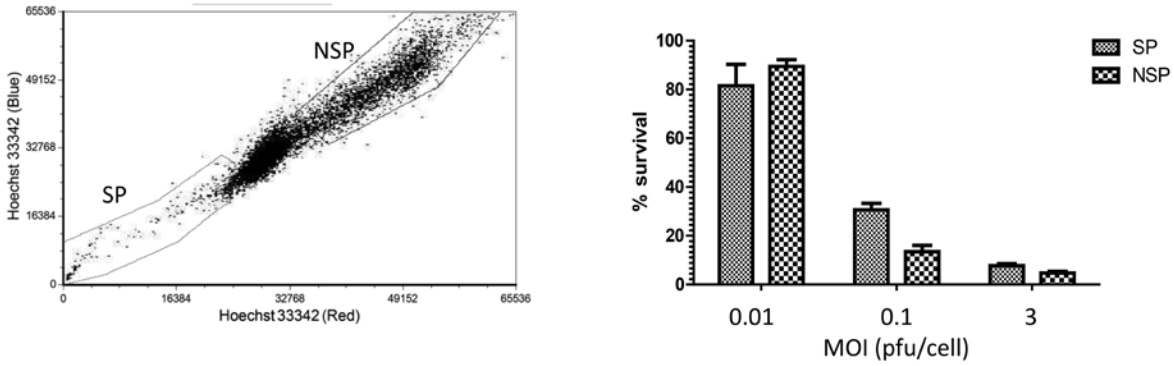
Figure 3.3. Replication of ΔF4L VACV is dependent on cellular RRM2 in MDA-MB-231 cells. (A) MDA-MB-231 cells were transfected with siRNA targeting RRM2 or non-specific siRNA. Cell lysates were collected 48 and 72 hours post-transfection and RRM2 levels were determined by Western blot analysis. RRM2 levels were normalized to β-tubulin and percentage knockdown relative to control was calculated using *Li-CoR* Odessey software. The percent knockdown shown is the mean of 3 experiments. **(B)** Cells were transfected with RRM2 siRNA or nonspecific control siRNA, then infected with WT or ΔF4L VACV at an MOI of 0.03 pfu/cell at 24 hours post-transfection. Plaque assays were used to determine the virus titers in lysates harvested at 48 and 72 hours post-infection. Each data point represents the mean of 3 independent experiments ± STD. (Student's *t*-test **P*<0.05, ***P*<0.01)


Isotype control for CD44-APC
0.51%
0.51%
97.31%
1.67%
APC
FITC
Isotype control for CD24-FITC
0.02%
0.25%
95.09%
4.64%
APC
FITC
38.21%
61.71%
CD44+CD24-/low
CD44+CD24+
0.07%
0.01%
CD44-APC
CD24-FITC

(A)

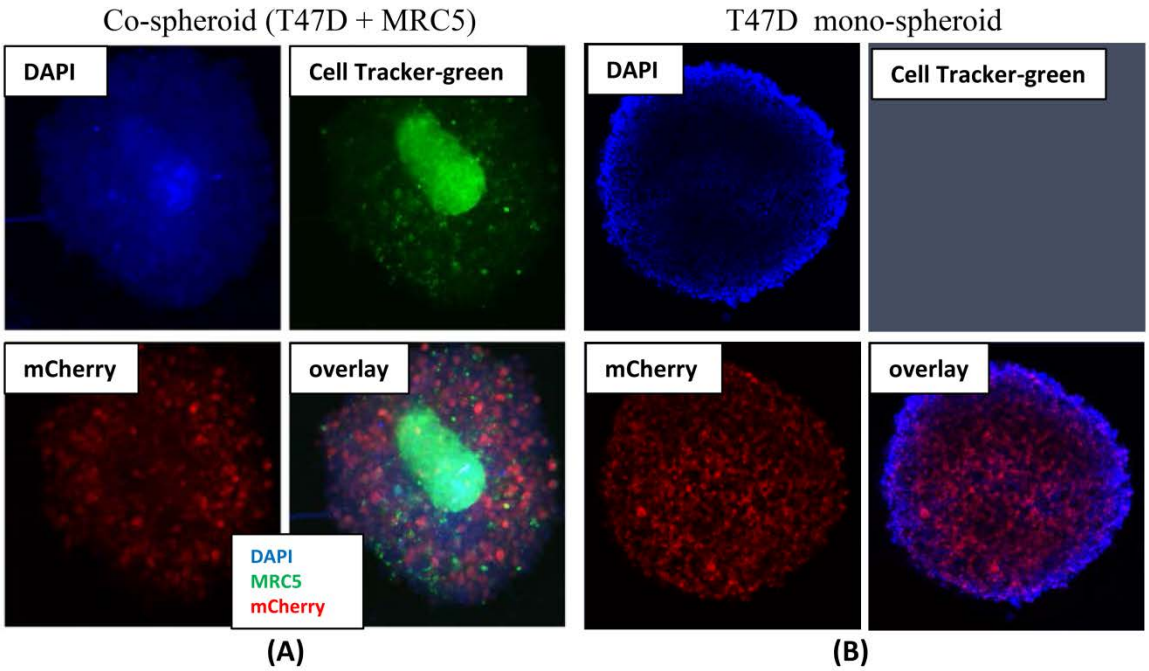

Fold change in pfu/cell
1000
100
10
1
48
72
Time post-infection (hours)
% survival
100
80
60
40
20
0
0.01
0.1
3
MOI (pfu/cell)
CD44+CD24-/low
CD44+CD24+/high

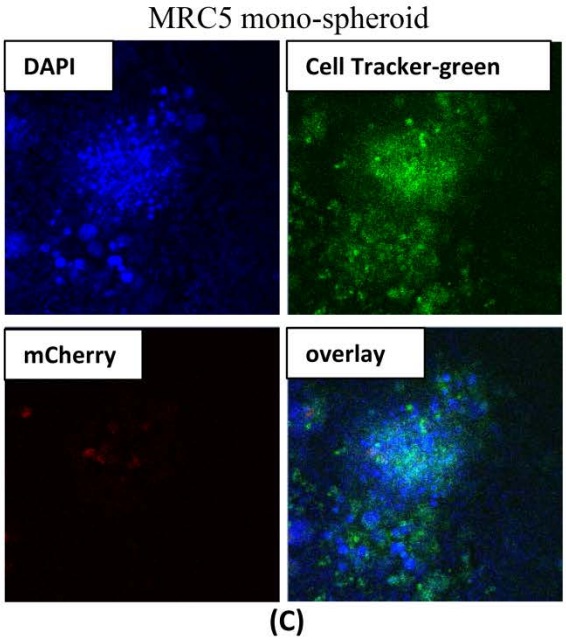
(B) **(C)**

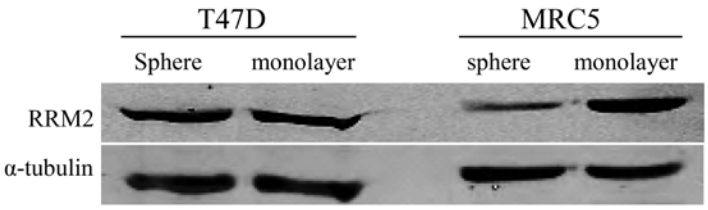


(D) **(E)**

Figure 3.4. CSC and non-CSC populations are both susceptible to ΔF4L VACV infection. (A) Sum-149 cells were stained with CD24 and CD44 antibodies and isotype controls were used for gating. $CD44^{+ve}CD24^{-ve/low}$ cells and $CD44^{+ve}CD24^{+ve/high}$ populations were separated by FACS. **(B)** Immediately after sorting, cell populations were infected with ΔF4L VACV at an moi of 0.1 pfu/cell. Virus yields were determined at 48 and 72 hours post-infection and compared to the input virus. **(C)** Immediately after sorting, cell populations were infected with ΔF4L VACV at the indicated MOI. Cell survival was determined 72 hours after infection. **(D)** SUM-149 cells were stained with Hoechst 33342 and the viability dye 7-AAD. Live cells with low Hoechst staining, side population (SP), and high Hoechst staining, non-side population (NSP), were sorted using FACS. **(E)** The two sorted populations were immediately infected with ΔF4L VACV for 1 hour. Cell viability relative to mock-infected cells was determined 72 hours post-infection using Alamar blue assay. Each data point represents the average of triplicate infections ± STD. The experiments have been performed 2 times.

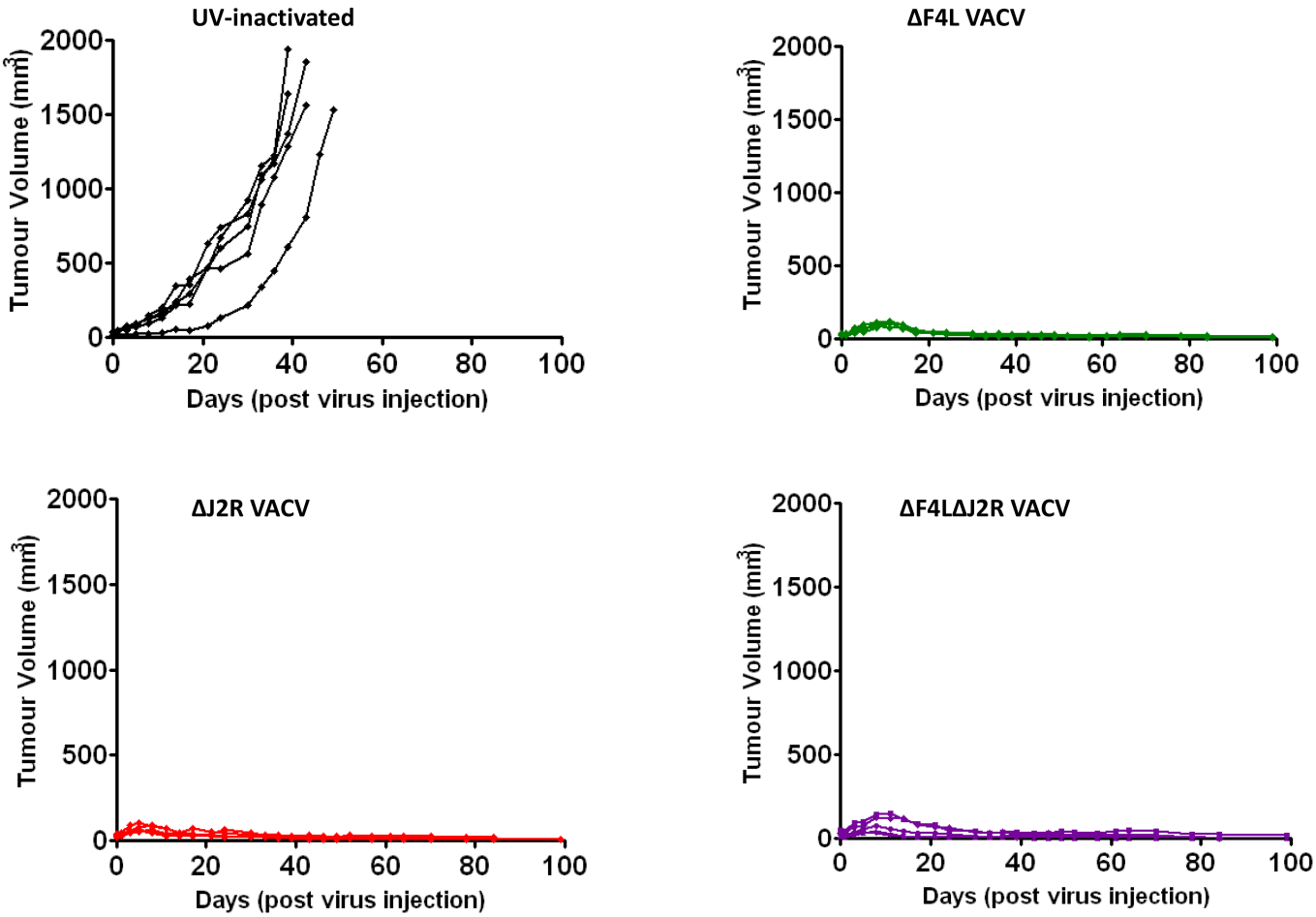

Co-spheroid (T47D + MRC5)
T47D mono-spheroid
DAPI
Cell Tracker-green
DAPI
Cell Tracker-green
mCherry
overlay
mCherry
overlay
DAPI
MRC5
mCherry
(A)
(B)


MRC5 mono-spheroid
DAPI
Cell Tracker-green
mCherry
overlay
(C)

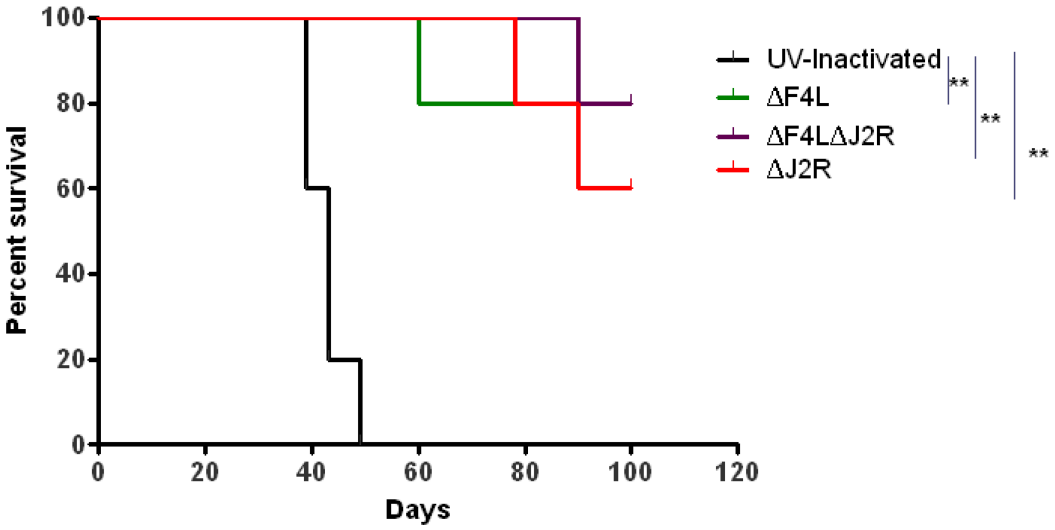


(D)

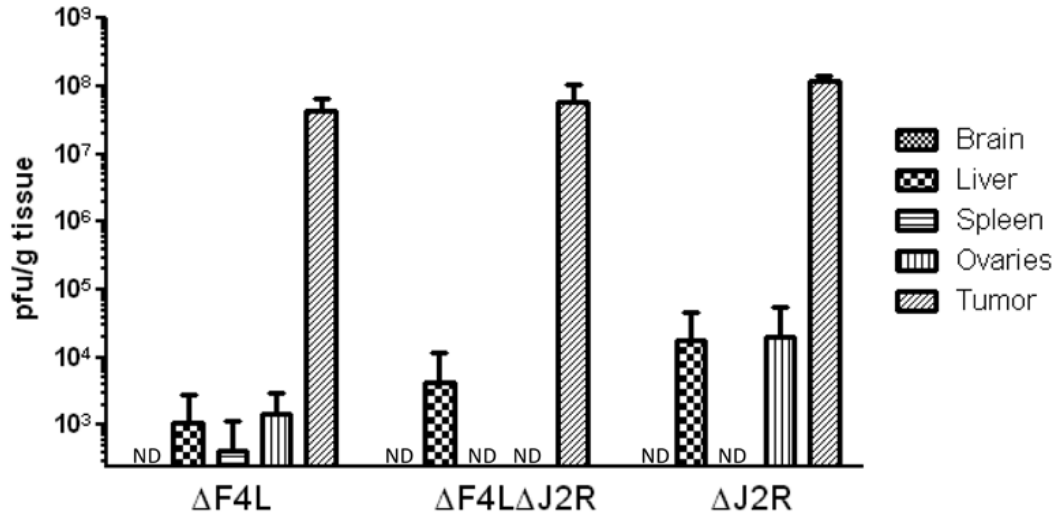
Figure 3.5. Cancer specificity of ΔF4L VACV in spheroid culture models. (A) Co-spheroids (~300 μm in diameter) made of T47D BrCa cells and MRC5 normal cells (labelled with Cell-Tracker green) were infected with ΔF4L VACV at an MOI of ~20 pfu/cell. 48 hours post-infection, spheroids were fixed in formalin, stained with DAPI and imaged using confocal microscopy (Z-stack images were acquired at 25 μm intervals). The mCherry signal indicates cells infected with ΔF4L VACV. Shown is a representative planar image from Z-stack at 150 μm depth. **(B)** & **(C)** Mono-spheroids of T47D and MRC5 cells, respectively, were infected and imaged as in (A). Virus infection throughout the sphere including the inner core is indicated by the mCherry signal. Shown are representative planar images from Z-stacks at 150 μM depth. **(D)** T47D and MRC5 cells were cultured as monolayers or 3-dimensional mono-spheroids prior to harvest. The levels of RRM2 were compared between the two culture systems by Western blot analysis. Reductions in RRM2 levels of >90% and ~25% were observed in MRC5 and T47D spheroids, respectively, compared to their respective monolayer cultures. Experiments (A), (B) and (C) have been performed 3 times in triplicate and (C) has been performed two times.


UV-inactivated
ΔF4L VACV
ΔJ2R VACV
ΔF4LΔJ2R VACV
Tumour Volume (mm3)
Days (post virus injection)
0
500
1000
1500
2000
0
20
40
60
80
100

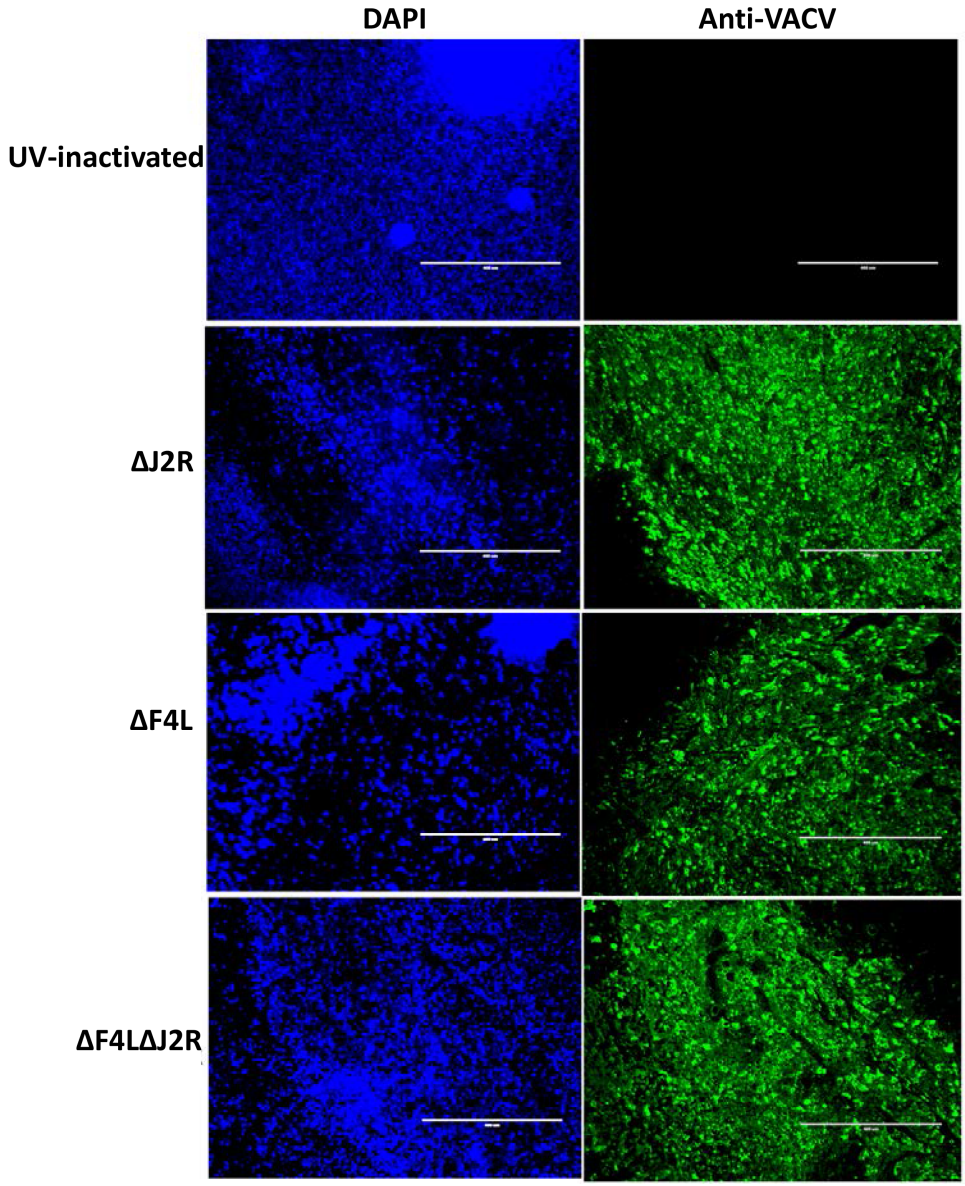
(A)


100
80
60
40
20
0
Percent survival
0
20
40
60
80
100
120
Days
UV-Inactivated
ΔF4L
ΔF4LΔJ2R
ΔJ2R
**
**
**

(B)

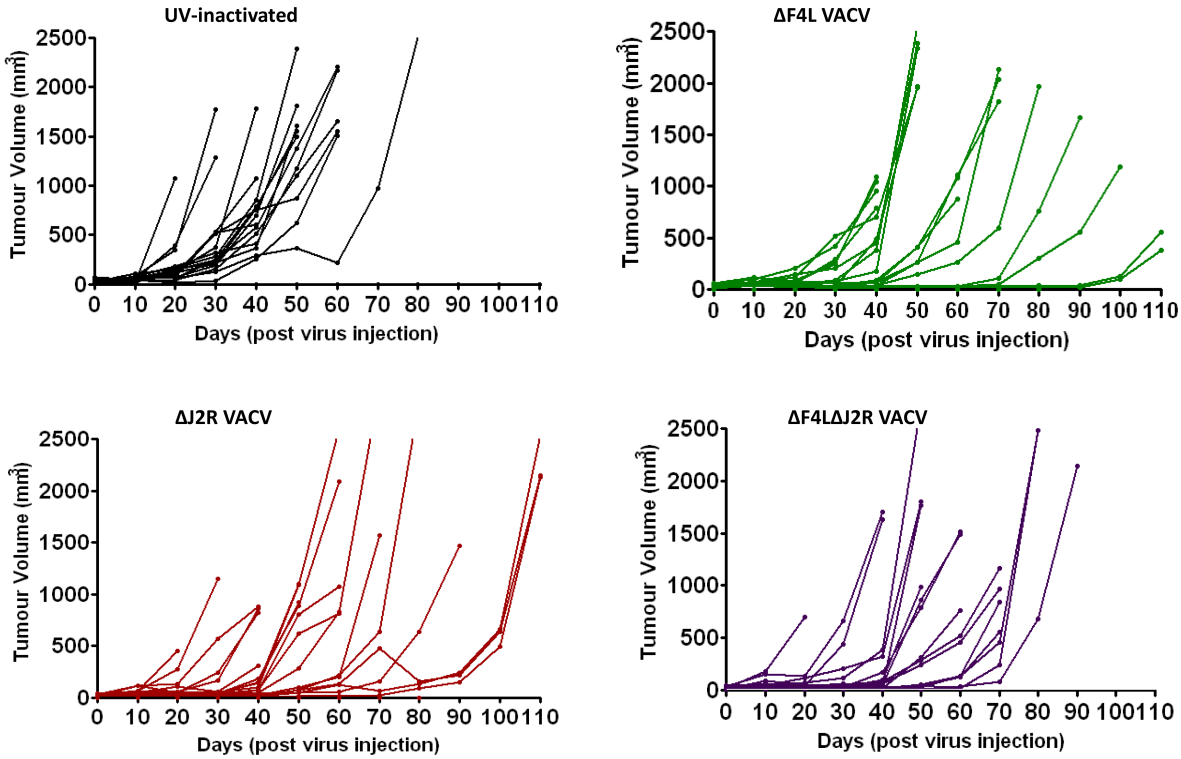

10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
pfu/g tissue
ND
ND
ND
ND
ND
ND
ΔF4L
ΔF4LΔJ2R
ΔJ2R
Brain
Liver
Spleen
Ovaries
Tumor

(C)

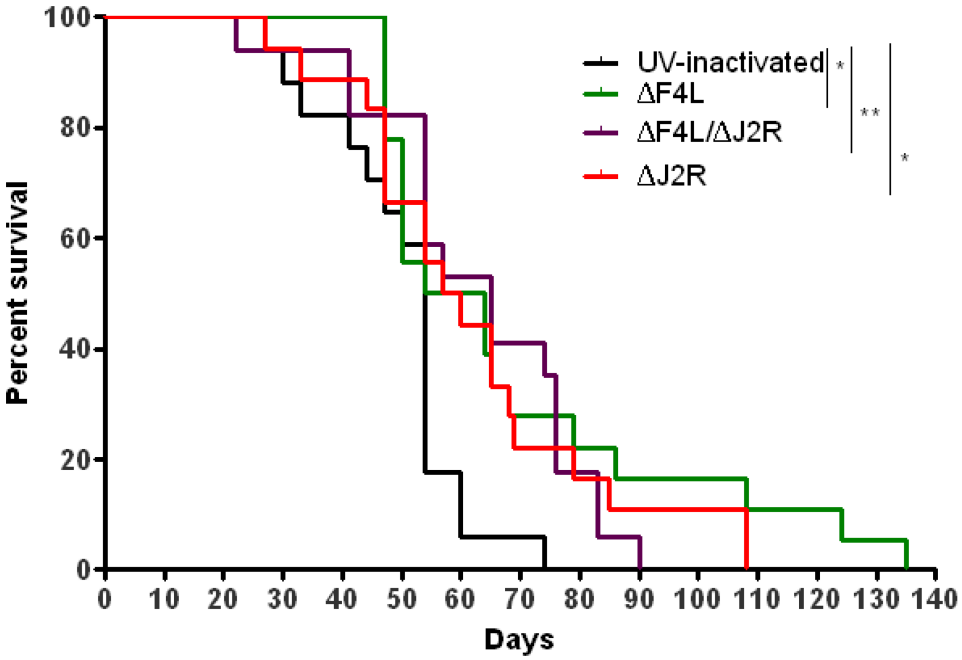


(D)

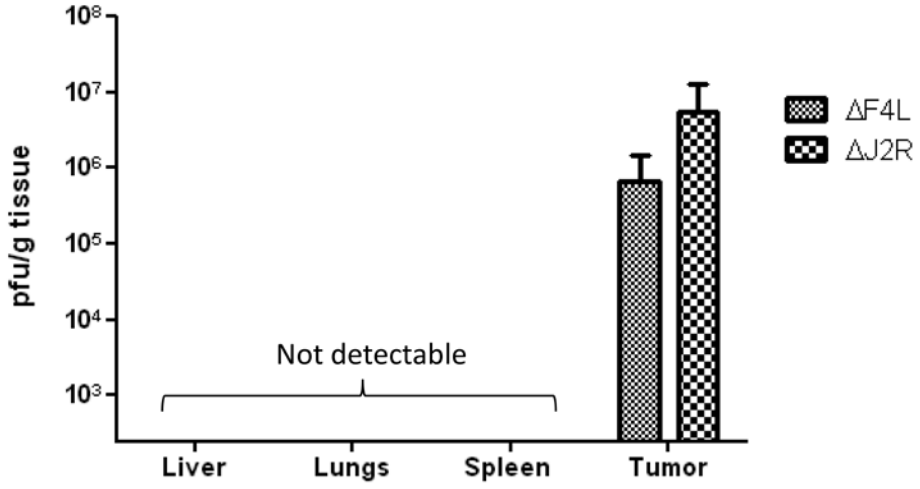
Figure 3.6. F4L-mutant VACVs efficiently control tumor growth and increase survival in a human breast cancer xenograft model. Nude mice bearing MDA-MB-231 xenografts in the mammary fat pad were injected intratumorally with $1x10^6$ pfu of UV-inactivated, ΔJ2R, ΔF4L, and ΔF4LΔJ2R VACVs on each of days 1, 3 and 5 (n=5 mice/group). **(A)** Growth of individual tumors following VACV treatment was monitored by tumor volume measurements using calipers. Each line represents tumor growth in a single animal. **(B)** Survival of mice following treatment with live or dead virus is shown. (**=$p<0.0001$). NB: All control virus-treated mice were euthanized when the tumor size exceeded 1500 mm^3. Four mice treated with live viruses did not survive in this experiment, but their deaths were unrelated to tumor growth or virus spread as confirmed by a veterinarian. **(C)** Biodistribution of mutant VACVs in nude mice bearing MDA-MB-231 xenografts. Mice bearing MDA-MB-231 orthotopic tumors were given 3 injections of the mutant viruses (10^6 pfu/injection) on each of days 1, 3 and 5. The mice were euthanized on day 7 and tumors along with other normal organs were harvested. Harvested organs were homogenized and virus titers in the homogenates were determined by plaque assay. ND, virus was not detectable. (**D**) Tumors were harvested from mice on day 7 after first virus injection and stained for late viral protein (A27) to determine virus replication in the tumor sections.


UV-inactivated
ΔF4L VACV
ΔJ2R VACV
ΔF4LΔJ2R VACV
Tumour Volume (mm3)
Days (post virus injection)
0
500
1000
1500
2000
2500
0 10 20 30 40 50 60 70 80 90 100 110

(A)

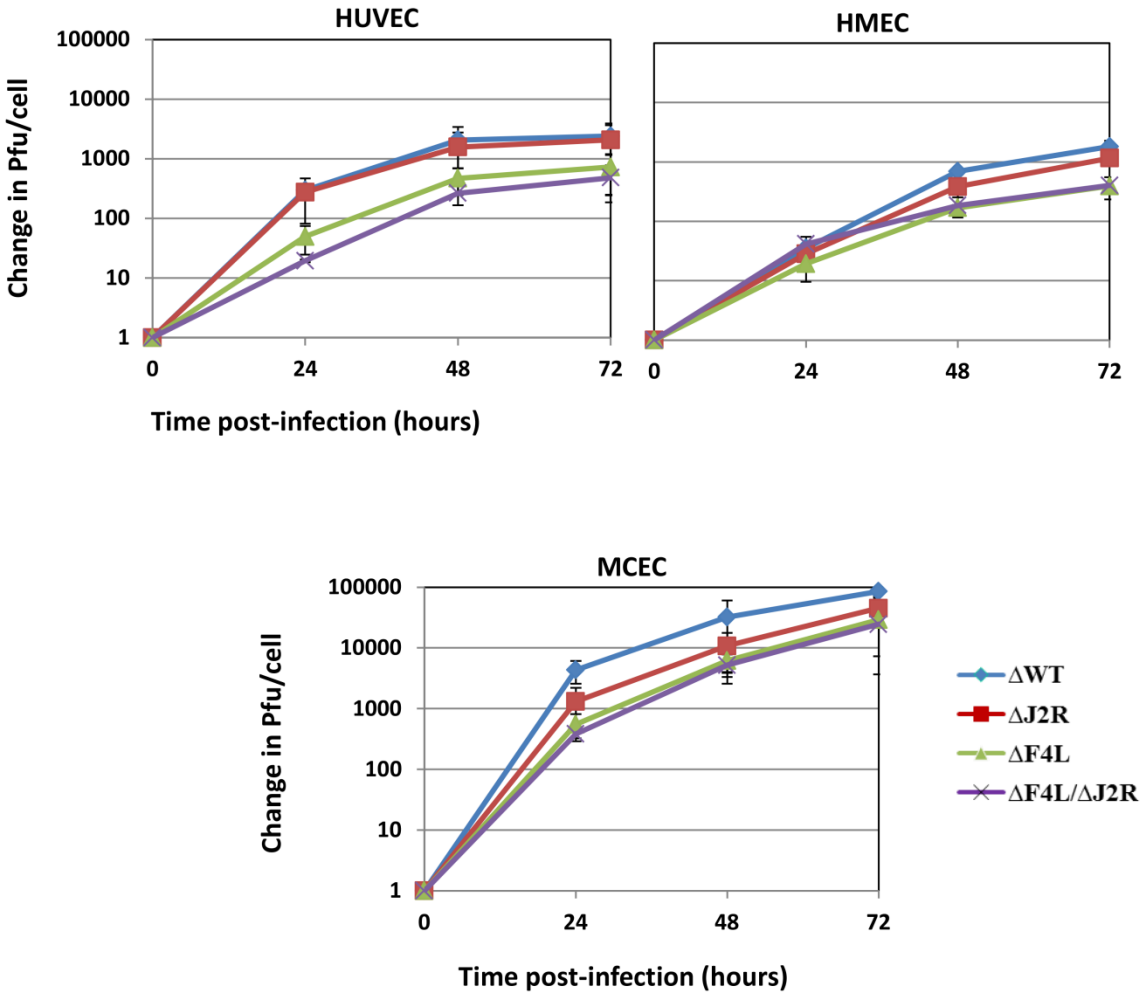

Percent survival
Days
UV-inactivated
ΔF4L
ΔF4L/ΔJ2R
ΔJ2R
*
**
*

(B)

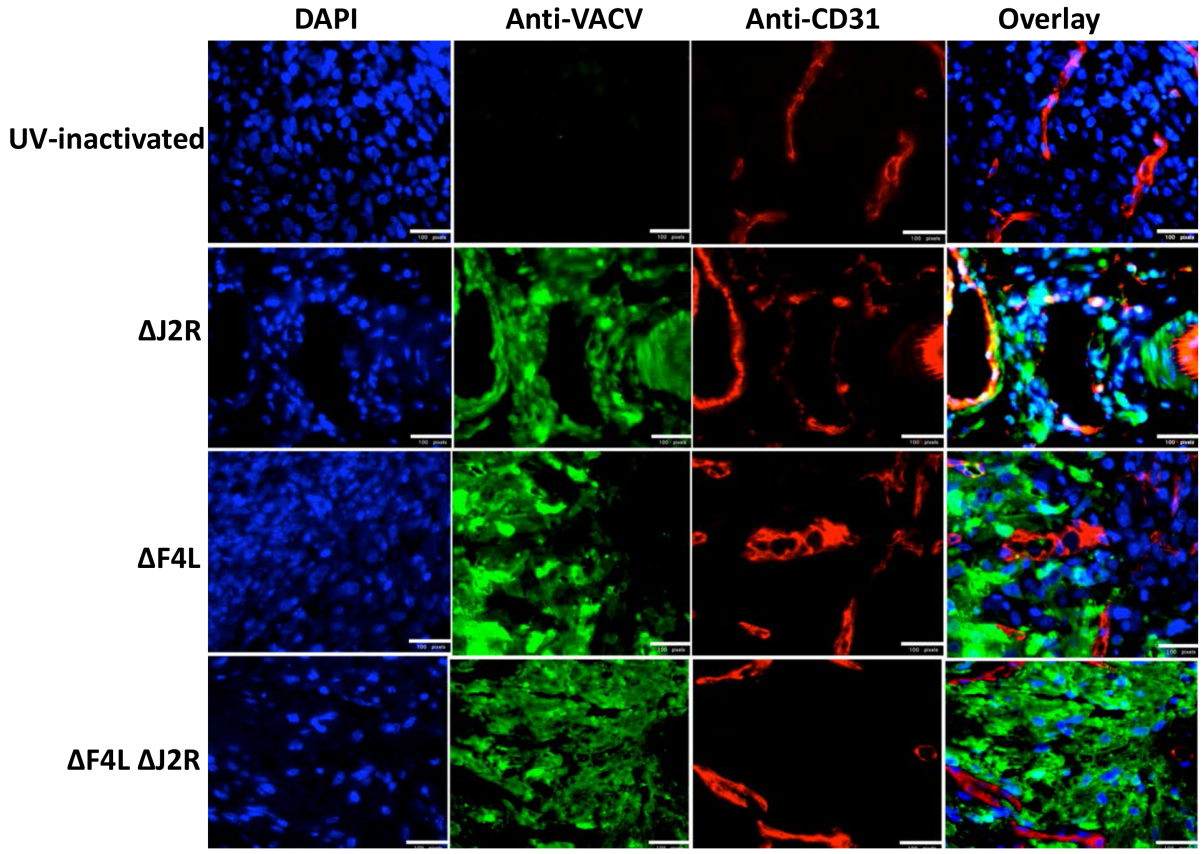

pfu/g tissue
Not detectable
Liver
Lungs
Spleen
Tumor
ΔF4L
ΔJ2R

(C)

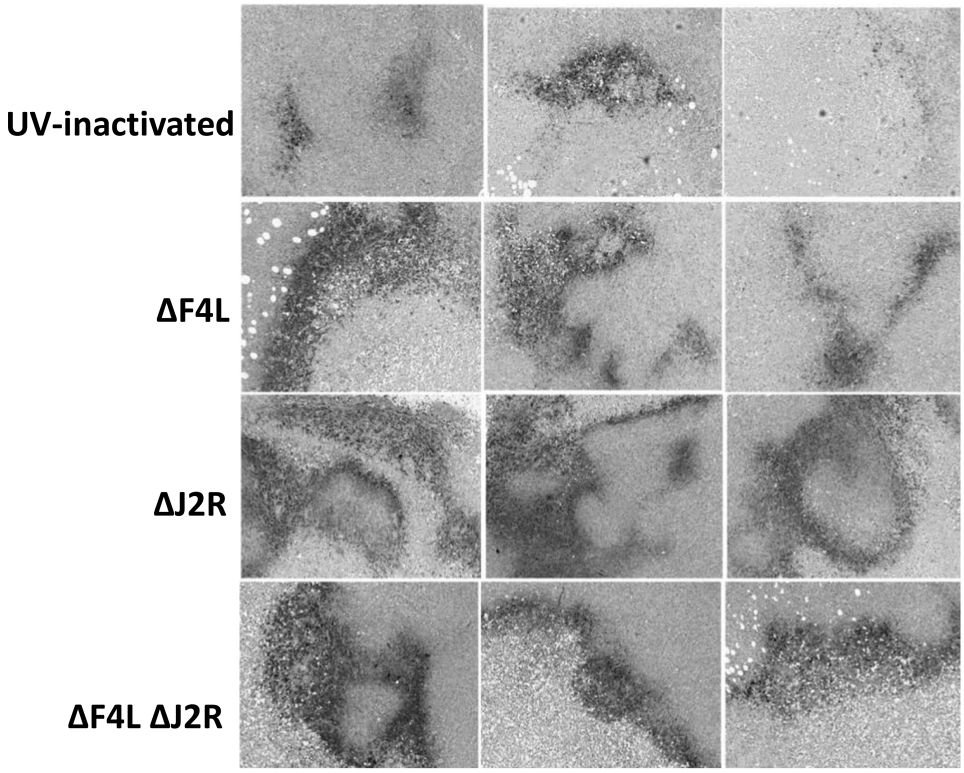
Figure 3.7. ΔF4L and ΔF4LΔJ2R VACVs show anti-tumor activity in a syngeneic BrCa model in mice. Immune-competent mice bearing orthotopic MTHJ tumors were injected intratumorally with 1x108 pfu of UV-inactivated, ΔJ2R, ΔF4L, and ΔF4LΔJ2R VACVs on each of days 1, 3 and 5 (n=20 mice/group). **(A)** Growth of individual tumors following VACV treatment was monitored by tumor volume measurements. Each line represents tumor growth in a single animal. **(B)** Mice were euthanized when the tumor size exceeded 1500 mm^3 and survival of the mice among the groups were compared (*=$p<0.05$, **=$p<0.001$). **(C)** Biodistribution of mutant VACVs in immune-competent mice bearing MTHJ tumors. Mice bearing MTHJ orthotopic tumors were given 3 injections of the mutant viruses (108 pfu/injection). The mice were euthanized on day 14 and tumors along with other normal organs were harvested. Harvested organs were homogenized and virus titers in the homogenates were determined by plaque assay (n=3).


HUVEC
HMEC
MCEC
Change in Pfu/cell
100000
10000
1000
100
10
1
0
24
48
72
Time post-infection (hours)
ΔWT
ΔJ2R
ΔF4L
ΔF4L/ΔJ2R

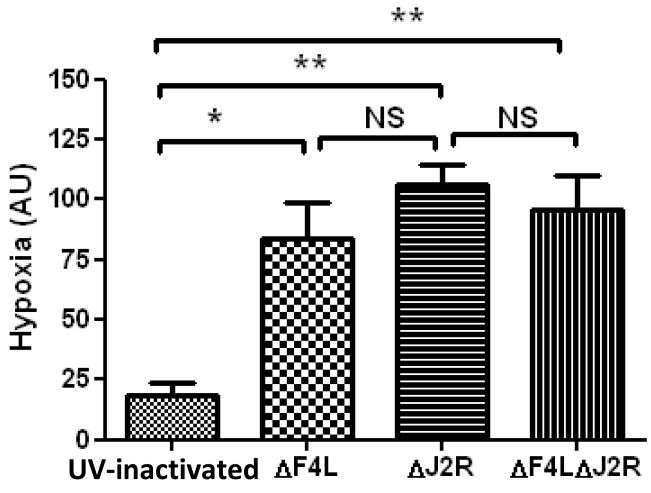
(A)


DAPI
Anti-VACV
Anti-CD31
Overlay
UV-inactivated
ΔJ2R
ΔF4L
ΔF4L ΔJ2R

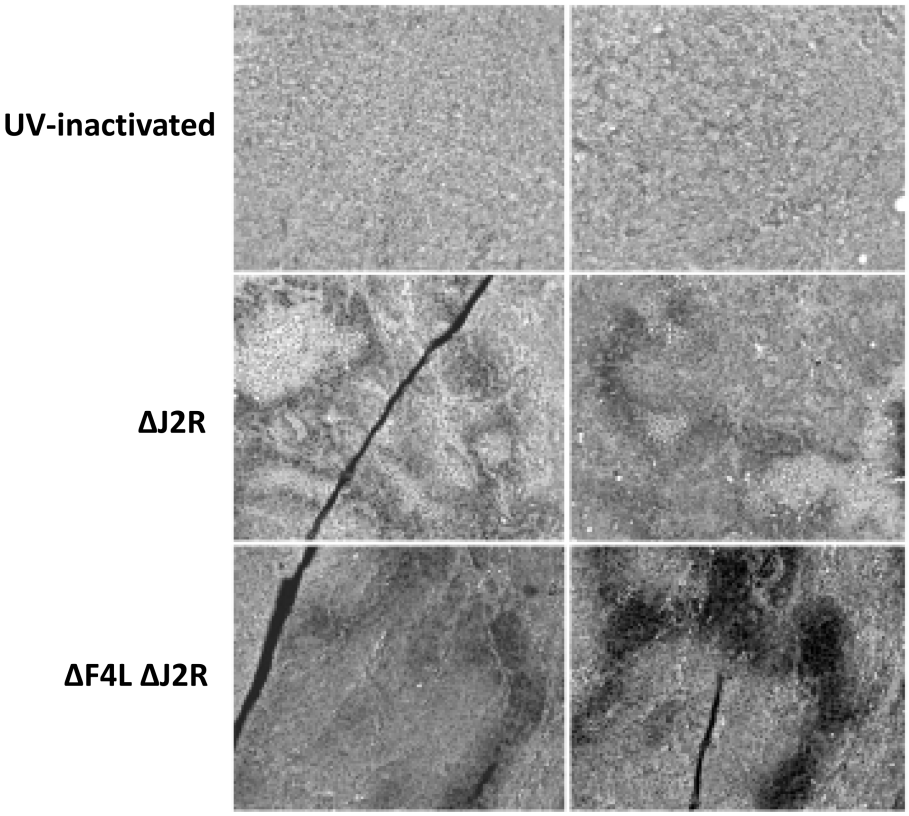
(B)


UV-inactivated
ΔF4L
ΔJ2R
ΔF4L ΔJ2R

(C)


**
**
*
NS
NS
150
125
100
75
50
25
0
Hypoxia (AU)
UV-inactivated
ΔF4L
ΔJ2R
ΔF4LΔJ2R

(D)



(E)

Figure 3.8. ΔF4L and ΔF4LΔJ2R VACVs robustly grow in endothelial cells *in vitro* and increase tumor hypoxia. (A) Human endothelial cells (HUVEC & HMEC) and mouse endothelial cells (MCEC) were infected with WT and mutant VACVs at an MOI of 0.03 pfu/cell. Virus titers produced at the indicated time points were determined by plaque assay. **(B** and **C)** Nude mice bearing MDA-MB-231 xenografts were injected intratumorally with $1x10^6$ pfu of UV-inactivated, ΔJ2R, ΔF4L, and ΔF4LΔJ2R VACVs (n=3) on each of days 1, 3 and 5. On day 7, mice were injected with Hypoxyprobe and were euthanized 30 minutes later. Tumors were harvested, formalin fixed, paraffin embedded and sectioned into 5 μm thick slices. **(B)** The tumor sections were stained with DAPI, vaccinia virus (antibody against A27 viral protein) and endothelial cells (antibody against CD31) as described in the material and method section. (**C**) Tumor sections were stained for hypoxic regions according to manufacturer's protocol (Dark staining indicates the hypoxic area). **(D)** The hypoxic area was quantified using 12 images from each treatment group (4 images per tumor section) by ImageJ software. The hypoxic areas (arbitrary units) among treatment groups were plotted and compared (One way ANOVA; *= $p<0.05$, **=$p<0.01$)**(E)** MTHJ tumor bearing mice were treated with UV-inactivated (n=1), ΔJ2R (n=2) or ΔF4LΔJ2R (n=1) VACV and tumor sections were stained for hypoxia as in **(C)**.

Chapter 4: General Discussion and Future Directions

Breast cancer has been one of the most studied cancers; consequently huge advancements have been made in the diagnosis and treatment of this malignancy over the last few decades. Despite these advancements, BrCa continues to claim more lives of women than any other malignancies worldwide (http://globocan.iarc.fr). While the success rate of treating primary BrCa is high, it is still dismal for the treatment of metastatic disease. Most of the BrCa mortalities result from metastatic disease (Gupta and Massague 2006). Chemo-, radiation- and endocrine-therapies are used to treat metastatic BrCa however, with limited success. Furthermore, the toxicities associated with these therapies often degrade the quality of patients' lives even if the therapies increase the overall survival.

Gene therapy, a therapeutic approach that is still in its infancy, was initially proposed as a promising approach for the treatment of monogenic disorders. However, later studies in the 70s and 80s showed that this approach has potential for use in cancer therapy. Given the dismal success rate of conventional cancer therapies and dire need for novel therapeutics, the concept of gene therapy soon became a hot topic in cancer research which is evident from the fact that out of approximately 2000 approved gene therapy clinical trials to date, more than two-thirds are focused on cancer (http://www.abedia.com/wiley/indications.php; accessed April, 2016). So far, only one gene therapy product has been successful in achieving regulatory approval in Western countries, interestingly, not for cancer. Glybera, an adeno-associated virus encoding lipoprotein lipase for the treatment of lipoprotein lipase deficiency was approved as the first gene therapy product in 2012, in Europe (Yla-Herttuala 2012). Several gene therapy products are currently under advanced phases of clinical trials (see chapter 1).

Oncolytic viruses represent another class of novel bio-therapeutics that holds promise for better treatment of cancer. Although the concept of oncolytic virotherapy is not new, the field has gained momentum recently as advancements in technology have made it feasible to study viruses thoroughly and to analyze the safety as well as their anti-tumor efficacy (Russell, Peng et al. 2012). Hundreds of studies have reported anti-tumor effects of a wide range of viruses in preclinical studies, and many of these have entered clinical trials. Very recently the first (outside of China) oncolytic virus, Talimogene laherparepvec (T-Vec), a herpes virus encoding GM-CSF, was approved by the US FDA (Greig 2016).

4.1 Breast Cancer: Immunogenicity and Prospect of Immunotherapy

Recent studies have pointed out that the response of a tumor to immunotherapy depends on the immunogenicity of the tumor (Blankenstein, Coulie et al. 2012; Lechner, Karimi et al. 2013). Certain types of cancer such as metastatic melanoma and renal cell carcinoma are thought to be highly immunogenic based on the following evidences: (i) occasional spontaneous regression (Komenaka, Hoerig et al. 2004; Janiszewska, Poletajew et al. 2013), (ii) improved survival associated with infiltrating T-lymphocytes (Day, Sober et al. 1981; Belldegrun, Muul et al. 1988), (iii) response to non-antigen-specific immunotherapies such as interferon-alpha, IL-2 and anti-CTLA4 (Itsumi and Tatsugami 2010; Kaufman 2012), (iv) higher incidence of these malignancies in immune-suppressed individuals (Jensen, Hansen et al. 1999; Itsumi and Tatsugami 2010), and (v) presence of tumor-associated antigens, as well as human leukocyte antigen (HLA)-restricted epitopes within these antigens (Bernhard, Maeurer et al. 1996; Rosenberg 1996; Kaufman 2012).

It has long been realized that the immune system is significantly impaired in BrCa patients (Stewart 1996; Rao, Dyer et al. 2006). However, the incidence of BrCa is not elevated in immune-suppressed individuals, suggesting that BrCa is a poorly immunogenic disease (Penn 1988). The low-immunogenicity has been suggested to be the result of one or more of the following events: (i) heterogeneous expression of tumor antigens, (ii) modulation in the antigen profile during the course of tumor progression, (iii) low levels of antigens and/or MHC proteins, and (iv) release of immunosuppressive factors in the tumor milieu that precludes optimal antigen presentation and has a negative effect on immune response (Reviewed in (Mittendorf, Peoples et al. 2007; Criscitiello, Esposito et al. 2014)).

Nevertheless, BrCa shows several characteristics that make the investigation of immunotherapy for BrCa worthwhile. First, increased tumor infiltration by lymphocytes correlates with better prognosis and increased overall survival in BrCa patients (Menard, Tomasic et al. 1997; Savas, Salgado et al. 2016). Second, several tumor-associated antigens have been identified in BrCa, including mammaglobin, mucin1, carcinoembryonic antigen, and HER2, which could be potential targets for immunotherapy (Tanaka, Amos et al. 2003; Criscitiello 2012). Third, a significantly higher rate of disease relapse is seen in patients with reduced levels of serum IL-2, an immune-modulatory cytokine, compared to the patients with normal levels of serum IL-2 (Arduino, Tessarolo et al. 1996). Fourth, and perhaps the best evidence that the immune system can control BrCa, comes from the fact that the first case of cancer transmission from a living organ donor was a BrCa transmitted from the kidney of a donor wife to her transplant-recipient husband (Myron Kauffman, McBride et al. 2002; Chapman, Webster

et al. 2013). Six months post-transplantation, the recipient, who was on immuno-suppressive drugs, developed bone and brain metastases which were of breast adenocarcinoma origin. The donor had suffered BrCa in the past but was cancer-free for at least 8 years at the time of organ donation. The absence of tumor in the immune-competent donor at the time of organ donation and growth of donor-derived tumor in the immune-compromised recipient strongly suggests that this donor's BrCa was immunogenic and being kept at bay by her immune system (Myron Kauffman, McBride et al. 2002; Chapman, Webster et al. 2013). Fifth, although the anti-tumor effect of chemo- and radiotherapy was previously thought to be solely the result of direct killing of tumor cells, recent studies strongly suggest that those classical treatments heavily rely on the immune system for their anti-tumor efficacy. A recent study by Apetoh et al. elegantly shows that the anti-tumor efficacy of chemo- and radiotherapy in mice as well as in humans is dictated by toll like receptor-4 (TLR-4) which is expressed by dendritic cells and thought to be crucial in processing and cross-presentation of antigens from dying cancer cells (Apetoh, Ghiringhelli et al. 2007). In this study, the authors showed that a loss-of-function mutation in the TLR-4 gene greatly reduces the efficacy of chemotherapies such as anthracyclines and taxanes as well as X-ray therapy in treating breast tumors in mice. Furthermore, in a retrospective study analyzing 280 BrCa patients who were treated with anthracyclines following local surgery and radiotherapy, the authors found that patients who had inactivating mutation in the TLR-4 gene relapsed quickly compared to those carrying a normal TLR-4 gene (Apetoh, Ghiringhelli et al. 2007). With the realization that BrCa is not as poorly immunogenic as perceived in the past, several immunotherapeutic strategies, especially strategies to inhibit check points

using inhibitors of programmed death 1 and its ligand or inhibitors of cytotoxic T-lymphocyte-associated protein-4, are currently under various phases of clinical trials for BrCa (https://clinicaltrials.gov). In chapter 2 of this dissertation, we evaluated the feasibility of transcriptionally targeted IL-2 gene therapy for the treatment of BrCa.

4.1.1 IL-2 in Breast Cancer Therapy

IL-2 is one of the most widely studied cytokines as a therapeutic for different malignancies, and has been approved for the treatment of renal cell carcinoma and malignant melanoma (Coventry and Ashdown 2012). The finding in early 1980s that IL-2 has the ability to mediate T cell survival and functioning *in vitro*, suggested that its administration could potentially stimulate functional T cells *in vivo* and help in clearance of cancer cells (Kurnick, Gronvik et al. 1979). It was noted that exposure to IL-2 could generate cytotoxic cells, *in vitro*, from mouse splenocytes or human peripheral blood mononuclear cells (Grimm, Mazumder et al. 1982). These cells, later called lymphokine activated killer (LAK) cells, were found to recognize and kill cultured cancer cells and fresh human cancer cells *in vitro* without the need for further stimulation (Yron, Wood et al. 1980; Grimm, Mazumder *et al.* 1982; Yang, Mule et al. 1986). In mice, rIL-2 showed anti-tumor effect, however only at doses that resulted into significant toxicities (Rosenberg, Mule et al. 1985).

One of the major limitations of IL-2 in cancer therapy is the severe toxicity associated with the high systemic dose that is used in clinic. IL-2-induced vascular leak syndrome (VLS) is a particularly severe form of toxicity which is marked by accumulation of extravascular fluid in vital organs such lungs and liver (Nakagawa, Miller et al. 1996; Epstein, Mizokami et al. 2003). Currently there is no treatment for

VLS and the only option is to discontinue IL-2 therapy (Laurent, Touvrey et al. 2013). Furthermore, IL-2 administered by intra-venous bolus injection has an initial half-life of ~13 minutes followed by a slower phase with a half-life of 85 min for up to 4 hours (Konrad, Hemstreet et al. 1990). Because of the short half-life, maintaining the therapeutically effective serum concentration of IL-2 over a sustained period is very difficult. Thus, the rapid systemic clearance and the narrow therapeutic window of effective concentrations greatly limit the use of IL-2 in cancer therapy (Rao, Driver et al. 2004).

Previous studies have shown that restricting high levels of IL-2 within the tumor micro-environment could circumvent IL-2 related toxicities. Only a few approaches have been studied in order to minimize IL-2 associated toxicities while maintaining the therapeutic concentration. One of these approaches is fusing IL-2 to antibodies that recognize an antigen expressed on the cancer cells (Sabzevari, Gillies et al. 1994; Becker, Pancook et al. 1996; Xiang, Lode et al. 1997; Lode, Xiang et al. 1998). For example, IL-2 fused to an antibody recognizing a glycolipid (GD2), expressed on the surface of melanoma and neuroblastoma cells, has been shown to accumulate in the tumor microenvironment after intra-venous injection in mouse models. This fusion protein was found to inhibit growth of established metastases of human melanoma and neuroblastoma in mice (Sabzevari, Gillies et al. 1994; Becker, Pancook et al. 1996). Intra-tumoral administration of antibody-IL-2 fusion proteins has been shown to be safer and more effective compared to systemic administration (Christ, Seiter et al. 2001). Currently, antibody-IL-2 fusion proteins are in phase I and II clinical trials for different types of cancer (www.clinicaltrials.gov; accessed on 9th April 2016).

Gene therapy is another approach that has been previously studied to harness the therapeutic benefits of IL-2. In the first part of this dissertation, we have explored the safety and efficacy of IL-2 gene therapy for BrCa. However, this is not the first study of IL-2 gene therapy for BrCa. Addison *et al.* studied the efficacy of adenovirus mediated IL-2 gene therapy in mouse models of BrCa and they found that the therapy resulted in complete tumor regression in one-third of mice treated intra-tumorally (Addison, Braciak et al. 1995). The adenovirus vector in this study encoded human IL-2 under the control of the constitutively active human CMV promoter. However, in this study the authors did not report IL-2 mediated toxicity in mice. Another study by the same group reported that the dose of Ad-hCMV-IL-2 required to obtain an anti-tumor effect killed more than one-third of treated mice within 6-10 days after intra-tumoral injection (Addison, Bramson et al. 1998). Death of the treated mice resulted from extensive damage to the liver and spleen which, the authors concluded, could have been due to dissemination of the vector from the tumor to other normal organs (Addison, Bramson et al. 1998). Indeed, studies have shown that even after intra-tumoral injection, a significant amount of adenovirus escapes the tumor and reaches the liver (Bramson, Hitt et al. 1997; Wang, Hu et al. 2003). In the liver, adenoviruses are sequestered by Kupffer cells which not only increase the risk of liver toxicity but also reduce the vector concentration in the target tissue (Tao, Gao et al. 2001). Another study by Toloza *et al.* also found that an IL-2-encoding adenovirus vector was ineffective in controlling BrCa in mice at lower doses, and at higher doses the virus was lethally toxic. This study also used the constitutively active hCMV promoter to drive IL-2 gene (Toloza, Hunt et al. 1996).

The novelty of our study is that we have employed the transcriptional targeting approach with the aim of restricting IL-2 expression to BrCa cells in order to minimize toxicity. To our knowledge, this is the first study using transcriptionally targeted IL-2 for BrCa gene therapy. Furthermore, our use of mammaglobin promoter/enhancer (MPE2) elements for transcriptional targeting of a therapeutic gene is novel. Our data show that the MPE2 and MCMV promoters are similarly active in driving a reporter gene (luciferase) in human and murine BrCa cell lines *in vitro*. Not surprisingly, the activity of the MCMV promoter was high in both BrCa and normal cells. However, the ability of the MPE2 sequence to drive reporter gene expression in normal cells was at least 100-fold less than that in BrCa cells. Interestingly, MPE2 was found to be as potent as, or better than, the most commonly used tumor specific promoter, hTERT, in driving the IL-2 gene. While the hTERT promoter was found to be BrCa-specific only in cells of human origin, the MPE2 promoter was found to have excellent cancer specificity in cells of both human and murine origin. Intriguingly, while MPE2 and MCMV promoters were found to be equally active in driving luciferase gene expression in BrCa, MPE2 activity was found to be 100 to 1000-fold less than that of the MCMV promoter in driving the IL-2 gene both *in vitro* and in mouse tumors.

This discrepancy in the ability of MPE2 to drive different genes was surprising. We postulated that IL-2 may have an inhibitory effect on the MPE2 promoter causing a negative feedback loop ultimately reducing IL-2 transcription. However our preliminary studies suggest that the MPE2 promoter is not down-regulated by IL-2-induced signaling pathways. An alternative possibility is that specific sequences in the transgene inserts are responsible for the differential activity of the promoter.

It should be noted that, like most other studies, our vectors encode IL-2 cDNA and not the IL-2 gene. The use of cDNAs for expression of transgenes is very common as they are smaller in size compared to their respective genes (Nott, Meislin et al. 2003). However, studies have shown that introns could play an important role in the overall expression of a gene and many eukaryotic genes require one or more introns for optimal expression (Niu and Yang 2011). While the presence of introns is not a universal requirement, addition of introns has been found to generally boost the expression of genes (Buchman and Berg 1988). A study by Nott *et al.* demonstrated that addition of a single intron (from a different gene) in the open reading frame of *Renilla* luciferase cDNA greatly enhanced the expression of luciferase (Nott, Meislin et al. 2003). Addition of the intron was found to enhance mRNA accumulation and translational yield compared to the intron-less transcripts. Several other studies have shown that addition of an intron from the same gene or a different gene to a cDNA can enhance the expression (Brinster, Allen et al. 1988; Nott, Le Hir et al. 2004). In fact, intron-containing genes produce more copies of mRNA, and highly expressed genes are associated with higher intron densities compared to weakly expressed genes in mammalian cells (Comeron 2004; Shabalina, Ogurtsov et al. 2010). Given the importance of introns in the overall expression of genes, it might be logical to speculate that delivering the entire sequence containing introns and exons instead of just the cDNA through our adenovirus vector may result in higher expression of IL-2 and overcome the issue of low IL-2 expression from MPE2 promoter. The full length human IL-2 gene excluding promoter (~5 kb) together with the MPE2 promoter (~2.5 kb) is still within the range of the first generation adenovirus vector's cloning capacity (~8.2 kb).

In our mouse experiments, Ad-MPE2-IL-2 was found to be safe whereas Ad-MCMV-IL-2 was found to be toxic resulting in the death of one-third of treated animals, which is similar to what was observed by Addison *et al.* (Addison, Braciak et al. 1995). Furthermore, the safety profile of our Ad-MPE2-IL-2 vector is in agreement with the previous study, by Bui *et al*., of transcriptionally targeted adenovirus-mediated IL-2 gene therapy for hepatocellular carcinoma (Bui, Butterfield et al. 1997). Bui *et al*. used the promoter from the murine alpha-fetoprotein (AFP) gene to direct mouse IL-2 expression to hepatocellular carcinoma. They used SCID mice bearing human hepatocellular carcinoma xenografts to compare the safety and anti-tumor efficacy of this vector with an adenovirus vector encoding IL-2 under the control of the hCMV promoter. Unlike our study where we used fully immune-competent mice, Bui *et al*. used immune-compromised mice. Similar to our study and the study by Addison *et al*., the study by Bui *et al.* found that the Ad-hCMV-IL-2 was highly toxic even when administered intra-tumorally (~90% mice died due to IL-2-related toxicity). Furthermore, they found that the AFP promoter was as potent as the CMV promoter in driving luciferase expression in mouse tumors, which is in accordance with our data. However, in their study they did not compare the activity of the AFP promoter with that of the CMV promoter in driving IL-2 gene expression. Interestingly, the transcriptionally targeted (AFP promoter driven) IL-2 was shown to be safe, no mortality was found at doses of Ad-hCMV-IL-2 that killed ~90% mice. Measurement of anti-tumor efficacy of IL-2 vectors was not the primary goal of this study, however, some anti-tumor effect was observed by the IL-2 encoding vectors which presumably was due to activation of macrophages and natural killer cells, or a direct anti-BrCa effect, as SCID mice lack T cells.

While our Ad-MPE2-IL-2 vector demonstrated an excellent safety profile, the anti-tumor efficacy of this vector was mild at best: the vector significantly delayed tumor growth in mice, no complete tumor regression was observed and all the mice eventually succumbed to tumor burden. There could be several possible reasons accounting for the failure of our Ad-MPE2-IL-2 vector to elicit a strong anti-tumor effect. First, IL-2 has been shown to have a narrow therapeutic window. In previous studies, the anti-tumor effect of IL-2 in mice was achievable only at doses that resulted in significant toxicities (Rosenberg, Mule et al. 1985; Addison, Braciak et al. 1995). Given the weak activity of the MPE2 promoter compared to the MCMV promoter, it is likely that low levels of IL-2 expression by Ad-MPE2-IL-2 may be below the therapeutic window of IL-2. Second, PyMidT over-expressing BrCa cells form rapidly growing tumors which may represent a subset of BrCa that would be less responsive to IL-2. Indeed, a study by Jan Vaage has shown that IL-2 is more effective in controlling slow growing tumors (Vaage 1988). As discussed in Chapter 1, animal models are not always good representatives of human disease. Many potential drugs that show promising therapeutic benefits in animal models often fail in clinical trials. Conversely, some drugs that would have therapeutic benefits in humans never make it to clinical trials because they fail to show therapeutic benefits in animal models. It should be noted that there is a huge discrepancy in the growth rate of tumors in animal models and humans: while the tumor doubling time in mice is usually in the range of days to weeks, in humans it is in the range of months to years (Friberg and Mattson 1997). This rapid growth of tumors in animal models increases susceptibility to chemotherapeutic agents that target dividing cells, but, on the other hand, these tumors

are less likely to respond to therapeutics such as IL-2 that are more effective in eliminating slow growing tumors (Vaage 1988; Francia, Cruz-Munoz et al. 2011).

Previous studies have shown that IL-2, as mono-therapy, has sub-optimal therapeutic efficacy in BrCa both in animal models and in clinical trials (Rao, Dyer et al. 2006), however, IL-2 in combination with other agents has been more effective. For example, in a study by Putzer *et al.* a single dose of 1.8×10^8 pfu Ad vector expressing IL-2 (human CMV promoter-controlled) in combination with a single dose of 10^9 pfu of Ad-p53 resulted in complete tumor regression in 50% of mice (n=20) bearing PyMidT tumors (Putzer, Bramson et al. 1998). Single agents caused tumor growth delays but no complete tumor regressions in that study, which is in accordance with our results where we see delayed tumor growth in a fraction of mice treated with Ad-MPE2-IL-2 or Ad-MCMV-IL-2 vector. One problem with delivering exogenous p53 by an Ad vector is that only a fraction of tumor cells (as little as 20%) are transduced with the virus, even after intra-tumoral injection with as high as $\sim 10^9$ pfu of virus (Putzer, Bramson et al. 1998). Unlike IL-2, p53 is not a secreted protein; therefore its effect would be limited to the transduced cells. In contrast, a small molecule drug such as PAC1 should have better access to the tumor cells. Like p53, a pro-apoptotic drug such as PAC1 would induce apoptosis in tumor cells potentially revealing tumor antigens that would be recognized by cytotoxic T cells, after which IL-2 in the tumor milieu could help to rapidly amplify these T cells. Therefore, Ad-IL-2 in combination with PAC1 should result in better anti-tumor effects than either agent alone.

PAC1 has been shown to be highly toxic to many types of cancer cells *in vitro* and induces significant anti-tumor responses in animal models for several types of tumors

(Putt, Chen et al. 2006). The dose of PAC1 used in this study (100 mg/kg) was selected because higher doses were reported to induce transient neuro-toxicity in mice (Peterson, Hsu et al. 2010). Although we observed high levels of PAC1-induced apoptosis *in vitro*, few, if any, apoptotic cells were observed in tumor sections of mice treated with PAC1 alone. While Ad-MPE2-IL-2 and Ad-MCMV-IL-2 vectors alone significantly delayed tumor growth *in vivo* compared to control, PAC1 alone failed to do so. Unfortunately, the addition of PAC1 did not boost the anti-tumor effect of Ad-MPE2-IL-2 vector, suggesting that the PAC1 treatment used might not have released enough tumor antigens to stimulate immunity. Notably, no sign of toxicity was observed in mice treated with the Ad-MPE2-IL-2 vector in combination with PAC1, suggesting that it may be possible to combine Ad-MPE2-IL-2 with chemotherapeutics to harness the combined therapeutic effect without additional toxicities.

Taken together, data presented in the second chapter of this dissertation suggest that adenovirus mediated IL-2 gene therapy utilizing the MPE2 promoter/enhancer is an excellent approach for BrCa therapy from the safety perspective. However, this approach as a mono-therapy or even in combination with the experimental drug PAC1 has sub-optimal anti-tumor efficacy. Strategies to enhance the strength of MPE2 promoter in driving IL-2 gene and/or combination with a strong pro-apoptotic drug might result in better treatment of BrCa.

4.2 Oncolytic Vaccinia Virus as a Potential Therapeutic for BrCa

Existing cancer therapies are not sufficient to cure cancer and they often cause a variety of debilitating side effects severely affecting the quality of patients' lives. Hence, there is a need for better therapeutics. Oncolytic viruses represent a novel class of bio-therapeutics that has been heavily studied in last few decades. Vaccinia virus is one of the most commonly studied viruses for its oncolytic properties. Several strategies have been used to render the virus cancer specific.

4.2.1 Vaccinia Virus Deleted of F4L and/or J2R Show Oncolytic Activities in Breast Cancer

Vaccinia virus encodes a large number of genes, several of which make proteins that are homologous to cellular proteins. Interestingly, the cellular homologs of some of the viral genes are over-expressed in cancer cells, a feature which can be exploited to make the virus cancer-selective. DNA viruses with large genomes such as herpes simplex virus and vaccinia virus encode genes required for dNTP synthesis to ensure that the viruses can replicate to high levels even in non-cycling cells which often have paucity of dNTPs. High demands of dNTPs for these viruses and high activity of dNTP synthesis machinery in cancer cells provide opportunity to make the virus cancer-selective by specifically deleting the viral genes involved in dNTP synthesis. Thymidine kinase (TK) is an enzyme that is involved in the salvage of dTTPs. TK is a cell cycle regulated protein in normal cells, but is constitutively expressed in cancer cells (Hengstschlager, Knofler et al. 1994). Furthermore, high levels of TK have been shown to correlate with large tumor size, higher histological grades and disease recurrence (O'Neill, Hoper et al. 1992; Broet, Romain et al. 2001). Therefore, viral TK is an attractive target for deletion in order to

make the virus cancer-selective. Indeed, deletion of the viral TK from a herpes simplex virus was the first evidence that a virus could be rendered oncolytic by genetic mutation (Martuza, Malick et al. 1991). Since then, deletion of viral TK from herpes simplex virus and vaccinia virus has been the most commonly employed strategy to make them oncolytic. Pexa-Vec, the clinically most advanced oncolytic VACV, is deleted of *J2R* (viral TK) and it also carries a transgene encoding human granulocyte macrophage-colony stimulating factor (GM-CSF) in order to promote host anti-tumour immune response (Heo, Reid et al. 2013). Pexa-Vec has completed phase I and II clinical trials and results have shown that both intra-tumoral and intra-venous injections of this virus were well tolerated and resulted in significant increase in overall survival of patients with liver cancer (Heo, Reid et al. 2013). However, several studies have shown that this virus can replicate, albeit at low levels, in normal cells (Lun, Chan et al. 2010; Parato, Breitbach et al. 2012). Furthermore, studies by our group have found that *J2R*-deleted WR strain of VACV is not sufficiently attenuated in normal cells *in vitro* and occasionally results in death of nude mice even after intra-tumoral injection of as low as 1 million pfu of virus. It should be noted that TK (viral J2) is involved only in the salvage of a single dNTP *i.e.* dTTP, however, there are other enzymes which are crucial for the synthesis of all 4 dNTPs (Engstrom, Eriksson et al. 1985; Mathews 2006). For example, ribonucleotide reductase (RR) is required for the conversion of nucleoside diphosphate into deoxy-ribonucleoside diphosphate and is, perhaps, the most important enzyme in dNTP synthesis pathways (Nordlund and Reichard 2006). RR is involved in the biogenesis and maintenance of a balanced pool of dNTPs in cells (Figure 1.4) (Engstrom, Eriksson et al. 1985; Mathews 2006). Functional RR is a hetero-tetramer

($RRM1_2RRM2_2$) comprised of dimers of large (RRM1) and small (RRM2) subunits. In normal cells, RRM1 levels remain stable throughout the cell cycle. However, RRM2 levels peak during S-phase and the protein gets rapidly degraded at G2-M transition. Consequently, RRM2 with a half-life of ~3 hours is the rate limiting factor in dNTP synthesis (Engstrom, Eriksson et al. 1985). Interestingly, BrCa cells have been found to express high levels of RRM2, which has led researchers to propose the use of cellular RRM2 promoter for transcriptional control of transgenes in BrCa gene therapy (Yun, Cho et al. 2008). Interestingly, VACV encodes the *F4L* gene whose product is homologous to cellular RRM2. Given the crucial role of RRM2 in dNTPs synthesis, it is logical to surmise that deletion of *F4L* from VACV should result in cancer-selectivity of the virus comparable to, or better than, cancer-selectivity obtained by *J2R* deletion. Therefore, we studied the oncolytic potential of *F4L* deletion either alone or in combination with *J2R* deletion.

Indeed, data presented in the third chapter of this dissertation suggest that deletion of *F4L* alone makes the virus highly cancer-selective. Cancer-selectivity of the *F4L*-deleted VACV was found to be better than the *J2R*-deleted virus, *in vitro*. However, *in vivo* cancer selectivity of the *F4L*-deleted VACV and *J2R*-deleted VACV were found to be similar, as determined by bio-distribution of the viruses in both nude and immune-competent mice after intra-tumoral injection. Interestingly, although replication of the double-deleted VACV (*F4L*- and *J2R*-deleted) showed cancer cell-selectivity *in vitro* similar to the single *F4L*-deleted virus, recovery of the double-deleted virus from tumors of immune-competent mice 2 weeks post-injection was much lower than that of the single-deletion viruses. This suggests that the double-deleted virus either has reduced

replication ability *in vivo* or is cleared faster by the immune system. Nevertheless, all the three mutant viruses were able to exert similar levels of anti-tumor activity in xenograft and syngeneic mouse models of BrCa.

When using viruses as therapeutics, there must be a second level of safety measures to ensure that virus production could be halted in the unlikely event of unwanted virus growth or spread. One of the most commonly used drugs against DNA viruses is cidofovir which has been approved for AIDS-associated cytomegalovirus retinitis (Plosker and Noble 1999; Biron 2006). Cidofovir is a nucleoside analog that has been shown to inhibit the replication of DNA viruses from a wide diversity of virus families such as herpesviridae (*e.g.,* HSV1), adenoviridae (*e.g.,* adenovirus type 2 and 5) and poxviridae (*e.g.,* vaccinia virus) (De Clercq 1996). Recent studies have demonstrated the utility of this drug in the treatment of diverse orthopoxvirus infections (Bray, Martinez et al. 2000; Baker, Bray et al. 2003). Because cidofovir is highly effective in controlling orthopoxviruses *in vitro* and in animal models, some authorities have opined that this drug be stockpiled for use in an unfortunate outbreak of smallpox (Andrei, Gammon *et al.* 2006). Interestingly, our group has previously shown that deletion of the *F4L* gene makes the virus hypersensitive to cidofovir (Gammon, Gowrishankar et al. 2010). Cidofovir competes with dCTP during DNA synthesis and is highly efficient in blocking the VACV E9 DNA polymerase (Magee, Hostetler et al. 2005; Magee, Aldern et al. 2008). Because the *F4L*-deleted VACV is unable to synthesize dNTPs, the dNTP concentration in cells infected with *F4L*-deleted VACV at any time point is, presumably, lower than that in cells infected with WT VACV. Hence, in cells infected with *F4L*-deleted VACV, there is higher chance of cidofovir incorporation in the viral genome

which may explain the hypersensitivity of the *F4L*-deleted VACV to cidofovir. Unlike the *F4L*-deletion, several studies have shown that *J2R*-deletion does not alter the sensitivity of VACV to cidofovir (Prichard, Keith et al. 2007; Gammon, Gowrishankar et al. 2010). Therefore, our *F4L*-mutant viruses appear to offer improved safety compared to the *J2R*-deleted VACV.

4.2.2 Oncolytic Viruses and Cancer Stem Cells

Early diagnosis and subsequent resection of primary tumors have significantly improved the overall survival of BrCa patients. However, although several chemo- and endocrine-therapies seem to work initially, patients often develop resistance to these therapies (Jones 2008). The concept of 'cancer stem cells (CSC)' appears to explain how BrCa becomes resistant to therapies and why disease relapse is so common. For example, CSCs in BrCa have been shown to be significantly more resistant to radiation and chemo-therapies (Phillips, McBride et al. 2006; Yu, Ramena et al. 2012). Furthermore, there is a growing body of evidence implicating CSC in cancer relapse after initial regression (Yu, Ramena et al. 2012). It is, therefore, important to develop therapies that would not only kill the differentiated cancer cells making up the bulk of tumor but also kill the CSCs that make up a very small proportion of the tumor.

Oncolytic viruses represent a completely different class of therapeutics employing wide varieties of mechanisms to kill cancer cells; hence CSCs that are resistant to conventional therapies may still be susceptible to oncolytic virotherapy. For example, over-expression of multi-drug resistant (MDR) gene is considered one of the most common causes of drug resistant in CSC, however, MDR over-expression is likely to

have no effect on oncolytic viruses, hence the CSC should be susceptible to the viruses (Dean, Fojo et al. 2005).

Indeed, several studies have shown that oncolytic viruses could efficiently kill chemo-resistant CSCs. Eriksson *et al.* showed that an oncolytic adenovirus efficiently kills both differentiated and CSC populations isolated from BrCa cell lines (Eriksson, Guse et al. 2007). In this study the authors considered $CD44^{+}CD24^{-/low}$ cells as CSCs, and they also selected a CSC-enriched population (called the side population, SP) based on the ability of SP to exclude the fluorescent dye Hoechst 33342. Likewise, Marcato *et al.* have shown that their oncolytic reovirus is equally efficient in killing CSC and non-CSC populations of a xenografted breast tumor in immune-compromised mice (Marcato, Dean et al. 2009). Levels of activated Ras, a determinant of cellular susceptibility to oncolysis by reovirus, were found to be similar in sorted CSCs and non-sorted tumor cells (Strong, Coffey et al. 1998; Marcato, Shmulevitz et al. 2007; Marcato, Dean et al. 2009). $CD44^{+}CD24^{-/low}$ cells over expressing aldehyde dehydrogenase were considered as CSC in this study. Unlike some chemotherapeutic treatments that result in enrichment of the CSC population in tumors, intra-tumoral injection of the oncolytic reovirus was found to reduce both the CSC and non-CSC populations in the tumor, ultimately resulting in tumor regression (Dylla, Beviglia et al. 2008; Marcato, Dean et al. 2009; Rizzo, Hersey et al. 2011).

Furthermore, a study by Cuddington *et al.* has demonstrated the susceptibility of BrCa stem cells to an oncolytic herpes virus (Cuddington, Dyer et al. 2013). In this study the authors have shown that an oncolytic bovine herpesvirus type 1 efficiently kills both differentiated and stem-like cells isolated from BrCa cell lines. In this study Hoechst

33342 dye exclusion was solely used to isolate "stem-like cells". Interestingly, in this study the authors found that the virus efficiently killed cancer stem-like cells without undergoing productive infection, so the exact mechanism of cell death was not clear.

In line with all these studies, we have found that our *F4L*-deleted vaccinia virus has similar killing activities in CSC and non-CSC populations isolated from a BrCa cell line, SUM-149. In our study, like most other studies, we considered $CD44^{+}CD24^{-/low}$ cells as CSC. However, some studies have indicated that the expression patterns of CD44 and CD24 may not be reliably used for the identification of CSCs in BrCa (Jaggupilli and Elkord 2012; Liu, Nenutil et al. 2014; Zhong, Shen et al. 2014). For example, in contrast to many studies in which $CD44^{+}CD24^{-/low}$ cells were considered CSC in BrCa, a study by Wang *et al.* evaluating oncolytic properties of a mutant vaccinia virus in BrCa stem cells found aldehyde dehydrogenase positive $CD44^{+}CD24^{+/high}$ cells, isolated from the BrCa cell line GI-101A, to be more tumorigenic and more stem-like than aldehyde dehydrogenase negative $CD44^{+}CD24^{-/low}$ (Wang, Chen et al. 2012). Nonetheless, the oncolytic virus in this study was found to kill both stem-like (tumorigenic) and differentiated cells.

Because of the discrepancies in the use of CD24 and CD44 markers for BrCa stem cells, we also used the Hoechst 33342 exclusion method to isolate side and non-side population and then compared the growth of *F4L*-deleted virus, and the resulting cytotoxicities thereof. Interestingly, regardless of the method used to isolate CSC and non-CSC in our study, we found that our mutant virus grew to similar levels and resulted in similar cell killing *in vitro* in both the populations. Further experiments need to be done in order to determine if the virus equally kills CSC and non-CSC in *in vivo* tumors.

4.3 Future Directions

4.3.1 Increasing the Strength of the Mammaglobin Promoter Might Enhance the Anti-Tumor Effect of Ad-IL-2 Vector

Data presented in the 2nd chapter of this dissertation show that the MPE2 promoter is active specifically in BrCa cells *in vitro* and that the Ad vector encoding IL-2 under the control of MPE2 regulatory sequences is safe in mice. However, the expression of IL-2 driven by MPE2 regulatory sequences was found to be much lower than that driven by MCMV promoter *in vivo*. The weak expression of IL-2 may be responsible for the sub-optimal anti-tumor activity of the MPE2-IL-2 vector.

Strategies to further enhance the transcriptional strength of the MPE2 promoter in driving IL-2 expression might result in a better therapeutic outcome. Studies have shown that enhancer sequences (~240 bp) obtained from the early gene of SV40 virus can highly enhance the transcriptional strength of tissue/tumor specific promoters, such as the hTERT and human heparanase promoters, without compromising their specificity (Song 2004; Chen, Chen et al. 2013). Furthermore, Boshart *et al.* have previously reported that enhancer from the immediate early gene of human CMV has little cell-type or species specificity and its activity is much stronger than the SV40 enhancer (Boshart, Weber et al. 1985). This suggests that enhancer sequences from human CMV (400 bp) could possibly be used to enhance transcriptional strength of a tissue/tumor specific promoter. Indeed, Kurane *et al.* made a chimeric promoter by fusing the enhancer from human CMV upstream of the tissue/tumor specific promoter, carcinoembryonic antigen (CEA) promoter. This chimeric promoter was shown to be much stronger than the original CEA promoter in driving gene expression specifically in lung and colorectal cancer cells

(Kurane, Krauss et al. 1998). The small size of the CMV enhancer would allow incorporation of multiple such enhancers upstream of the mammaglobin promoter without exceeding the packaging capacity of the first generation adenovirus vectors. Therefore, it would be interesting to determine if addition of CMV enhancer elements to MPE2 or the minimal mammaglobin promoter could result in stronger expression of IL-2 and ultimately better therapeutic effect.

4.3.2 Breast Cancer-Specific Replication-competent Ad Vector Encoding Transcriptionally Targeted IL-2

Another strategy for increasing the IL-2 expression from an adenovirus vector could be to use replication-competent virus. A bi-cistronic expression cassette in which viral E1A and the IL-2 gene are controlled by a single mammaglobin (or MPE2) promoter through the use of an internal ribosome entry site (IRES) could be used to construct such a virus. Unlike, the first generation vector used in this study, this replication-competent virus should be able to not only produce more IL-2 but also kill cancer cells through direct lysis. However, safety of such a replicating virus armed with IL-2 would be a major concern, therefore, a strategy must be included to ensure that in the unlikely event of untoward virus growth and IL-2-related toxicity, both the virus growth and IL-2 expression can be inhibited. One strategy could be to insert *loxp* sequences at both ends of the cloned cassette (Ad sequence-loxp-MPE2→E1A-IRES-IL2-loxP-Ad-sequence) in the virus. A separate non-replicating adenovirus vector encoding *Cre*-recombinase could be administered which should allow recombination between the two *loxp* sites in the previous virus causing the release of the entire cloned

cassette ultimately stopping both the virus replication and IL-2 expression. Furthermore, cidofovir could also be used to inhibit replication of the virus.

4.3.3 Potential of F4L-mutant Oncolytic VACV in Controlling Metastatic Breast Cancer

Data presented in the 3rd chapter of this dissertation provides the proof-of-principle that *F4L*-deleted VACV has oncolytic potential against BrCa. However, in our study we have only tested the oncolytic effect of our viruses in controlling primary tumors using an intra-tumoral route of virus administration. Because treatment of metastatic BrCa is the major problem, our viruses should be tested for their ability to control metastatic disease. In order to facilitate virus' dissemination to the metastatic lesions, systemic delivery of the virus might be more desirable. MDA-MB-231 and 4T1 cells have been shown to form both primary and metastatic tumors efficiently after injection of the cells into mammary fat pad of mice (Aslakson and Miller 1992; Fantozzi and Christofori 2006). However, if the metastatic tumors are formed late, the primary tumor may grow too large, requiring the mice to be euthanized. As an alternative, the cells could be injected through the tail vein of the mice to ensure early establishment of metastasis (Zeng, Yang et al. 2010). Establishment of metastatic lesions could be confirmed by bioluminescence imaging if luciferase-tagged cancer cells are used. The route of choice of virus delivery would be intra-venous.

4.3.4 Arming F4L-mutant VACV with Cytokines to Enhance its Oncolytic Effect

Our study shows that *F4L*-deleted VACVs could completely control BrCa in a xenograft model, as does the *J2R*-deleted VACV. In a syngeneic mouse model, our viruses were able to significantly slow down the growth of tumors compared to control tumors, however, the anti-tumor effect of the viruses were less profound in the syngeneic model compared to that in the xenograft model. Our result is in accordance with a study by Guo *et al.*, who found that an oncolytic WR VACV lacking the anti-apoptotic gene *SPI-1* and *SPI-2*, was less potent in controlling colon tumors in syngeneic mouse model than in xenograft model (Guo, Naik et al. 2005). Likewise, another study by Hou *et al.*, found that an oncolytic WR VACV lacking the genes for viral TK and vaccinia growth factor, was better in controlling breast tumors in xenograft mouse model compared to that in the syngeneic model (Hou, Chen et al. 2014). Therefore, modifications in first generation oncolytic viruses need to be done in order to enhance their anti-tumor effect in immune-competent models. Several oncolytic viruses that are currently in clinical trials are armed with immune-modulatory cytokines in order to enhance their anti-tumor effect. For example, the oncolytic HSV T-Vec and the oncolytic VACV Pexa-Vec both encode the cytokine GM-CSF (Table 1.2). This cytokine is important in the stimulation of dendritic cells for antigen presentation (Sallusto and Lanzavecchia 1994). Interestingly, studies have shown that high concentrations of GM-CSF could cause immune-suppression rather than activation through the recruitment of myeloid derived suppressor cells (Serafini, Carbley et al. 2004; Marigo, Bosio et al. 2010).

IL-15 is another immuno-stimulatory cytokine with potential to be used in arming oncolytic viruses. Unlike, human GM-CSF, human IL-15 retains its biological activities

in mice which allow the use of murine models for studying the anti-tumor effect of the IL-15-armed oncolytic VACV (Nishijima, Nakahata et al. 1995). There are many mechanisms suggested for the anti-tumor activity of IL-15. Similar to GM-CSF, IL-15 can activate dendritic cells, which would enhance the presentation of tumor antigens to CD4+ and CD8+ T cells (Dubsky, Saito et al. 2007). Moreover, IL-15 has been shown to induce long term maintenance of memory T cells, and therefore, IL-15 could potentially minimize tumor recurrence by maintaining tumor-antigen specific memory T cells (Fehniger and Caligiuri 2001)**.** IL-15 can also induce the activation of natural killer (NK) cells. NK cells have the potential to inhibit tumor formation, metastasis and recurrence; therefore, IL-15 could exert NK cell-mediated strong anti-tumor effect (Terme, Ullrich et al. 2008; Liu, Engels et al. 2012). Because of these promising anti-tumor activities, recombinant IL-15 was recently approved for clinical trials for different malignancies (NCT01021059; 13-c-0045).

Unlike with adenovirus vectors, it is not feasible to transcriptionally target VACV-encoding IL-15 using cellular promoters, as the VACV completes its entire life-cycle within the cytoplasm of infected cells where specific transcription factors required for the tumor/tissue specific promoters are not available. Furthermore, IL-15 has been shown to have potent anti-viral activity resulting into rapid clearance of VACV encoding IL-15 transgene, in mice (Perera, Goldman et al. 2001; Foong, Jans et al. 2009). Therefore, it would be highly desirable to use a regulatable IL-15 whose function would be suppressed initially to allow maximal oncolysis by the virus followed by activation of IL-15 expression that would enhance the anti-tumor immunity. For instance, one such regulatable system was employed by Chen *et al.* to express IL-2 from oncolytic vaccinia

virus (Chen, Sampath et al. 2013). In their study, the authors expressed IL-2 fused to a protein-destabilizing domain that led to quick degradation of the nascent IL-2 in mice. However, administration of a cell permeable small molecule called shield-1 that specifically binds to the protein-destabilizing domain, was shown to protect the fusion protein from degradation and the biological activities of IL-2 was restored. It would be interesting to study if this regulatable system for conditional expression of IL-15 from the oncolytic VACV could increase the overall anti-tumor effect of the virus.

4.4 Conclusions

Engineered viruses have the potential to be used as cancer therapeutics. We have studied two different engineered DNA viruses for their safety and anti-cancer potency in the treatment of BrCa. Our data show that the MPE2 promoter has high specificity for BrCa cells and can be used for targeted expression of a reporter/therapeutic gene in cells of BrCa origin. Also, we have shown that an adenoviral vector expressing human IL-2 cDNA under the control of the MPE2 promoter is safe and exerts significant anti-tumor activity in murine models of BrCa. Attempts to enhance the anti-tumor activities of the Ad-MPE2-IL-2 vector with PAC1, which showed a very weak activity against BrCa *in vivo*, were unsuccessful. Further studies are required to determine whether other chemotherapeutics with strong apoptotic activity could enhance the anti-tumor activities of Ad-MPE2-IL-2. Likewise, we have shown that *F4L*-deleted vaccinia virus is highly attenuated in normal cells but robustly replicates in BrCa cells. While *F4L*-deleted oncolytic vaccinia viruses were able to completely control breast tumors in nude mice, their anti-tumor activities were found to be sub-optimal in syngeneic mouse models.

Further studies combining the *F4L*-deleted viruses with chemotherapeutics, or arming the viruses with immune-modulatory cytokines, are required to determine whether the anti-tumor activities of these oncolytic viruses could be further enhanced.

References

Addison, C. L., T. Braciak, et al. (1995). "Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model." Proc Natl Acad Sci U S A **92**(18): 8522-8526.

Addison, C. L., J. L. Bramson, et al. (1998). "Intratumoral coinjection of adenoviral vectors expressing IL-2 and IL-12 results in enhanced frequency of regression of injected and untreated distal tumors." Gene Ther **5**(10): 1400-1409.

Addison, C. L., M. Hitt, et al. (1997). "Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors." J Gen Virol **78 (Pt 7)**: 1653-1661.

Ahmad, A. (2013). "Pathways to breast cancer recurrence." ISRN Oncol **2013**: 290568.

Al-Hajj, M., M. S. Wicha, et al. (2003). "Prospective identification of tumorigenic breast cancer cells." Proc Natl Acad Sci U S A **100**(7): 3983-3988.

Al-Husein, B., M. Abdalla, et al. (2012). "Antiangiogenic therapy for cancer: an update." Pharmacotherapy **32**(12): 1095-1111.

Alvarez, M., M. N. Bouchlaka, et al. (2014). "Increased antitumor effects using IL-2 with anti-TGF-beta reveals competition between mouse NK and CD8 T cells." J Immunol **193**(4): 1709-1716.

Ambriovic-Ristov, A., J. Gabrilovac, et al. (2004). "Increased adenoviral transduction efficacy in human laryngeal carcinoma cells resistant to cisplatin is associated with increased expression of integrin alphavbeta3 and coxsackie adenovirus receptor." Int J Cancer **110**(5): 660-667.

Amer, M. H. (2014). "Gene therapy for cancer: present status and future perspective." Mol Cell Ther **2**: 27.

Anampa, J., D. Makower, et al. (2015). "Progress in adjuvant chemotherapy for breast cancer: an overview." BMC Med **13**: 195.

Anders, C. and L. A. Carey (2008). "Understanding and treating triple-negative breast cancer." Oncology (Williston Park) **22**(11): 1233-1239; discussion 1239-1240, 1243.

Anderson, B. D., T. Nakamura, et al. (2004). "High CD46 receptor density determines preferential killing of tumor cells by oncolytic measles virus." Cancer Res **64**(14): 4919-4926.

Anderson, D. D., C. M. Quintero, et al. (2011). "Identification of a de novo thymidylate biosynthesis pathway in mammalian mitochondria." Proc Natl Acad Sci U S A **108**(37): 15163-15168.

Andre, F. and C. C. Zielinski (2012). "Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents." Ann Oncol **23 Suppl 6**: vi46-51.

Andreansky, S., B. He, et al. (1998). "Treatment of intracranial gliomas in immunocompetent mice using herpes simplex viruses that express murine interleukins." Gene Ther **5**(1): 121-130.

Andrei, G., D. B. Gammon, et al. (2006). "Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice." J Virol **80**(19): 9391-9401.

Angarita, F. A., S. A. Acuna, et al. (2013). "Mounting a strategic offense: fighting tumor vasculature with oncolytic viruses." Trends Mol Med **19**(6): 378-392.

Apetoh, L., F. Ghiringhelli, et al. (2007). "Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy." Nat Med **13**(9): 1050-1059.

Arduino, S., M. Tessarolo, et al. (1996). "Reduced IL-2 level concentration in patients with breast cancer as a possible risk factor for relapse." Eur J Gynaecol Oncol **17**(6): 535-537.

Artenstein, A. W. (2008). "New generation smallpox vaccines: a review of preclinical and clinical data." Rev Med Virol **18**(4): 217-231.

Aslakson, C. J. and F. R. Miller (1992). "Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor." Cancer Res **52**(6): 1399-1405.

Atkins, M. B. (2002). "Interleukin-2: clinical applications." Semin Oncol **29**(3 Suppl 7): 12-17.

Atkins, M. B., M. Regan, et al. (2004). "Update on the role of interleukin 2 and other cytokines in the treatment of patients with stage IV renal carcinoma." Clin Cancer Res **10**(18 Pt 2): 6342S-6346S.

Aufderklamm, S., T. Todenhofer, et al. (2012). "Thymidine kinase and cancer monitoring." Cancer Lett **316**(1): 6-10.

Bai, F. L., Y. H. Yu, et al. (2014). "Genetically engineered Newcastle disease virus expressing interleukin-2 and TNF-related apoptosis-inducing ligand for cancer therapy." Cancer Biol Ther **15**(9): 1226-1238.

Bajszar, G., R. Wittek, et al. (1983). "Vaccinia virus thymidine kinase and neighboring genes: mRNAs and polypeptides of wild-type virus and putative nonsense mutants." J Virol **45**(1): 62-72.

Baker, R. O., M. Bray, et al. (2003). "Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections." Antiviral Res **57**(1-2): 13-23.

Baldick, C. J., Jr. and B. Moss (1993). "Characterization and temporal regulation of mRNAs encoded by vaccinia virus intermediate-stage genes." J Virol **67**(6): 3515-3527.

Baltimore, D. (1978). "Limiting science: a biologist's perspective." Daedalus **107**(2): 37-45.

Baluna, R. and E. S. Vitetta (1997). "Vascular leak syndrome: a side effect of immunotherapy." Immunopharmacology **37**(2-3): 117-132.

Balzar, M., M. J. Winter, et al. (1999). "The biology of the 17-1A antigen (Ep-CAM)." J Mol Med (Berl) **77**(10): 699-712.

Bannerji, R., C. D. Arroyo, et al. (1994). "The role of IL-2 secreted from genetically modified tumor cells in the establishment of antitumor immunity." J Immunol **152**(5): 2324-2332.

Barretina, J., G. Caponigro, et al. (2012). "The Cancer Cell Line Encyclopedia enables predictive modelling of anticancer drug sensitivity." Nature **483**(7391): 603-607.

Baselga, J., E. A. Perez, et al. (2006). "Adjuvant trastuzumab: a milestone in the treatment of HER-2-positive early breast cancer." Oncologist **11 Suppl 1**: 4-12.

Becker, J. C., J. D. Pancook, et al. (1996). "Eradication of human hepatic and pulmonary melanoma metastases in SCID mice by antibody-interleukin 2 fusion proteins." Proc Natl Acad Sci U S A **93**(7): 2702-2707.

Becker, J. C., N. Varki, et al. (1996). "An antibody-interleukin 2 fusion protein overcomes tumor heterogeneity by induction of a cellular immune response." Proc Natl Acad Sci U S A **93**(15): 7826-7831.

Bell, J. and G. McFadden (2014). "Viruses for tumor therapy." Cell Host Microbe **15**(3): 260-265.

Belldegrun, A., L. M. Muul, et al. (1988). "Interleukin 2 expanded tumor-infiltrating lymphocytes in human renal cell cancer: isolation, characterization, and antitumor activity." Cancer Res **48**(1): 206-214.

Belongia, E. A. and A. L. Naleway (2003). "Smallpox vaccine: the good, the bad, and the ugly." Clin Med Res **1**(2): 87-92.

Benencia, F., M. C. Courreges, et al. (2005). "Oncolytic HSV exerts direct antiangiogenic activity in ovarian carcinoma." Hum Gene Ther **16**(6): 765-778.

Bergelson, J. M., J. A. Cunningham, et al. (1997). "Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5." Science **275**(5304): 1320-1323.

Berget, S. M., C. Moore, et al. (1977). "Spliced segments at the 5' terminus of adenovirus 2 late mRNA." Proc Natl Acad Sci U S A **74**(8): 3171-3175.

Berk, A. J. (2007). Adenoviridae: The viruses and their replication. Fields Virology. H. D. Knipe, P., Ed. Philadelphia, Wolters Kluwer Health/Lippincott Williams & Wilkins**:** 2355-2394.

Berk, A. J. and D. A. Clayton (1973). "A genetically distinct thymidine kinase in mammalian mitochondria. Exclusive labeling of mitochondrial deoxyribonucleic acid." J Biol Chem **248**(8): 2722-2729.

Bernhard, H., M. J. Maeurer, et al. (1996). "Recognition of human renal cell carcinoma and melanoma by HLA-A2-restricted cytotoxic T lymphocytes is mediated by shared peptide epitopes and up-regulated by interferon-gamma." Scand J Immunol **44**(3): 285-292.

Bett, A. J., W. Haddara, et al. (1994). "An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3." Proc Natl Acad Sci U S A **91**(19): 8802-8806.

Bett, A. J., L. Prevec, et al. (1993). "Packaging capacity and stability of human adenovirus type 5 vectors." J Virol **67**(10): 5911-5921.

Biron, K. K. (2006). "Antiviral drugs for cytomegalovirus diseases." Antiviral Res **71**(2-3): 154-163.

Blaese, R. M., K. W. Culver, et al. (1995). "T lymphocyte-directed gene therapy for ADA-SCID: initial trial results after 4 years." Science **270**(5235): 475-480.

Blankenstein, T., P. G. Coulie, et al. (2012). "The determinants of tumour immunogenicity." Nat Rev Cancer **12**(4): 307-313.

Boone, R. F. and B. Moss (1978). "Sequence complexity and relative abundance of vaccinia virus mRNA's synthesized in vivo and in vitro." J Virol **26**(3): 554-569.

Boshart, M., F. Weber, et al. (1985). "A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus." Cell **41**(2): 521-530.

Bramson, J. L., M. Hitt, et al. (1997). "Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination." Gene Ther **4**(10): 1069-1076.

Bray, M., M. Martinez, et al. (2000). "Cidofovir protects mice against lethal aerosol or intranasal cowpox virus challenge." J Infect Dis **181**(1): 10-19.

Breitbach, C. J., R. Arulanandam, et al. (2013). "Oncolytic vaccinia virus disrupts tumor-associated vasculature in humans." Cancer Res **73**(4): 1265-1275.

Breitbach, C. J., J. Burke, et al. (2011). "Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans." Nature **477**(7362): 99-102.

Breitbach, C. J., N. S. De Silva, et al. (2011). "Targeting tumor vasculature with an oncolytic virus." Mol Ther **19**(5): 886-894.

Breitbach, C. J., J. M. Paterson, et al. (2007). "Targeted inflammation during oncolytic virus therapy severely compromises tumor blood flow." Mol Ther **15**(9): 1686-1693.

Brinster, R. L., J. M. Allen, et al. (1988). "Introns increase transcriptional efficiency in transgenic mice." Proc Natl Acad Sci U S A **85**(3): 836-840.

Brisken, C. and B. O'Malley (2010). "Hormone action in the mammary gland." Cold Spring Harb Perspect Biol **2**(12): a003178.

Britton, K. M., J. A. Kirby, et al. (2011). "Cancer stem cells and side population cells in breast cancer and metastasis." Cancers (Basel) **3**(2): 2106-2130.

Broet, P., S. Romain, et al. (2001). "Thymidine kinase as a proliferative marker: clinical relevance in 1,692 primary breast cancer patients." J Clin Oncol **19**(11): 2778-2787.

Buchman, A. R. and P. Berg (1988). "Comparison of intron-dependent and intron-independent gene expression." Mol Cell Biol **8**(10): 4395-4405.

Bui, L. A., L. H. Butterfield, et al. (1997). "In vivo therapy of hepatocellular carcinoma with a tumor-specific adenoviral vector expressing interleukin-2." Hum Gene Ther **8**(18): 2173-2182.

Buller, R. M. and G. J. Palumbo (1991). "Poxvirus pathogenesis." Microbiol Rev **55**(1): 80-122.

Burns, A. R., C. W. Smith, et al. (2003). "Unique structural features that influence neutrophil emigration into the lung." Physiol Rev **83**(2): 309-336.

Burstein, H. J. and M. Morrow (2015). "Nodal Irradiation after Breast-Cancer Surgery in the Era of Effective Adjuvant Therapy." N Engl J Med **373**(4): 379-381.

Cairns, J. (1960). "The initiation of vaccinia infection." Virology **11**: 603-623.

Cancer Genome Atlas, N. (2012). "Comprehensive molecular portraits of human breast tumours." Nature **490**(7418): 61-70.

Carey, L. A., E. C. Dees, et al. (2007). "The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes." Clin Cancer Res **13**(8): 2329-2334.

Cavallo, F., M. Giovarelli, et al. (1992). "Role of neutrophils and CD4+ T lymphocytes in the primary and memory response to nonimmunogenic murine mammary adenocarcinoma made immunogenic by IL-2 gene." J Immunol **149**(11): 3627-3635.

Cawood, R., H. H. Chen, et al. (2009). "Use of tissue-specific microRNA to control pathology of wild-type adenovirus without attenuation of its ability to kill cancer cells." PLoS Pathog **5**(5): e1000440.

Cen, H., F. Mao, et al. (2008). "DEVD-NucView488: a novel class of enzyme substrates for real-time detection of caspase-3 activity in live cells." FASEB J **22**(7): 2243-2252.

Chaffer, C. L., I. Brueckmann, et al. (2011). "Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state." Proc Natl Acad Sci U S A **108**(19): 7950-7955.

Chang, H. Y. and X. Yang (2000). "Proteases for cell suicide: functions and regulation of caspases." Microbiol Mol Biol Rev **64**(4): 821-846.

Chapman, J. R., A. C. Webster, et al. (2013). "Cancer in the transplant recipient." Cold Spring Harb Perspect Med **3**(7).

Charafe-Jauffret, E., C. Ginestier, et al. (2009). "Breast cancer cell lines contain functional cancer stem cells with metastatic capacity and a distinct molecular signature." Cancer Res **69**(4): 1302-1313.

Chaurasiya, S. and M. M. Hitt (2016). Adenoviral Vector Construction I: Mammalian Systems. Adenoviral Vectors for Gene Therapy D. T. Curiel. San Diego, Academic Press**:** 85-112.

Chen, B., X. P. Chen, et al. (2013). "Simian virus 40 enhancer does not affect the tumor specificity of human heparanase gene promoter." Biomed Rep **1**(1): 41-46.

Chen, H., P. Sampath, et al. (2013). "Regulating cytokine function enhances safety and activity of genetic cancer therapies." Mol Ther **21**(1): 167-174.

Chen, Y., P. Emtage, et al. (2001). "Induction of ErbB-2/neu-specific protective and therapeutic antitumor immunity using genetically modified dendritic cells: enhanced efficacy by cotransduction of gene encoding IL-12." Gene Ther **8**(4): 316-323.

Chimploy, K. and C. K. Mathews (2001). "Mouse ribonucleotide reductase control: influence of substrate binding upon interactions with allosteric effectors." J Biol Chem **276**(10): 7093-7100.

Choi, S. Y., D. Lin, et al. (2014). "Lessons from patient-derived xenografts for better in vitro modeling of human cancer." Adv Drug Deliv Rev **79-80**: 222-237.

Christ, O., S. Seiter, et al. (2001). "Efficacy of local versus systemic application of antibody-cytokine fusion proteins in tumor therapy." Clin Cancer Res **7**(4): 985-998.

Christgen, M., M. Ballmaier, et al. (2012). "Detection of putative cancer stem cells of the side population phenotype in human tumor cell cultures." Methods Mol Biol **878**: 201-215.

Chung, C. S., J. C. Hsiao, et al. (1998). "A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate." J Virol **72**(2): 1577-1585.

Ciccone, E., O. Viale, et al. (1988). "Antigen recognition by human T cell receptor gamma-positive lymphocytes. Specific lysis of allogeneic cells after activation in mixed lymphocyte culture." J Exp Med **167**(4): 1517-1522.

Ciruelos, E., T. Pascual, et al. (2014). "The therapeutic role of fulvestrant in the management of patients with hormone receptor-positive breast cancer." Breast **23**(3): 201-208.

Clarke, M. (2006). "Meta-analyses of adjuvant therapies for women with early breast cancer: the Early Breast Cancer Trialists' Collaborative Group overview." Ann Oncol **17 Suppl 10**: x59-62.

Clarke, M. F., J. E. Dick, et al. (2006). "Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells." Cancer Res **66**(19): 9339-9344.

Coffey, M. C., J. E. Strong, et al. (1998). "Reovirus therapy of tumors with activated Ras pathway." Science **282**(5392): 1332-1334.

Cohen, G. M. (1997). "Caspases: the executioners of apoptosis." Biochem J **326 (Pt 1)**: 1-16.

Cohen, M. H., Y. L. Shen, et al. (2009). "FDA drug approval summary: bevacizumab (Avastin) as treatment of recurrent glioblastoma multiforme." Oncologist **14**(11): 1131-1138.

Comeron, J. M. (2004). "Selective and mutational patterns associated with gene expression in humans: influences on synonymous composition and intron presence." Genetics **167**(3): 1293-1304.

Cong, Y. S., J. Wen, et al. (1999). "The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter." Hum Mol Genet **8**(1): 137-142.

Cordier, L., M. T. Duffour, et al. (1995). "Complete recovery of mice from a pre-established tumor by direct intratumoral delivery of an adenovirus vector harboring the murine IL-2 gene." Gene Ther **2**(1): 16-21.

Cotlar, A. M., J. J. Dubose, et al. (2003). "History of surgery for breast cancer: radical to the sublime." Curr Surg **60**(3): 329-337.

Cotrim, A. P. and B. J. Baum (2008). "Gene therapy: some history, applications, problems, and prospects." Toxicol Pathol **36**(1): 97-103.

Coventry, B. J. and M. L. Ashdown (2012). "The 20th anniversary of interleukin-2 therapy: bimodal role explaining longstanding random induction of complete clinical responses." Cancer Manag Res **4**: 215-221.

Creighton, C. J., X. Li, et al. (2009). "Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features." Proc Natl Acad Sci U S A **106**(33): 13820-13825.

Criscitiello, C. (2012). "Tumor-associated antigens in breast cancer." Breast Care (Basel) **7**(4): 262-266.

Criscitiello, C., A. Esposito, et al. (2014). "Immune approaches to the treatment of breast cancer, around the corner?" Breast Cancer Res **16**(1): 204.

Cuddington, B. P., A. L. Dyer, et al. (2013). "Oncolytic bovine herpesvirus type 1 infects and kills breast tumor cells and breast cancer-initiating cells irrespective of tumor subtype." Cancer Gene Ther **20**(5): 282-289.

D'Angiolella, V., V. Donato, et al. (2012). "Cyclin F-mediated degradation of ribonucleotide reductase M2 controls genome integrity and DNA repair." Cell **149**(5): 1023-1034.

Danthinne, X. and M. J. Imperiale (2000). "Production of first generation adenovirus vectors: a review." Gene Ther **7**(20): 1707-1714.

Dardousis, K., C. Voolstra, et al. (2007). "Identification of differentially expressed genes involved in the formation of multicellular tumor spheroids by HT-29 colon carcinoma cells." Mol Ther **15**(1): 94-102.

Das, S. K., M. E. Menezes, et al. (2015). "Gene Therapies for Cancer: Strategies, Challenges and Successes." J Cell Physiol **230**(2): 259-271.

Davis, C. B. and S. D. Gillies (2003). "Immunocytokines: amplification of anti-cancer immunity." Cancer Immunol Immunother **52**(5): 297-308.

Day, C. L., Jr., A. J. Sober, et al. (1981). "A prognostic model for clinical stage I melanoma of the upper extremity. The importance of anatomic subsites in predicting recurrent disease." Ann Surg **193**(4): 436-440.

De Clercq, E. (1996). "Therapeutic potential of Cidofovir (HPMPC, Vistide) for the treatment of DNA virus (i.e. herpes-, papova-, pox- and adenovirus) infections." Verh K Acad Geneeskd Belg **58**(1): 19-47; discussion 47-19.

De Clercq, E. (2002). "Cidofovir in the treatment of poxvirus infections." Antiviral Res **55**(1): 1-13.

Dean, M., T. Fojo, et al. (2005). "Tumour stem cells and drug resistance." Nat Rev Cancer **5**(4): 275-284.

Defer, C., M. T. Belin, et al. (1990). "Human adenovirus-host cell interactions: comparative study with members of subgroups B and C." J Virol **64**(8): 3661-3673.

Den Otter, W., J. J. Jacobs, et al. (2008). "Local therapy of cancer with free IL-2." Cancer Immunol Immunother **57**(7): 931-950.

DeRose, Y. S., G. Wang, et al. (2011). "Tumor grafts derived from women with breast cancer authentically reflect tumor pathology, growth, metastasis and disease outcomes." Nat Med **17**(11): 1514-1520.

Desilva, A., M. Wuest, et al. (2012). "Comparative functional evaluation of immunocompetent mouse breast cancer models established from PyMT-tumors using small animal PET with [(18)F]FDG and [(18)F]FLT." Am J Nucl Med Mol Imaging **2**(1): 88-98.

Devos, R., G. Plaetinck, et al. (1983). "Molecular cloning of human interleukin 2 cDNA and its expression in E. coli." Nucleic Acids Res **11**(13): 4307-4323.

Diehn, M., R. W. Cho, et al. (2009). "Association of reactive oxygen species levels and radioresistance in cancer stem cells." Nature **458**(7239): 780-783.

Dixon, L. K., D. A. Chapman, et al. (2013). "African swine fever virus replication and genomics." Virus Res **173**(1): 3-14.

Dmitriev, I., V. Krasnykh, et al. (1998). "An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism." J Virol **72**(12): 9706-9713.

Dock, G. (1904). "The influence of complicating diseases upon leukemia." Am J Med Sci **127**: 563–592.

Dono, M., P. Ferro, et al. (2009). "Molecular detection of human mammaglobin in cerebrospinal fluid from breast cancer patient with leptomeningeal carcinomatosis." J Neurooncol **91**(3): 295-298.

Douglas, J. T. (2007). "Adenoviral vectors for gene therapy." Mol Biotechnol **36**(1): 71-80.

Dranoff, G. (2004). "Cytokines in cancer pathogenesis and cancer therapy." Nat Rev Cancer **4**(1): 11-22.

Driss, A., K. O. Asare, et al. (2009). "Sickle Cell Disease in the Post Genomic Era: A Monogenic Disease with a Polygenic Phenotype." Genomics Insights **2009**(2): 23-48.

Dubsky, P., H. Saito, et al. (2007). "IL-15-induced human DC efficiently prime melanoma-specific naive CD8+ T cells to differentiate into CTL." Eur J Immunol **37**(6): 1678-1690.

Dufau, I., C. Frongia, et al. (2012). "Multicellular tumor spheroid model to evaluate spatio-temporal dynamics effect of chemotherapeutics: application to the gemcitabine/CHK1 inhibitor combination in pancreatic cancer." BMC Cancer **12**: 15.

Duxbury, M. S., H. Ito, et al. (2004). "RNA interference targeting the M2 subunit of ribonucleotide reductase enhances pancreatic adenocarcinoma chemosensitivity to gemcitabine." Oncogene **23**(8): 1539-1548.

Dylla, S. J., L. Beviglia, et al. (2008). "Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy." PLoS One **3**(6): e2428.

Early Breast Cancer Trialists' Collaborative, G. (2005). "Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials." Lancet **365**(9472): 1687-1717.

Edmondson, R., J. J. Broglie, et al. (2014). "Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors." Assay Drug Dev Technol **12**(4): 207-218.

Elmore, S. (2007). "Apoptosis: a review of programmed cell death." Toxicol Pathol **35**(4): 495-516.

Engstrom, Y., S. Eriksson, et al. (1985). "Cell cycle-dependent expression of mammalian ribonucleotide reductase. Differential regulation of the two subunits." J Biol Chem **260**(16): 9114-9116.

Epstein, A. L., M. M. Mizokami, et al. (2003). "Identification of a protein fragment of interleukin 2 responsible for vasopermeability." J Natl Cancer Inst **95**(10): 741-749.

Epstein, C. J., D. R. Cox, et al. (1983). "Recent developments in the prenatal diagnosis of genetic diseases and birth defects." Annu Rev Genet **17**: 49-83.

Eriksson, M., K. Guse, et al. (2007). "Oncolytic adenoviruses kill breast cancer initiating CD44+CD24-/low cells." Mol Ther **15**(12): 2088-2093.

Eroles, P., A. Bosch, et al. (2012). "Molecular biology in breast cancer: intrinsic subtypes and signaling pathways." Cancer Treat Rev **38**(6): 698-707.

Everts, B. and H. G. van der Poel (2005). "Replication-selective oncolytic viruses in the treatment of cancer." Cancer Gene Ther **12**(2): 141-161.

Evgin, L., M. Vaha-Koskela, et al. (2010). "Potent oncolytic activity of raccoonpox virus in the absence of natural pathogenicity." Mol Ther **18**(5): 896-902.

Fan, H., C. Villegas, et al. (1996). "Ribonucleotide reductase R2 component is a novel malignancy determinant that cooperates with activated oncogenes to determine transformation and malignant potential." Proc Natl Acad Sci U S A **93**(24): 14036-14040.

Fantozzi, A. and G. Christofori (2006). "Mouse models of breast cancer metastasis." Breast Cancer Res **8**(4): 212.

Fasullo, M., A. D. Burch, et al. (2009). "Hypoxia enhances the replication of oncolytic herpes simplex virus in p53- breast cancer cells." Cell Cycle **8**(14): 2194-2197.

Fearon, E. R., D. M. Pardoll, et al. (1990). "Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response." Cell **60**(3): 397-403.

Fehniger, T. A. and M. A. Caligiuri (2001). "Interleukin 15: biology and relevance to human disease." Blood **97**(1): 14-32.

Fenner, F. (1982). "A successful eradication campaign. Global eradication of smallpox." Rev Infect Dis **4**(5): 916-930.

Fenner, J. J. E. a. F. (2001). Poxviruses. Fields Virology. D. M. H. Knipe, P.M. Philadelphia, Williams & Wilkins.

Fillmore, C. M. and C. Kuperwasser (2008). "Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy." Breast Cancer Res **10**(2): R25.

Fink, D., H. Schlagbauer-Wadl, et al. (2001). "Elevated procaspase levels in human melanoma." Melanoma Res **11**(4): 385-393.

Fischer, K., A. C. von Brunneck, et al. (2014). "Differential expression of secretoglobins in normal ovary and in ovarian carcinoma--overexpression of mammaglobin-1 is linked to tumor progression." Arch Biochem Biophys **547**: 27-36.

Fisher, B., J. H. Jeong, et al. (2002). "Twenty-five-year follow-up of a randomized trial comparing radical mastectomy, total mastectomy, and total mastectomy followed by irradiation." N Engl J Med **347**(8): 567-575.

Fisher, R. I., S. A. Rosenberg, et al. (2000). "Long-term survival update for high-dose recombinant interleukin-2 in patients with renal cell carcinoma." Cancer J Sci Am **6 Suppl 1**: S55-57.

Foldvari, M., D. W. Chen, et al. (2015). "Non-viral gene therapy: Gains and challenges of non-invasive administration methods." J Control Release.

Foong, Y. Y., D. A. Jans, et al. (2009). "Interleukin-15 mediates potent antiviral responses via an interferon-dependent mechanism." Virology **393**(2): 228-237.

Francia, G., W. Cruz-Munoz, et al. (2011). "Mouse models of advanced spontaneous metastasis for experimental therapeutics." Nat Rev Cancer **11**(2): 135-141.

Franken, M., A. Estabrooks, et al. (1996). "Epstein-Barr virus-driven gene therapy for EBV-related lymphomas." Nat Med **2**(12): 1379-1382.

Friberg, S. and S. Mattson (1997). "On the growth rates of human malignant tumors: implications for medical decision making." J Surg Oncol **65**(4): 284-297.

Friedrich, J., R. Ebner, et al. (2007). "Experimental anti-tumor therapy in 3-D: spheroids--old hat or new challenge?" Int J Radiat Biol **83**(11-12): 849-871.

Furuta, E., H. Okuda, et al. (2010). "Metabolic genes in cancer: their roles in tumor progression and clinical implications." Biochim Biophys Acta **1805**(2): 141-152.

Gaffen, S. L. and K. D. Liu (2004). "Overview of interleukin-2 function, production and clinical applications." Cytokine **28**(3): 109-123.

Gammon, D. B., B. Gowrishankar, et al. (2010). "Vaccinia virus-encoded ribonucleotide reductase subunits are differentially required for replication and pathogenesis." PLoS Pathog **6**(7): e1000984.

Garber, K. (2006). "China approves world's first oncolytic virus therapy for cancer treatment." J Natl Cancer Inst **98**(5): 298-300.

Garber, K. (2009). "From human to mouse and back: 'tumorgraft' models surge in popularity." J Natl Cancer Inst **101**(1): 6-8.

Garon, C. F., K. W. Berry, et al. (1972). "A unique form of terminal redundancy in adenovirus DNA molecules." Proc Natl Acad Sci U S A **69**(9): 2391-2395.

Georgiades, J., T. Zielinski, et al. (1959). "Research on the oncolytic effect of APC viruses in cancer of the cervix uteri; preliminary report." Biul Inst Med Morsk Gdansk **10**: 49-57.

Gey, C., Coffman W and Kubicek, M (1952). "Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium." *Cancer Res* **12**: 264-265.

Gibson, L., D. Lawrence, et al. (2009). "Aromatase inhibitors for treatment of advanced breast cancer in postmenopausal women." Cochrane Database Syst Rev(4): CD003370.

Goedegebuure, P. S., M. A. Watson, et al. (2004). "Mammaglobin-based strategies for treatment of breast cancer." Curr Cancer Drug Targets **4**(6): 531-542.

Goldman, C. K., B. E. Rogers, et al. (1997). "Targeted gene delivery to Kaposi's sarcoma cells via the fibroblast growth factor receptor." Cancer Res **57**(8): 1447-1451.

Goran Akusjarvi, U. P. a. R. J. R. (1986). "Structure and function of the adenovirus-2 genome." Adenovirus DNA: The Viral Genome and Its Expression: 53-96.

Gorczynski, R. M., Z. Chen, et al. (2013). "Cure of metastatic growth of EMT6 tumor cells in mice following manipulation of CD200:CD200R signaling." Breast Cancer Res Treat **142**(2): 271-282.

Gordon, H. L., T. J. Bardos, et al. (1968). "Comparative study of the thymidine kinase and thymidylate kinase activities and of the feedbach inhibition of thymidine kinase in normal and neoplastic human tissue." Cancer Res **28**(10): 2068-2077.

Graham (1984). Transformation by and oncogenicity of human adenoviruses. . The adenoviruses. G. H. S. New York, N.Y, Plenum Press**:** pp. 339–398.

Gravekamp, C., R. Sypniewska, et al. (2004). "Behavior of metastatic and nonmetastatic breast tumors in old mice." Exp Biol Med (Maywood) **229**(7): 665-675.

Greig, S. L. (2016). "Talimogene Laherparepvec: First Global Approval." Drugs **76**(1): 147-154.

Grimm, E. A., A. Mazumder, et al. (1982). "Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin 2-activated autologous human peripheral blood lymphocytes." J Exp Med **155**(6): 1823-1841.

Gu, J., M. Andreeff, et al. (2002). "hTERT promoter induces tumor-specific Bax gene expression and cell killing in syngenic mouse tumor model and prevents systemic toxicity." Gene Ther **9**(1): 30-37.

Guo, Z. S., A. Naik, et al. (2005). "The enhanced tumor selectivity of an oncolytic vaccinia lacking the host range and antiapoptosis genes SPI-1 and SPI-2." Cancer Res **65**(21): 9991-9998.

Gupta, G. P. and J. Massague (2006). "Cancer metastasis: building a framework." Cell **127**(4): 679-695.

Gupta, P. B., C. M. Fillmore, et al. (2011). "Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells." Cell **146**(4): 633-644.

Guse, K., V. Cerullo, et al. (2011). "Oncolytic vaccinia virus for the treatment of cancer." Expert Opin Biol Ther **11**(5): 595-608.

Guse, K., M. Sloniecka, et al. (2010). "Antiangiogenic arming of an oncolytic vaccinia virus enhances antitumor efficacy in renal cell cancer models." J Virol **84**(2): 856-866.

Guy, C. T., R. D. Cardiff, et al. (1992). "Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease." Mol Cell Biol **12**(3): 954-961.

Guy, C. T., M. A. Webster, et al. (1992). "Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease." Proc Natl Acad Sci U S A **89**(22): 10578-10582.

Hagemann, I. S., J. D. Pfeifer, et al. (2013). "Mammaglobin expression in gynecologic adenocarcinomas." Hum Pathol **44**(4): 628-635.

Hagiwara, E., F. Abbasi, et al. (1995). "Phenotype and frequency of cells secreting IL-2, IL-4, IL-6, IL-10, IFN and TNF-alpha in human peripheral blood." Cytokine **7**(8): 815-822.

Han, Z. Q., M. Assenberg, et al. (2007). "Development of a second-generation oncolytic Herpes simplex virus expressing TNFalpha for cancer therapy." J Gene Med **9**(2): 99-106.

Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.

Harada, S., R. Yalamanchili, et al. (1998). "Residues 231 to 280 of the Epstein-Barr virus nuclear protein 2 are not essential for primary B-lymphocyte growth transformation." J Virol **72**(12): 9948-9954.

Hart, I. R. (1996). "Tissue specific promoters in targeting systemically delivered gene therapy." Semin Oncol **23**(1): 154-158.

Hartkopf, A. D., T. Fehm, et al. (2011). "Oncolytic virotherapy of breast cancer." Gynecol Oncol **123**(1): 164-171.

Hashiro, G., P. C. Loh, et al. (1977). "The preferential cytotoxicity of reovirus for certain transformed cell lines." Arch Virol **54**(4): 307-315.

Hay, J. G. (2005). "The potential impact of hypoxia on the success of oncolytic virotherapy." Curr Opin Mol Ther **7**(4): 353-358.

Hay, M., D. W. Thomas, et al. (2014). "Clinical development success rates for investigational drugs." Nat Biotechnol **32**(1): 40-51.

He, P., Z. Y. Tang, et al. (1999). "The targeted expression of the human interleukin-2/interferon alpha2b fused gene in alpha-fetoprotein-expressing hepatocellular carcinoma cells." J Cancer Res Clin Oncol **125**(2): 77-82.

Heiber, J. F. and G. N. Barber (2011). "Vesicular stomatitis virus expressing tumor suppressor p53 is a highly attenuated, potent oncolytic agent." J Virol **85**(20): 10440-10450.

Hengstschlager, M., M. Knofler, et al. (1994). "Different regulation of thymidine kinase during the cell cycle of normal versus DNA tumor virus-transformed cells." J Biol Chem **269**(19): 13836-13842.

Heo, J., T. Reid, et al. (2013). "Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer." Nat Med **19**(3): 329-336.

Herrmann, R., W. Fayad, et al. (2008). "Screening for compounds that induce apoptosis of cancer cells grown as multicellular spheroids." J Biomol Screen **13**(1): 1-8.

Higgins, M. J. and J. Baselga (2011). "Targeted therapies for breast cancer." J Clin Invest **121**(10): 3797-3803.

Higgins, M. J. and A. C. Wolff (2008). "Therapeutic options in the management of metastatic breast cancer." Oncology (Williston Park) **22**(6): 614-623; discussion 623, 627-619.

Hiley, C. T., M. Yuan, et al. (2010). "Lister strain vaccinia virus, a potential therapeutic vector targeting hypoxic tumours." Gene Ther **17**(2): 281-287.

Hiltunen, M. O., M. P. Turunen, et al. (2000). "Biodistribution of adenoviral vector to nontarget tissues after local in vivo gene transfer to arterial wall using intravascular and periadventitial gene delivery methods." FASEB J **14**(14): 2230-2236.

Hiraga, T., S. Ito, et al. (2011). "Side population in MDA-MB-231 human breast cancer cells exhibits cancer stem cell-like properties without higher bone-metastatic potential." Oncol Rep **25**(1): 289-296.

Hirschhaeuser, F., H. Menne, et al. (2010). "Multicellular tumor spheroids: an underestimated tool is catching up again." J Biotechnol **148**(1): 3-15.

Hitt, M. M., C. L. Addison, et al. (1997). "Human adenovirus vectors for gene transfer into mammalian cells." Adv Pharmacol **40**: 137-206.

Hitt, M. M. and J. Gauldie (2000). "Gene vectors for cytokine expression in vivo." Curr Pharm Des **6**(6): 613-632.

Hock, H., M. Dorsch, et al. (1993). "Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin 2, interleukin 4, interleukin 7, tumor necrosis factor, or interferon gamma." Proc Natl Acad Sci U S A **90**(7): 2774-2778.

Hoffmeyer, M. R., K. M. Wall, et al. (2005). "In vitro analysis of the invasive phenotype of SUM 149, an inflammatory breast cancer cell line." Cancer Cell Int **5**(1): 11.

HogenEsch, H. and A. Y. Nikitin (2012). "Challenges in pre-clinical testing of anti-cancer drugs in cell culture and in animal models." J Control Release **164**(2): 183-186.

Hollingshead, M. G. (2008). "Antitumor efficacy testing in rodents." J Natl Cancer Inst **100**(21): 1500-1510.

Horwitz, M. S. (1990). Adenoviridae: The viruses and their replication. D. M. K. "Virology" (B.N. Fields, *et al.*, Eds.) New York Raven Press 1679-1721.

Hoster, H. A., R. P. Zanes, Jr., et al. (1949). "Studies in Hodgkin's syndrome; the association of viral hepatitis and Hodgkin's disease; a preliminary report." Cancer Res **9**(8): 473-480.

Hou, W., H. Chen, et al. (2014). "Oncolytic vaccinia virus demonstrates antiangiogenic effects mediated by targeting of VEGF." Int J Cancer **135**(5): 1238-1246.

Howes, A. L., G. G. Chiang, et al. (2007). "The phosphatidylinositol 3-kinase inhibitor, PX-866, is a potent inhibitor of cancer cell motility and growth in three-dimensional cultures." Mol Cancer Ther **6**(9): 2505-2514.

Hruby, D. E., D. L. Lynn, et al. (1979). "Vaccinia virus replication requires active participation of the host cell transcriptional apparatus." Proc Natl Acad Sci U S A **76**(4): 1887-1890.

Hsiao, J. C., C. S. Chung, et al. (1999). "Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells." J Virol **73**(10): 8750-8761.

Hu, Z., C. Fan, et al. (2006). "The molecular portraits of breast tumors are conserved across microarray platforms." BMC Genomics **7**: 96.

Huard, J., H. Lochmuller, et al. (1995). "The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants." Gene Ther **2**(2): 107-115.

Hudis, C. A. and C. T. Dang (2004). "Adjuvant therapy for breast cancer: practical lessons from the early breast cancer trialists' collaborative group." Breast Dis **21**: 3-13.

Hughes, J., P. Wang, et al. (2015). "Lister strain vaccinia virus with thymidine kinase gene deletion is a tractable platform for development of a new generation of oncolytic virus." Gene Ther **22**(6): 476-484.

Hughes, R. M. (2004). "Strategies for cancer gene therapy." J Surg Oncol **85**(1): 28-35.

Huh, D., G. A. Hamilton, et al. (2011). "From 3D cell culture to organs-on-chips." Trends Cell Biol **21**(12): 745-754.

Hummel, J. L., E. Safroneeva, et al. (2005). "The role of ICP0-Null HSV-1 and interferon signaling defects in the effective treatment of breast adenocarcinoma." Mol Ther **12**(6): 1101-1110.

Ichihashi, Y. (1996). "Extracellular enveloped vaccinia virus escapes neutralization." Virology **217**(2): 478-485.

Itoi, T., A. Sofuni, et al. (2007). "Ribonucleotide reductase subunit M2 mRNA expression in pretreatment biopsies obtained from unresectable pancreatic carcinomas." J Gastroenterol **42**(5): 389-394.

Itsumi, M. and K. Tatsugami (2010). "Immunotherapy for renal cell carcinoma." Clin Dev Immunol **2010**: 284581.

Jaggupilli, A. and E. Elkord (2012). "Significance of CD44 and CD24 as cancer stem cell markers: an enduring ambiguity." Clin Dev Immunol **2012**: 708036.

Janiszewska, A. D., S. Poletajew, et al. (2013). "Spontaneous regression of renal cell carcinoma." Contemp Oncol (Pozn) **17**(2): 123-127.

Jared D. Evans, P. H. (2002). "Adenovirus replication". In *Adenoviral vectors for gene therapy.* D. T. Curiel. USA, Academic Press**:** 39-70.

Jelovac, D. and A. C. Wolff (2012). "The adjuvant treatment of HER2-positive breast cancer." Curr Treat Options Oncol **13**(2): 230-239.

Jemal, A., M. M. Center, et al. (2010). "Global patterns of cancer incidence and mortality rates and trends." Cancer Epidemiol Biomarkers Prev **19**(8): 1893-1907.

Jemal, A., R. Siegel, et al. (2010). "Cancer statistics, 2010." CA Cancer J Clin **60**(5): 277-300.

Jensen, P., S. Hansen, et al. (1999). "Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens." J Am Acad Dermatol **40**(2 Pt 1): 177-186.

Jensen, R. A., D. L. Page, et al. (1994). "Identification of genes expressed in premalignant breast disease by microscopy-directed cloning." Proc Natl Acad Sci U S A **91**(20): 9257-9261.

Jin, X. and P. Mu (2015). "Targeting Breast Cancer Metastasis." Breast Cancer (Auckl) **9**(Suppl 1): 23-34.

Jogler, C., D. Hoffmann, et al. (2006). "Replication properties of human adenovirus in vivo and in cultures of primary cells from different animal species." J Virol **80**(7): 3549-3558.

Johnson, J. I., S. Decker, et al. (2001). "Relationships between drug activity in NCI preclinical in vitro and in vivo models and early clinical trials." Br J Cancer **84**(10): 1424-1431.

Johnson, L. F., L. G. Rao, et al. (1982). "Regulation of thymidine kinase enzyme level in serum-stimulated mouse 3T6 fibroblasts." Exp Cell Res **138**(1): 79-85.

Joklik, W. K. and Y. Becker (1964). "The Replication and Coating of Vaccinia DNA." J Mol Biol **10**: 452-474.

Jones, S. E. (2008). "Metastatic breast cancer: the treatment challenge." Clin Breast Cancer **8**(3): 224-233.

Jung, J. (2014). "Human tumor xenograft models for preclinical assessment of anticancer drug development." Toxicol Res **30**(1): 1-5.

Kanai, R., H. Wakimoto, et al. (2011). "A novel oncolytic herpes simplex virus that synergizes with phosphoinositide 3-kinase/Akt pathway inhibitors to target glioblastoma stem cells." Clin Cancer Res **17**(11): 3686-3696.

Kane, R. C., A. T. Farrell, et al. (2009). "Sorafenib for the treatment of unresectable hepatocellular carcinoma." Oncologist **14**(1): 95-100.

Katsafanas, G. C. and B. Moss (2007). "Colocalization of transcription and translation within cytoplasmic poxvirus factories coordinates viral expression and subjugates host functions." Cell Host Microbe **2**(4): 221-228.

Kaufman, H. L. (2012). "Vaccines for melanoma and renal cell carcinoma." Semin Oncol **39**(3): 263-275.

Kaufman, H. L., F. J. Kohlhapp, et al. (2015). "Oncolytic viruses: a new class of immunotherapy drugs." Nat Rev Drug Discov **14**(9): 642-662.

Kaur, P., G. M. Nagaraja, et al. (2012). "A mouse model for triple-negative breast cancer tumor-initiating cells (TNBC-TICs) exhibits similar aggressive phenotype to the human disease." BMC Cancer **12**: 120.

Keck, J. G., C. J. Baldick, Jr., et al. (1990). "Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes." Cell **61**(5): 801-809.

Kelkar, S. A., K. K. Pfister, et al. (2004). "Cytoplasmic dynein mediates adenovirus binding to microtubules." J Virol **78**(18): 10122-10132.

Kelly, E. and S. J. Russell (2007). "History of oncolytic viruses: genesis to genetic engineering." Mol Ther **15**(4): 651-659.

Kim, H. S., Y. J. Sung, et al. (2015). "Cancer Cell Line Panels Empower Genomics-Based Discovery of Precision Cancer Medicine." Yonsei Med J **56**(5): 1186-1198.

Kim, J. B. (2005). "Three-dimensional tissue culture models in cancer biology." Semin Cancer Biol **15**(5): 365-377.

Kim, J. H., J. Y. Oh, et al. (2006). "Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF." Mol Ther **14**(3): 361-370.

Kim, M., K. R. Zinn, et al. (2002). "The therapeutic efficacy of adenoviral vectors for cancer gene therapy is limited by a low level of primary adenovirus receptors on tumour cells." Eur J Cancer **38**(14): 1917-1926.

Kim, N. W., M. A. Piatyszek, et al. (1994). "Specific association of human telomerase activity with immortal cells and cancer." Science **266**(5193): 2011-2015.

Kirn, D. H. and S. H. Thorne (2009). "Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer." Nat Rev Cancer **9**(1): 64-71.

Kirn, D. H., Y. Wang, et al. (2007). "Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus." PLoS Med **4**(12): e353.

Klonisch, T., E. Wiechec, et al. (2008). "Cancer stem cell markers in common cancers - therapeutic implications." Trends Mol Med **14**(10): 450-460.

Knipe, D. M. H., P.M. (2001). Fields Virology. Philadelphia, Lippincott Williams & Wilkins Publishers.

Knop, D. R. and H. Harrell (2007). "Bioreactor production of recombinant herpes simplex virus vectors." Biotechnol Prog **23**(3): 715-721.

Koga, S., S. Hirohata, et al. (2001). "FADD gene therapy using the human telomerase catalytic subunit (hTERT) gene promoter to restrict induction of apoptosis to tumors in vitro and in vivo." Anticancer Res **21**(3B): 1937-1943.

Kojaoghlanian, T., P. Flomenberg, et al. (2003). "The impact of adenovirus infection on the immunocompromised host." Rev Med Virol **13**(3): 155-171.

Kola, I. and J. Landis (2004). "Can the pharmaceutical industry reduce attrition rates?" Nat Rev Drug Discov **3**(8): 711-715.

Komata, T., Y. Kondo, et al. (2001). "Treatment of malignant glioma cells with the transfer of constitutively active caspase-6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) gene promoter." Cancer Res **61**(15): 5796-5802.

Komenaka, I., H. Hoerig, et al. (2004). "Immunotherapy for melanoma." Clin Dermatol **22**(3): 251-265.

Konrad, M. W., G. Hemstreet, et al. (1990). "Pharmacokinetics of recombinant interleukin 2 in humans." Cancer Res **50**(7): 2009-2017.

Kovacs, G. R., N. Vasilakis, et al. (2001). "Regulation of viral intermediate gene expression by the vaccinia virus B1 protein kinase." J Virol **75**(9): 4048-4055.

Krepela, E., J. Prochazka, et al. (2004). "Increased expression of Apaf-1 and procaspase-3 and the functionality of intrinsic apoptosis apparatus in non-small cell lung carcinoma." Biol Chem **385**(2): 153-168.

Kretschmer, C., A. Sterner-Kock, et al. (2011). "Identification of early molecular markers for breast cancer." Mol Cancer **10**(1): 15.

Kundig, T. M., H. Schorle, et al. (1993). "Immune responses in interleukin-2-deficient mice." Science **262**(5136): 1059-1061.

Kurane, S., J. C. Krauss, et al. (1998). "Targeted gene transfer for adenocarcinoma using a combination of tumor-specific antibody and tissue-specific promoter." Jpn J Cancer Res **89**(11): 1212-1219.

Kurnick, J. T., K. O. Gronvik, et al. (1979). "Long term growth in vitro of human T cell blasts with maintenance of specificity and function." J Immunol **122**(4): 1255-1260.

La Rosa, A. M., R. E. Champlin, et al. (2001). "Adenovirus infections in adult recipients of blood and marrow transplants." Clin Infect Dis **32**(6): 871-876.

Laliberte, J. P. and B. Moss (2009). "Appraising the apoptotic mimicry model and the role of phospholipids for poxvirus entry." Proc Natl Acad Sci U S A **106**(41): 17517-17521.

Lapidot, T., C. Sirard, et al. (1994). "A cell initiating human acute myeloid leukaemia after transplantation into SCID mice." Nature **367**(6464): 645-648.

Laurent, J., C. Touvrey, et al. (2013). "T-cell activation by treatment of cancer patients with EMD 521873 (Selectikine), an IL-2/anti-DNA fusion protein." J Transl Med **11**: 5.

Lechner, M. G., S. S. Karimi, et al. (2013). "Immunogenicity of murine solid tumor models as a defining feature of in vivo behavior and response to immunotherapy." J Immunother **36**(9): 477-489.

Lee, J., B. M. Fenton, et al. (1998). "Interleukin 2 expression by tumor cells alters both the immune response and the tumor microenvironment." Cancer Res **58**(7): 1478-1485.

Lee, Y., A. Vassilakos, et al. (2003). "GTI-2040, an antisense agent targeting the small subunit component (R2) of human ribonucleotide reductase, shows potent antitumor activity against a variety of tumors." Cancer Res **63**(11): 2802-2811.

Lenardo, M. J. (1991). "Interleukin-2 programs mouse alpha beta T lymphocytes for apoptosis." Nature **353**(6347): 858-861.

Levine, P. H., S. C. Steinhorn, et al. (1985). "Inflammatory breast cancer: the experience of the surveillance, epidemiology, and end results (SEER) program." J Natl Cancer Inst **74**(2): 291-297.

Lewis, A. M., Jr., S. G. Baum, et al. (1966). "Occurrence of adenovirus-SV40 hybrids among monkey kidney cell adapted strains of adenovirus." Proc Soc Exp Biol Med **122**(1): 214-218.

Lewis, A. M., Jr. and W. P. Rowe (1970). "Isolation of two plaque variants from the adenovirus type 2-simian virus 40 hybrid population which differ in their efficiency in yielding simian virus 40." J Virol **5**(4): 413-420.

Li, X., M. T. Lewis, et al. (2008). "Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy." J Natl Cancer Inst **100**(9): 672-679.

Li, Y., R. C. Pong, et al. (1999). "Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy." Cancer Res **59**(2): 325-330.

Liedtke, C. and A. Rody (2015). "New treatment strategies for patients with triple-negative breast cancer." Curr Opin Obstet Gynecol **27**(1): 77-84.

Lin, Z. P., M. F. Belcourt, et al. (2004). "Stable suppression of the R2 subunit of ribonucleotide reductase by R2-targeted short interference RNA sensitizes p53(-/-) HCT-116 colon cancer cells to DNA-damaging agents and ribonucleotide reductase inhibitors." J Biol Chem **279**(26): 27030-27038.

Liu, B. L., M. Robinson, et al. (2003). "ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties." Gene Ther **10**(4): 292-303.

Liu, H., J. H. Naismith, et al. (2003). "Adenovirus DNA replication." Curr Top Microbiol Immunol **272**: 131-164.

Liu, R. B., B. Engels, et al. (2012). "Densely granulated murine NK cells eradicate large solid tumors." Cancer Res **72**(8): 1964-1974.

Liu, T. C., T. Hwang, et al. (2008). "The targeted oncolytic poxvirus JX-594 demonstrates antitumoral, antivascular, and anti-HBV activities in patients with hepatocellular carcinoma." Mol Ther **16**(9): 1637-1642.

Liu, T. C., S. H. Thorne, et al. (2008). "Oncolytic adenoviruses for cancer gene therapy." Methods Mol Biol **433**: 243-258.

Liu, X., H. Zhang, et al. (2013). "Ribonucleotide reductase small subunit M2 serves as a prognostic biomarker and predicts poor survival of colorectal cancers." Clin Sci (Lond) **124**(9): 567-578.

Liu, Y., R. Nenutil, et al. (2014). "Lack of correlation of stem cell markers in breast cancer stem cells." Br J Cancer **110**(8): 2063-2071.

Lo, H. W., C. P. Day, et al. (2005). "Cancer-specific gene therapy." Adv Genet **54**: 235-255.

Lode, H. N., R. Xiang, et al. (1998). "Natural killer cell-mediated eradication of neuroblastoma metastases to bone marrow by targeted interleukin-2 therapy." Blood **91**(5): 1706-1715.

Lu, J., P. S. Steeg, et al. (2009). "Breast cancer metastasis: challenges and opportunities." Cancer Res **69**(12): 4951-4953.

Lun, X., J. Chan, et al. (2010). "Efficacy and safety/toxicity study of recombinant vaccinia virus JX-594 in two immunocompetent animal models of glioma." Mol Ther **18**(11): 1927-1936.

Lun, X., Y. Ruan, et al. (2013). "Double-deleted vaccinia virus in virotherapy for refractory and metastatic pediatric solid tumors." Mol Oncol **7**(5): 944-954.
Ma, J. and D. J. Waxman (2008). "Combination of antiangiogenesis with chemotherapy for more effective cancer treatment." Mol Cancer Ther **7**(12): 3670-3684.
Ma, X. J., R. Salunga, et al. (2003). "Gene expression profiles of human breast cancer progression." Proc Natl Acad Sci U S A **100**(10): 5974-5979.
Mackett, M. and L. C. Archard (1979). "Conservation and variation in Orthopoxvirus genome structure." J Gen Virol **45**(3): 683-701.
Magee, W. C., K. A. Aldern, et al. (2008). "Cidofovir and (S)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine are highly effective inhibitors of vaccinia virus DNA polymerase when incorporated into the template strand." Antimicrob Agents Chemother **52**(2): 586-597.
Magee, W. C., K. Y. Hostetler, et al. (2005). "Mechanism of inhibition of vaccinia virus DNA polymerase by cidofovir diphosphate." Antimicrob Agents Chemother **49**(8): 3153-3162.
Maglione, J. E., D. Moghanaki, et al. (2001). "Transgenic Polyoma middle-T mice model premalignant mammary disease." Cancer Res **61**(22): 8298-8305.
Malhotra, G. K., X. Zhao, et al. (2010). "Histological, molecular and functional subtypes of breast cancers." Cancer Biol Ther **10**(10): 955-960.
Mallon, E., P. Osin, et al. (2000). "The basic pathology of human breast cancer." J Mammary Gland Biol Neoplasia **5**(2): 139-163.
Marcato, P., C. A. Dean, et al. (2009). "Oncolytic reovirus effectively targets breast cancer stem cells." Mol Ther **17**(6): 972-979.
Marcato, P., M. Shmulevitz, et al. (2007). "Ras transformation mediates reovirus oncolysis by enhancing virus uncoating, particle infectivity, and apoptosis-dependent release." Mol Ther **15**(8): 1522-1530.
Marigo, I., E. Bosio, et al. (2010). "Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor." Immunity **32**(6): 790-802.
Martin, T. A., G. Watkins, et al. (2005). "The Coxsackie-adenovirus receptor has elevated expression in human breast cancer." Clin Exp Med **5**(3): 122-128.
Martuza, R. L., A. Malick, et al. (1991). "Experimental therapy of human glioma by means of a genetically engineered virus mutant." Science **252**(5007): 854-856.
Masson, D., A. Jarry, et al. (2001). "Overexpression of the CD155 gene in human colorectal carcinoma." Gut **49**(2): 236-240.
Massung, R. F., L. I. Liu, et al. (1994). "Analysis of the complete genome of smallpox variola major virus strain Bangladesh-1975." Virology **201**(2): 215-240.
Mathews, C. K. (2006). "DNA precursor metabolism and genomic stability." FASEB J **20**(9): 1300-1314.
Mautner, V., V. Steinthorsdottir, et al. (1995). "Enteric adenoviruses." Curr Top Microbiol Immunol **199 (Pt 3)**: 229-282.
McCart, J. A., J. M. Ward, et al. (2001). "Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes." Cancer Res **61**(24): 8751-8757.

McConnell, M. J. and M. J. Imperiale (2004). "Biology of adenovirus and its use as a vector for gene therapy." Hum Gene Ther **15**(11): 1022-1033.
McCrudden, C. M. and H. O. McCarthy (2014). "Current status of gene therapy for breast cancer: progress and challenges." Appl Clin Genet **7**: 209-220.
McDermott, S. P. and M. S. Wicha (2010). "Targeting breast cancer stem cells." Mol Oncol **4**(5): 404-419.
McFadden, G. (2005). "Poxvirus tropism." Nat Rev Microbiol **3**(3): 201-213.
Mehta, G., A. Y. Hsiao, et al. (2012). "Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy." J Control Release **164**(2): 192-204.
Menard, S., G. Tomasic, et al. (1997). "Lymphoid infiltration as a prognostic variable for early-onset breast carcinomas." Clin Cancer Res **3**(5): 817-819.
Merchlinsky, M. and B. Moss (1989). "Resolution of vaccinia virus DNA concatemer junctions requires late-gene expression." J Virol **63**(4): 1595-1603.
Mi, Q., J. M. Pezzuto, et al. (2009). "Use of the in vivo hollow fiber assay in natural products anticancer drug discovery." J Nat Prod **72**(3): 573-580.
Mittendorf, E. A., G. E. Peoples, et al. (2007). "Breast cancer vaccines: promise for the future or pipe dream?" Cancer **110**(8): 1677-1686.
Moore, A. (1952). " Viruses with oncolytic properties and their adaptation in tumours." Ann N Y Acad Sci USA **54**: 945–952.
Moore, M., N. Horikoshi, et al. (1996). "Oncogenic potential of the adenovirus E4orf6 protein." Proc Natl Acad Sci U S A **93**(21): 11295-11301.
Moran, E. (1993). "Interaction of adenoviral proteins with pRB and p53." FASEB J **7**(10): 880-885.
Moran, E. and M. B. Mathews (1987). "Multiple functional domains in the adenovirus E1A gene." Cell **48**(2): 177-178.
Morgan, D. A., F. W. Ruscetti, et al. (1976). "Selective in vitro growth of T lymphocytes from normal human bone marrows." Science **193**(4257): 1007-1008.
Morikawa, T., D. Maeda, et al. (2010). "Ribonucleotide reductase M2 subunit is a novel diagnostic marker and a potential therapeutic target in bladder cancer." Histopathology **57**(6): 885-892.
Moss, B. (2007). Poxviridae: the viruses and their replicaton. Fields Virology. D. M. H. Knipe, P.M. Philadelphia, Williams & Wilkins**:** 2905–2946.
Moss, B., B. Y. Ahn, et al. (1991). "Cytoplasmic transcription system encoded by vaccinia virus." J Biol Chem **266**(3): 1355-1358.
Moyer RW, A. B., Black DN, et al. (2000). Poxviridae. Virus taxonomy: Classification and nomenclature of viruses. F. C. Murphy FA, Bishop DHL, et and e. al. San Diego, Academic Press,**:** 137–157.
Mullen, J. T. and K. K. Tanabe (2002). "Viral oncolysis." Oncologist **7**(2): 106-119.
Myron Kauffman, H., M. A. McBride, et al. (2002). "Transplant tumor registry: donor related malignancies." Transplantation **74**(3): 358-362.
Nabholtz, J. M. and J. Gligorov (2005). "The role of taxanes in the treatment of breast cancer." Expert Opin Pharmacother **6**(7): 1073-1094.

Nafissi, N., C. H. Sum, et al. (2014). "Optimization of a one-step heat-inducible in vivo mini DNA vector production system." PLoS One **9**(2): e89345.

Nakagawa, K., F. N. Miller, et al. (1996). "Mechanisms of interleukin-2-induced hepatic toxicity." Cancer Res **56**(3): 507-510.

Narayanan, K., A. Jaramillo, et al. (2004). "Response of established human breast tumors to vaccination with mammaglobin-A cDNA." J Natl Cancer Inst **96**(18): 1388-1396.

Nayerossadat, N., T. Maedeh, et al. (2012). "Viral and nonviral delivery systems for gene delivery." Adv Biomed Res **1**: 27.

Nelson, B. H. and D. M. Willerford (1998). "Biology of the interleukin-2 receptor." Adv Immunol **70**: 1-81.

Nettelbeck, D. M., V. Jerome, et al. (1998). "A strategy for enhancing the transcriptional activity of weak cell type-specific promoters." Gene Ther **5**(12): 1656-1664.

Nguyen, L. V., R. Vanner, et al. (2012). "Cancer stem cells: an evolving concept." Nat Rev Cancer **12**(2): 133-143.

Nicklin, S. A., K. L. Dishart, et al. (2003). "Transductional and transcriptional targeting of cancer cells using genetically engineered viral vectors." Cancer Lett **201**(2): 165-173.

Nishijima, I., T. Nakahata, et al. (1995). "A human GM-CSF receptor expressed in transgenic mice stimulates proliferation and differentiation of hemopoietic progenitors to all lineages in response to human GM-CSF." Mol Biol Cell **6**(5): 497-508.

Nishikawa, M. and L. Huang (2001). "Nonviral vectors in the new millennium: delivery barriers in gene transfer." Hum Gene Ther **12**(8): 861-870.

Niu, D. K. and Y. F. Yang (2011). "Why eukaryotic cells use introns to enhance gene expression: splicing reduces transcription-associated mutagenesis by inhibiting topoisomerase I cutting activity." Biol Direct **6**: 24.

Nordlund, P. and P. Reichard (2006). "Ribonucleotide reductases." Annu Rev Biochem **75**: 681-706.

Nott, A., H. Le Hir, et al. (2004). "Splicing enhances translation in mammalian cells: an additional function of the exon junction complex." Genes Dev **18**(2): 210-222.

Nott, A., S. H. Meislin, et al. (2003). "A quantitative analysis of intron effects on mammalian gene expression." RNA **9**(5): 607-617.

O'Brien, T. P., B. H. Jeng, et al. (2009). "Acute conjunctivitis: truth and misconceptions." Curr Med Res Opin **25**(8): 1953-1961.

O'Donovan, N., J. Crown, et al. (2003). "Caspase 3 in breast cancer." Clin Cancer Res **9**(2): 738-742.

O'Neill, K. L., M. R. Buckwalter, et al. (2001). "Thymidine kinase: diagnostic and prognostic potential." Expert Rev Mol Diagn **1**(4): 428-433.

O'Neill, K. L., M. Hoper, et al. (1992). "Can thymidine kinase levels in breast tumors predict disease recurrence?" J Natl Cancer Inst **84**(23): 1825-1828.

Ostapchuk, P. and P. Hearing (2003). "Minimal cis-acting elements required for adenovirus genome packaging." J Virol **77**(9): 5127-5135.

Ottewell, P. D., R. E. Coleman, et al. (2006). "From genetic abnormality to metastases: murine models of breast cancer and their use in the development of anticancer therapies." Breast Cancer Res Treat **96**(2): 101-113.

Pan, C. X. and K. S. Koeneman (1999). "A novel tumor-specific gene therapy for bladder cancer." Med Hypotheses **53**(2): 130-135.

Pancook, J. D., J. C. Becker, et al. (1996). "Eradication of established hepatic human neuroblastoma metastases in mice with severe combined immunodeficiency by antibody-targeted interleukin-2." Cancer Immunol Immunother **42**(2): 88-92.

Parato, K. A., C. J. Breitbach, et al. (2012). "The oncolytic poxvirus JX-594 selectively replicates in and destroys cancer cells driven by genetic pathways commonly activated in cancers." Mol Ther **20**(4): 749-758.

Pardoll, D. M. (2012). "The blockade of immune checkpoints in cancer immunotherapy." Nat Rev Cancer **12**(4): 252-264.

Parker, J. N., G. Y. Gillespie, et al. (2000). "Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors." Proc Natl Acad Sci U S A **97**(5): 2208-2213.

Parker, R. F., L. H. Bronson, et al. (1941). "Further Studies of the Infectious Unit of Vaccinia." J Exp Med **74**(3): 263-281.

Payne, L. G. (1980). "Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia." J Gen Virol **50**(1): 89-100.

Pelner, L., G. A. Fowler, et al. (1958). "Effects of concurrent infections and their toxins on the course of leukemia." Acta Med Scand Suppl **338**: 1-47.

Penichet, M. L., J. S. Dela Cruz, et al. (2001). "A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/neu expressing tumors." Hum Antibodies **10**(1): 43-49.

Penn, I. (1988). "Tumors of the immunocompromised patient." Annu Rev Med **39**: 63-73.

Perera, L. P., C. K. Goldman, et al. (2001). "Comparative assessment of virulence of recombinant vaccinia viruses expressing IL-2 and IL-15 in immunodeficient mice." Proc Natl Acad Sci U S A **98**(9): 5146-5151.

Perou, C. M., T. Sorlie, et al. (2000). "Molecular portraits of human breast tumours." Nature **406**(6797): 747-752.

Perry, D. K., M. J. Smyth, et al. (1997). "Zinc is a potent inhibitor of the apoptotic protease, caspase-3. A novel target for zinc in the inhibition of apoptosis." J Biol Chem **272**(30): 18530-18533.

Peterson, Q. P., D. R. Goode, et al. (2009). "PAC-1 activates procaspase-3 in vitro through relief of zinc-mediated inhibition." J Mol Biol **388**(1): 144-158.

Peterson, Q. P., D. C. Hsu, et al. (2010). "Discovery and canine preclinical assessment of a nontoxic procaspase-3-activating compound." Cancer Res **70**(18): 7232-7241.

Phillips, T. M., W. H. McBride, et al. (2006). "The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation." J Natl Cancer Inst **98**(24): 1777-1785.

Picot, N., R. Guerrette, et al. (2015). "Mammaglobin 1 promotes breast cancer malignancy and confers sensitivity to anticancer drugs." Mol Carcinog.

Pierce, W. E., M. J. Rosenbaum, et al. (1968). "Live and inactivated adenovirus vaccines for the prevention of acute respiratory illness in naval recruits." Am J Epidemiol **87**(1): 237-246.

Pilder, S., M. Moore, et al. (1986). "The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs." Mol Cell Biol **6**(2): 470-476.

Pipiya, T., H. Sauthoff, et al. (2005). "Hypoxia reduces adenoviral replication in cancer cells by downregulation of viral protein expression." Gene Ther **12**(11): 911-917.

Plosker, G. L. and S. Noble (1999). "Cidofovir: a review of its use in cytomegalovirus retinitis in patients with AIDS." Drugs **58**(2): 325-345.

Pol, J., A. Buque, et al. (2016). "Trial Watch-Oncolytic viruses and cancer therapy." Oncoimmunology **5**(2): e1117740.

Politi, K. and W. Pao (2011). "How genetically engineered mouse tumor models provide insights into human cancers." J Clin Oncol **29**(16): 2273-2281.

Polyak, K. (2007). "Breast cancer: origins and evolution." J Clin Invest **117**(11): 3155-3163.

Polyak, K. (2011). "Heterogeneity in breast cancer." J Clin Invest **121**(10): 3786-3788.

Porteus, M. H., J. P. Connelly, et al. (2006). "A look to future directions in gene therapy research for monogenic diseases." PLoS Genet **2**(9): e133.

Prestwich, R. J., K. J. Harrington, et al. (2008). "Oncolytic viruses: a novel form of immunotherapy." Expert Rev Anticancer Ther **8**(10): 1581-1588.

Prichard, M. N., K. A. Keith, et al. (2007). "Selective phosphorylation of antiviral drugs by vaccinia virus thymidine kinase." Antimicrob Agents Chemother **51**(5): 1795-1803.

Puhlmann, M., C. K. Brown, et al. (2000). "Vaccinia as a vector for tumor-directed gene therapy: biodistribution of a thymidine kinase-deleted mutant." Cancer Gene Ther **7**(1): 66-73.

Putt, K. S., G. W. Chen, et al. (2006). "Small-molecule activation of procaspase-3 to caspase-3 as a personalized anticancer strategy." Nat Chem Biol **2**(10): 543-550.

Putzer, B. M., J. L. Bramson, et al. (1998). "Combination therapy with interleukin-2 and wild-type p53 expressed by adenoviral vectors potentiates tumor regression in a murine model of breast cancer." Hum Gene Ther **9**(5): 707-718.

Qian, C., X. Y. Liu, et al. (2006). "Therapy of cancer by cytokines mediated by gene therapy approach." Cell Res **16**(2): 182-188.

Qin, L., N. Favis, et al. (2015). "Evolution of and evolutionary relationships between extant vaccinia virus strains." J Virol **89**(3): 1809-1824.

Qin, L., C. Upton, et al. (2011). "Genomic analysis of the vaccinia virus strain variants found in Dryvax vaccine." J Virol **85**(24): 13049-13060.

Raguz, S. and E. Yague (2008). "Resistance to chemotherapy: new treatments and novel insights into an old problem." Br J Cancer **99**(3): 387-391.

Ramos-Vara, J. A. (2005). "Technical aspects of immunohistochemistry." Vet Pathol **42**(4): 405-426.

Rao, B. M., I. Driver, et al. (2004). "Interleukin 2 (IL-2) variants engineered for increased IL-2 receptor alpha-subunit affinity exhibit increased potency arising from a cell surface ligand reservoir effect." Mol Pharmacol **66**(4): 864-869.

Rao, V. S., C. E. Dyer, et al. (2006). "Potential prognostic and therapeutic roles for cytokines in breast cancer (Review)." Oncol Rep **15**(1): 179-185.

Reichard, P. and B. Estborn (1951). "Utilization of desoxyribosides in the synthesis of polynucleotides." J Biol Chem **188**(2): 839-846.

Reis-Filho, J. S. and L. Pusztai (2011). "Gene expression profiling in breast cancer: classification, prognostication, and prediction." Lancet **378**(9805): 1812-1823.

Rizzo, S., J. M. Hersey, et al. (2011). "Ovarian cancer stem cell-like side populations are enriched following chemotherapy and overexpress EZH2." Mol Cancer Ther **10**(2): 325-335.

Rockwell, S. C., R. F. Kallman, et al. (1972). "Characteristics of a serially transplanted mouse mammary tumor and its tissue-culture-adapted derivative." J Natl Cancer Inst **49**(3): 735-749.

Roder, G., O. Keil, et al. (2003). "Novel cGMP liposomal vectors mediate efficient gene transfer." Cancer Gene Ther **10**(4): 312-317.

Rofstad, E. K. (1995). "Metastatic behavior of human tumors in congenitally athymic nude mice: intrinsic properties of the tumor cells and host immune reactivity." Int J Cancer **63**(5): 744-749.

Rofstad, E. K. and H. Lyng (1996). "Xenograft model systems for human melanoma." Mol Med Today **2**(9): 394-403.

Rogers, S., A. Lowenthal, et al. (1973). "Induction of arginase activity with the Shope papilloma virus in tissue culture cells from an argininemic patient." J Exp Med **137**(4): 1091-1096.

Rosenberg, S. A. (1996). "Development of cancer immunotherapies based on identification of the genes encoding cancer regression antigens." J Natl Cancer Inst **88**(22): 1635-1644.

Rosenberg, S. A. (2014). "IL-2: the first effective immunotherapy for human cancer." J Immunol **192**(12): 5451-5458.

Rosenberg, S. A., M. T. Lotze, et al. (1989). "Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients." Ann Surg **210**(4): 474-484; discussion 484-475.

Rosenberg, S. A., J. J. Mule, et al. (1985). "Regression of established pulmonary metastases and subcutaneous tumor mediated by the systemic administration of high-dose recombinant interleukin 2." J Exp Med **161**(5): 1169-1188.

Rosenfeld, M. E. and D. T. Curiel (1996). "Gene therapy strategies for novel cancer therapeutics." Curr Opin Oncol **8**(1): 72-77.

Roth, J. A. and R. J. Cristiano (1997). "Gene therapy for cancer: what have we done and where are we going?" J Natl Cancer Inst **89**(1): 21-39.

Rowe, W. P., R. J. Huebner, et al. (1953). "Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture." Proc Soc Exp Biol Med **84**(3): 570-573.

Rubinchik, S., J. S. Norris, et al. (2002). "Construction, purification and characterization of adenovirus vectors expressing apoptosis-inducing transgenes." Methods Enzymol **346**: 529-547.

Russell, S. J., K. W. Peng, et al. (2012). "Oncolytic virotherapy." Nat Biotechnol **30**(7): 658-670.

Sabzevari, H., S. D. Gillies, et al. (1994). "A recombinant antibody-interleukin 2 fusion protein suppresses growth of hepatic human neuroblastoma metastases in severe combined immunodeficiency mice." Proc Natl Acad Sci U S A **91**(20): 9626-9630.

Sadeghi, H. and M. M. Hitt (2005). "Transcriptionally targeted adenovirus vectors." Curr Gene Ther **5**(4): 411-427.

Saito, Y., M. Sunamura, et al. (2006). "Oncolytic replication-competent adenovirus suppresses tumor angiogenesis through preserved E1A region." Cancer Gene Ther **13**(3): 242-252.

Sallusto, F. and A. Lanzavecchia (1994). "Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha." J Exp Med **179**(4): 1109-1118.

Salzman, N. P. (1960). "The rate of formation of vaccinia deoxyribonucleic acid and vaccinia virus." Virology **10**: 150-152.

Sanjuan, X., P. L. Fernandez, et al. (1996). "Overexpression of the 67-kD laminin receptor correlates with tumour progression in human colorectal carcinoma." J Pathol **179**(4): 376-380.

Sausville, E. A. and A. M. Burger (2006). "Contributions of human tumor xenografts to anticancer drug development." Cancer Res **66**(7): 3351-3354, discussion 3354.

Savas, P., R. Salgado, et al. (2016). "Clinical relevance of host immunity in breast cancer: from TILs to the clinic." Nat Rev Clin Oncol **13**(4): 228-241.

Schoenenberger, C. A., A. C. Andres, et al. (1988). "Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription." EMBO J **7**(1): 169-175.

Senzer, N. N., H. L. Kaufman, et al. (2009). "Phase II clinical trial of a granulocyte-macrophage colony-stimulating factor-encoding, second-generation oncolytic herpesvirus in patients with unresectable metastatic melanoma." J Clin Oncol **27**(34): 5763-5771.

Serafini, P., R. Carbley, et al. (2004). "High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells." Cancer Res **64**(17): 6337-6343.

Shabalina, S. A., A. Y. Ogurtsov, et al. (2010). "Distinct patterns of expression and evolution of intronless and intron-containing mammalian genes." Mol Biol Evol **27**(8): 1745-1749.

Shay, J. W. (1995). "Aging and cancer: are telomeres and telomerase the connection?" Mol Med Today **1**(8): 378-384.

Shen, B. H. and T. W. Hermiston (2005). "Effect of hypoxia on Ad5 infection, transgene expression and replication." Gene Ther **12**(11): 902-910.

Shen, Y. and J. Nemunaitis (2005). "Fighting cancer with vaccinia virus: teaching new tricks to an old dog." Mol Ther **11**(2): 180-195.

SHENK, T. (1996). Adenoviridae: The viruses and their replication. In *Virology.* B.N. Fields, D.M. Knipe, and P.M. Howley, eds., Lippincott-Raven**:** 2111–2148.

Shi, C. X., F. L. Graham, et al. (2006). "A convenient plasmid system for construction of helper-dependent adenoviral vectors and its application for analysis of the breast-cancer-specific mammaglobin promoter." J Gene Med **8**(4): 442-451.

Shi, C. X., M. A. Long, et al. (2004). "The human SCGB2A2 (mammaglobin-1) promoter/enhancer in a helper-dependent adenovirus vector directs high levels of transgene expression in mammary carcinoma cells but not in normal nonmammary cells." Mol Ther **10**(4): 758-767.

Shtrichman, R. and T. Kleinberger (1998). "Adenovirus type 5 E4 open reading frame 4 protein induces apoptosis in transformed cells." J Virol **72**(4): 2975-2982.

Siegel, J. P. and R. K. Puri (1991). "Interleukin-2 toxicity." J Clin Oncol **9**(4): 694-704.

Simonian, S. Z., T. P. Antonova, et al. (1979). "[Analysis of vaccinia virus genome with restriction endonucleases EcoRI, BamHI, KpnI and HindIII]." Mol Biol (Mosk) **13**(6): 1255-1261.

Siolas, D. and G. J. Hannon (2013). "Patient-derived tumor xenografts: transforming clinical samples into mouse models." Cancer Res **73**(17): 5315-5319.

Slabaugh, M., N. Roseman, et al. (1988). "Vaccinia virus-encoded ribonucleotide reductase: sequence conservation of the gene for the small subunit and its amplification in hydroxyurea-resistant mutants." J Virol **62**(2): 519-527.

Slamon, D., W. Eiermann, et al. (2011). "Adjuvant trastuzumab in HER2-positive breast cancer." N Engl J Med **365**(14): 1273-1283.

Slos, P., M. De Meyer, et al. (2001). "Immunotherapy of established tumors in mice by intratumoral injection of an adenovirus vector harboring the human IL-2 cDNA: induction of CD8(+) T-cell immunity and NK activity." Cancer Gene Ther **8**(5): 321-332.

Smith, A. L., T. P. Robin, et al. (2012). "Molecular pathways: targeting the TGF-beta pathway for cancer therapy." Clin Cancer Res **18**(17): 4514-4521.

Smith, G. L., A. de Carlos, et al. (1989). "Vaccinia virus encodes a thymidylate kinase gene: sequence and transcriptional mapping." Nucleic Acids Res **17**(19): 7581-7590.

Smith, G. L. and M. Law (2004). "The exit of vaccinia virus from infected cells." Virus Res **106**(2): 189-197.

Smith, G. L. and B. Moss (1983). "Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA." Gene **25**(1): 21-28.

Smith, G. L., B. J. Murphy, et al. (2003). "Vaccinia virus motility." Annu Rev Microbiol **57**: 323-342.

Smith, G. L. and A. Vanderplasschen (1998). "Extracellular enveloped vaccinia virus. Entry, egress, and evasion." Adv Exp Med Biol **440**: 395-414.

Smith, G. L., A. Vanderplasschen, et al. (2002). "The formation and function of extracellular enveloped vaccinia virus." J Gen Virol **83**(Pt 12): 2915-2931.

Smith, T. T., J. C. Roth, et al. (2014). "Oncolytic viral therapy: targeting cancer stem cells." Oncolytic Virother **2014**(3): 21-33.

Sneider, T. W. and V. R. Potter (1969). "Alternative de novo and "salvage" pathways to thymidine triphosphate synthesis: possible implications for cancer chemotherapy." Cancer Res **29**(12): 2398-2403.

Sodeik, B., R. W. Doms, et al. (1993). "Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks." J Cell Biol **121**(3): 521-541.

Song, J. S. (2004). "Activity of the human telomerase catalytic subunit (hTERT) gene promoter could be increased by the SV40 enhancer." Biosci Biotechnol Biochem **68**(8): 1634-1639.

Sorlie, T., C. M. Perou, et al. (2001). "Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications." Proc Natl Acad Sci U S A **98**(19): 10869-10874.

Sorlie, T., Y. Wang, et al. (2006). "Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms." BMC Genomics **7**: 127.

Souglakos, J., I. Boukovinas, et al. (2008). "Ribonucleotide reductase subunits M1 and M2 mRNA expression levels and clinical outcome of lung adenocarcinoma patients treated with docetaxel/gemcitabine." Br J Cancer **98**(10): 1710-1715.

Southam, C. M. (1960). "Present status of oncolytic virus studies." Trans N Y Acad Sci **22**: 657-673.

Southam, C. M. and A. E. Moore (1952). "Clinical studies of viruses as antineoplastic agents with particular reference to Egypt 101 virus." Cancer **5**(5): 1025-1034.

Stewart, T. H. (1996). "Evidence for immune facilitation of breast cancer growth and for the immune promotion of oncogenesis in breast cancer." Medicina (B Aires) **56 Suppl 1**: 13-24.

Stojdl, D. F., B. Lichty, et al. (2000). "Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus." Nat Med **6**(7): 821-825.

Strong, J. E., M. C. Coffey, et al. (1998). "The molecular basis of viral oncolysis: usurpation of the Ras signaling pathway by reovirus." EMBO J **17**(12): 3351-3362.

Suminami, Y., E. M. Elder, et al. (1995). "In situ interleukin-4 gene expression in cancer patients treated with genetically modified tumor vaccine." J Immunother Emphasis Tumor Immunol **17**(4): 238-248.

Suzuki, T., F. Sakurai, et al. (2008). "miR-122a-regulated expression of a suicide gene prevents hepatotoxicity without altering antitumor effects in suicide gene therapy." Mol Ther **16**(10): 1719-1726.

Tanaka, Y., K. D. Amos, et al. (2003). "Mammaglobin-A is a tumor-associated antigen in human breast carcinoma." Surgery **133**(1): 74-80.

Taniguchi, T., H. Matsui, et al. (1983). "Structure and expression of a cloned cDNA for human interleukin-2." Nature **302**(5906): 305-310.

Tao, N., G. P. Gao, et al. (2001). "Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver." Mol Ther **3**(1): 28-35.

Tatum, E. L. (1966). "Molecular biology, nucleic acids, and the future of medicine." Perspect Biol Med **10**(1): 19-32.

Temin, H. M. (1961). "Mixed infection with two types of Rous sarcoma virus." Virology **13**: 158-163.

Tengelsen, L. A., M. B. Slabaugh, et al. (1988). "Nucleotide sequence and molecular genetic analysis of the large subunit of ribonucleotide reductase encoded by vaccinia virus." Virology **164**(1): 121-131.

Tentler, J. J., A. C. Tan, et al. (2012). "Patient-derived tumour xenografts as models for oncology drug development." Nat Rev Clin Oncol **9**(6): 338-350.

Terheggen, H. G., A. Lowenthal, et al. (1975). "Unsuccessful trial of gene replacement in arginase deficiency." Z Kinderheilkd **119**(1): 1-3.

Terme, M., E. Ullrich, et al. (2008). "Natural killer cell-directed therapies: moving from unexpected results to successful strategies." Nat Immunol **9**(5): 486-494.

Thelander, L. and P. Reichard (1979). "Reduction of ribonucleotides." Annu Rev Biochem **48**: 133-158.

Thirunavukarasu, P., M. Sathaiah, et al. (2013). "A rationally designed A34R mutant oncolytic poxvirus: improved efficacy in peritoneal carcinomatosis." Mol Ther **21**(5): 1024-1033.

Thomas, G. P. and M. B. Mathews (1980). "DNA replication and the early to late transition in adenovirus infection." Cell **22**(2 Pt 2): 523-533.

Thorne, S. H., T. H. Hwang, et al. (2007). "Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963." J Clin Invest **117**(11): 3350-3358.

Tollefson, A. E., J. S. Ryerse, et al. (1996). "The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants." Virology **220**(1): 152-162.

Toloza, E. M., K. Hunt, et al. (1996). "In vivo cancer gene therapy with a recombinant interleukin-2 adenovirus vector." Cancer Gene Ther **3**(1): 11-17.

Toneff, M. J., Z. Du, et al. (2010). "Somatic expression of PyMT or activated ErbB2 induces estrogen-independent mammary tumorigenesis." Neoplasia **12**(9): 718-726.

Torre, L. A., F. Bray, et al. (2015). "Global cancer statistics, 2012." CA Cancer J Clin **65**(2): 87-108.

Treves, S., P. L. Trentini, et al. (1994). "Apoptosis is dependent on intracellular zinc and independent of intracellular calcium in lymphocytes." Exp Cell Res **211**(2): 339-343.

Truong-Tran, A. Q., R. E. Ruffin, et al. (2000). "Visualization of labile zinc and its role in apoptosis of primary airway epithelial cells and cell lines." Am J Physiol Lung Cell Mol Physiol **279**(6): L1172-1183.

Tulman, E. R., G. Delhon, et al. (2006). "Genome of horsepox virus." J Virol **80**(18): 9244-9258.

Turner, G. S. (1982). "Jenner's smallpox vaccine-the riddle of vaccinia, virus and its origin by Derrick Baxby, Heineman Educational Books Ltd, 1981. pound8.50 (xiii + 214 pages) ISBN 0 435 54047 2." Immunol Today **3**(1): 26.

Vaage, J. (1988). "Local interleukin 2 therapy of mouse mammary tumors of various immunogenicities." Cancer Res **48**(8): 2193-2197.

Vaha-Koskela, M. J., J. E. Heikkila, et al. (2007). "Oncolytic viruses in cancer therapy." Cancer Lett **254**(2): 178-216.

Vallis, K. A. and I. F. Tannock (2004). "Postoperative radiotherapy for breast cancer: growing evidence for an impact on survival." J Natl Cancer Inst **96**(2): 88-89.

Vanderplasschen, A., E. Mathew, et al. (1998). "Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope." Proc Natl Acad Sci U S A **95**(13): 7544-7549.

Verbanac, K. M., C. J. Min, et al. (2010). "Long-term follow-up study of a prospective multicenter sentinel node trial: molecular detection of breast cancer sentinel node metastases." Ann Surg Oncol **17 Suppl 3**: 368-377.

Vile, R., N. Miller, et al. (1994). "A comparison of the properties of different retroviral vectors containing the murine tyrosinase promoter to achieve transcriptionally targeted expression of the HSVtk or IL-2 genes." Gene Ther **1**(5): 307-316.

Vogelzang, N. J., S. I. Benowitz, et al. (2012). "Clinical cancer advances 2011: Annual Report on Progress Against Cancer from the American Society of Clinical Oncology." J Clin Oncol **30**(1): 88-109.

Wack, S., S. Rejiba, et al. (2008). "Telomerase transcriptional targeting of inducible Bax/TRAIL gene therapy improves gemcitabine treatment of pancreatic cancer." Mol Ther **16**(2): 252-260.

Wadell, G., M. L. Hammarskjold, et al. (1980). "Genetic variability of adenoviruses." Ann N Y Acad Sci **354**: 16-42.

Wang, H., N. G. Chen, et al. (2012). "Oncolytic vaccinia virus GLV-1h68 strain shows enhanced replication in human breast cancer stem-like cells in comparison to breast cancer cells." J Transl Med **10**: 167.

Wang, K., T. Guan, et al. (2000). "Regulation of adenovirus membrane penetration by the cytoplasmic tail of integrin beta5." J Virol **74**(6): 2731-2739.

Wang, L. M., F. F. Lu, et al. (2012). "Overexpression of catalytic subunit M2 in patients with ovarian cancer." Chin Med J (Engl) **125**(12): 2151-2156.

Wang, X. (2001). "The expanding role of mitochondria in apoptosis." Genes Dev **15**(22): 2922-2933.

Wang, Y., J. K. Hu, et al. (2003). "Systemic dissemination of viral vectors during intratumoral injection." Mol Cancer Ther **2**(11): 1233-1242.

Watson, M. A., C. Darrow, et al. (1998). "Structure and transcriptional regulation of the human mammaglobin gene, a breast cancer associated member of the uteroglobin gene family localized to chromosome 11q13." Oncogene **16**(6): 817-824.

Watson, M. A., S. Dintzis, et al. (1999). "Mammaglobin expression in primary, metastatic, and occult breast cancer." Cancer Res **59**(13): 3028-3031.

Watson, M. A. and T. P. Fleming (1994). "Isolation of differentially expressed sequence tags from human breast cancer." Cancer Res **54**(17): 4598-4602.

Watson, M. A. and T. P. Fleming (1996). "Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer." Cancer Res **56**(4): 860-865.

Weibel, S., V. Raab, et al. (2011). "Viral-mediated oncolysis is the most critical factor in the late-phase of the tumor regression process upon vaccinia virus infection." BMC Cancer **11**: 68.

Weller, T. H., F. C. Robbins, et al. (1949). "Cultivation of poliomyelitis virus in cultures of human foreskin and embryonic tissues." Proc Soc Exp Biol Med **72**(1): 153-155.

White, J., R. Achuthan, et al. (2011). "Breast conservation surgery: state of the art." Int J Breast Cancer **2011**: 107981.

Wilding, J. L. and W. F. Bodmer (2014). "Cancer cell lines for drug discovery and development." Cancer Res **74**(9): 2377-2384.

Williamson, J. D., R. W. Reith, et al. (1990). "Biological characterization of recombinant vaccinia viruses in mice infected by the respiratory route." J Gen Virol **71 (Pt 11)**: 2761-2767.

Wittek, R. (2006). "Vaccinia immune globulin: current policies, preparedness, and product safety and efficacy." Int J Infect Dis **10**(3): 193-201.

Wolff, A. C., M. E. Hammond, et al. (2014). "Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update." Arch Pathol Lab Med **138**(2): 241-256.

Wolfson, J. and D. Dressler (1972). "Adenovirus-2 DNA contains an inverted terminal repetition." Proc Natl Acad Sci U S A **69**(10): 3054-3057.

Xiang, R., H. N. Lode, et al. (1997). "Elimination of established murine colon carcinoma metastases by antibody-interleukin 2 fusion protein therapy." Cancer Res **57**(21): 4948-4955.

Xiong, B., L. Ma, et al. (2014). "Characterization of side population cells isolated from the colon cancer cell line SW480." Int J Oncol **45**(3): 1175-1183.

Yang, G., D. C. Pevear, et al. (2005). "An orally bioavailable antipoxvirus compound (ST-246) inhibits extracellular virus formation and protects mice from lethal orthopoxvirus Challenge." J Virol **79**(20): 13139-13149.

Yang, J., S. A. Mani, et al. (2004). "Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis." Cell **117**(7): 927-939.

Yang, J. C., J. J. Mule, et al. (1986). "Murine lymphokine-activated killer (LAK) cells: phenotypic characterization of the precursor and effector cells." J Immunol **137**(2): 715-722.

Yang, Z., L. Maruri-Avidal, et al. (2013). "Cascade regulation of vaccinia virus gene expression is modulated by multistage promoters." Virology **447**(1-2): 213-220.

Ye, Z., X. Wang, et al. (2006). "Oncolytic adenovirus-mediated E1A gene therapy induces tumor-cell apoptosis and reduces tumor angiogenesis leading to inhibition of hepatocellular carcinoma growth in animal model." Cancer Biother Radiopharm **21**(3): 225-234.

Yla-Herttuala, S. (2012). "Endgame: glybera finally recommended for approval as the first gene therapy drug in the European union." Mol Ther **20**(10): 1831-1832.

Yron, I., T. A. Wood, Jr., et al. (1980). "In vitro growth of murine T cells. V. The isolation and growth of lymphoid cells infiltrating syngeneic solid tumors." J Immunol **125**(1): 238-245.

Yu, Y., G. Ramena, et al. (2012). "The role of cancer stem cells in relapse of solid tumors." Front Biosci (Elite Ed) **4**: 1528-1541.

Yun, H. J., Y. H. Cho, et al. (2008). "Transcriptional targeting of gene expression in breast cancer by the promoters of protein regulator of cytokinesis 1 and ribonuclease reductase 2." Exp Mol Med **40**(3): 345-353.

Zeh, H. J., S. Downs-Canner, et al. (2015). "First-in-man study of western reserve strain oncolytic vaccinia virus: safety, systemic spread, and antitumor activity." Mol Ther **23**(1): 202-214.

Zehentner, B. K. and D. Carter (2004). "Mammaglobin: a candidate diagnostic marker for breast cancer." Clin Biochem **37**(4): 249-257.

Zeng, Q., Z. Yang, et al. (2010). "Treating triple-negative breast cancer by a combination of rapamycin and cyclophosphamide: an in vivo bioluminescence imaging study." Eur J Cancer **46**(6): 1132-1143.

Zhang, H., X. Liu, et al. (2014). "Prognostic and therapeutic significance of ribonucleotide reductase small subunit M2 in estrogen-negative breast cancers." BMC Cancer **14**: 664.

Zhang, Q. X., M. Nakhaei-Nejad, et al. (2011). "Glomerular endothelial PI3 kinase-alpha couples to VEGFR2, but is not required for eNOS activation." Am J Physiol Renal Physiol **301**(6): F1242-1250.

Zhang, Y. W., T. L. Jones, et al. (2009). "Implication of checkpoint kinase-dependent up-regulation of ribonucleotide reductase R2 in DNA damage response." J Biol Chem **284**(27): 18085-18095.

Zhao, M. and B. Ramaswamy (2014). "Mechanisms and therapeutic advances in the management of endocrine-resistant breast cancer." World J Clin Oncol **5**(3): 248-262.

Zhong, Y., S. Shen, et al. (2014). "ALDH1 is a better clinical indicator for relapse of invasive ductal breast cancer than the CD44+/CD24- phenotype." Med Oncol **31**(3): 864.

Zhou, B. S., P. Tsai, et al. (1998). "Overexpression of transfected human ribonucleotide reductase M2 subunit in human cancer cells enhances their invasive potential." Clin Exp Metastasis **16**(1): 43-49.

Zips, D., H. D. Thames, et al. (2005). "New anticancer agents: in vitro and in vivo evaluation." In Vivo **19**(1): 1-7.

Appendix A: Pathologist's Report on Liver Injury in Mice Treated with Ad-IL-2 Vectors in Combination with PAC1

ANIMAL PATHOLOGY SERVICES (APS) LTD.
POSTMORTEM REPORT

CASE NUMBER: 15-017 DATE EXAMINED: 7 November 2014

SUBMITTED BY: Shyam Chaurasiya

PRINCIPAL INVESTIGATOR: Dr. M. Hitt

HISTORY: Liver samples from animals on a study of regression of orthotopic murine breast tumors following treatment with an Ad vector encoding the cytokine IL-2 alone or in combination with PAC1, a small molecular weight compound that induces apoptosis. Livers were collected post treatment, processed and stained with H&E. Are there any microscopic signs of liver toxicity?

COMMENTS: There is a reaction in livers CMV-IL-2+HPBCD and PAC1 + CMV – IL -2 Liver 2 which is similar in nature and is almost certainly associated with the experimental treatment.

Artifactual vacuolation of hepatocyte cytoplasm was present in all of the livers and is of no experimental significance. It is most likely associated with handling of the samples, and could be due to the time elapsed from euthanasia of the animals to placing the livers in formalin, if it was more than half an hour. Alternatively, if the livers were placed in saline or other solutions prior to fixation, that could account for it as well.

HISTOPATHOLOGY:

HPBCD+D170-3: There is artifactual vacuolation of hepatocyte cytoplasm.

PAC1+D170: There is artifactual vacuolation of hepatocyte cytoplasm.

CMV-IL-2+HPBCD: Throughout the liver there are numerous clustered infiltrates of small lymphocytes and neutrophils. These are located both in the parenchyma and around portal triads. In the parenchyma they are very often associated with necrotic hepatocytes. In some areas of the liver, there are as many as three necrotic hepatocytes per 200X field. Hepatocyte nuclei are generally enlarged and have an open chromatin network. There is a background of artifactual vacuolation of hepatocyte cytoplasm.

PAC + MPE2, Liver 1: There is artifactual vacuolation of hepatocyte cytoplasm.

PAC + MPE2 Liver 2: There is artifactual vacuolation of hepatocyte cytoplasm.

PAC1 + CMV – IL -2 Liver 1: There is artifactual vacuolation of hepatocyte cytoplasm.

PAC1 + CMV – IL -2 Liver 2: Microscopic findings in this liver are qualitatively the same as in liver CMV-IL-2+HPBCD but are qualitatively less severe. The cellular infiltrates are smaller and less numerous and there are only occasional necrotic hepatocytes.

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Appendix B: Adenoviral Vector Construction I: Mammalian Systems

Shyambabu Chaurasiya, Mary M. Hitt

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Contributions:

I wrote this book chapter and Dr. Mary Hitt edited it.

Adenoviral Vector Construction I: Mammalian Systems

4

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1. Introduction

Rowe and colleagues first discovered adenovirus (Ad) in 1953 while trying to culture human adenoid tissue in the laboratory.[1] Following the discovery of human Ad, nonhuman Ads have been isolated from a number of species including dog, mouse, chimpanzee, and pigs as well as other mammalian and avian species.[2,3] After their discovery, Ads were extensively studied as a model system to understand basic eukaryotic cellular processes such as DNA replication, transcription, RNA splicing, and translation.[4] The study of Ad led Sharp and colleagues to discover the existence of introns and the process of mRNA splicing.[5] During late 1960s it was found that adenoviruses can recombine during growth in culture. This finding ultimately set the stage for the use of Ad as a vector for gene delivery to cells both in vitro and in vivo.[6–8]

Ads have many features that make them a suitable vector for gene therapy including: (1) the viral genome is relatively easy to manipulate by recombinant DNA technology; (2) scaling up and purification of the recombinant virus for use in the clinic are relatively easy; (3) the virus infects both quiescent and dividing cells with high efficiency; (4) recombinant viruses are fairly stable as the viral genome does not undergo rearrangement at a high rate; (5) in permissive cells the virus replicates to high levels producing up to 10,000 plaque-forming units (pfu) per infected cell; and (6) high levels of transgene expression are achieved. Moreover, the viral genome is maintained as an episome in the infected cell and rarely integrates into the cellular genome. This increases the safety of adenoviral vectors as the risk of insertional mutagenesis is quite low. However, because of the episomal nature of the vector genome, transgene expression is transient in dividing cells (reviewed in Sadeghi and Hitt[9]). These features have made Ad a vector of choice for gene therapy, which is evident from the fact that adenoviral vectors have been used in almost a quarter of all the gene therapy clinical trials performed to date.[10]

1.1 Adenovirus Biology

More than 100 serotypes of Ad are known, 51 among which are isolated from humans. Based on sequence homology and their ability to agglutinate red blood cells, the 51 serotypes of human Ads have been classified into six groups: A to F.[3] The serotypes most widely studied and most commonly used as vectors for gene therapy are Ad2

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and Ad5, both of which belong to group C.[2,11] This chapter will focus mainly on the biology of these two serotypes of Ad. The adenovirion is a nonenveloped icosahedral particle about 70–90 nm in size containing a linear double-stranded DNA genome of approximately 36 kilobase pairs (kbp). The facets of the icosahedral capsid of the virion are composed mainly of trimers of hexon protein, and some other minor proteins. The vertices of the capsid are composed of penton bases anchoring the fiber proteins that are responsible for the primary attachment of the virion to the cell surface.

The first event in virus infection is the binding of fiber protein to the coxsackievirus adenovirus receptor (CAR) on the cell surface. This is followed by a secondary interaction between virion penton and $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins, leading to internalization of the virion by clathrin-dependent endocytosis.[12,13] The levels of primary (CAR) and secondary (integrins) receptors present on the cell surface determine the efficiency with which the cell will be infected with adenovirus.[14] After internalization, the acidic environment of the endosome leads to escape of the virion to the cytoplasm. Here the virion is trafficked by dynein along microtubules toward the nucleus.[15] During translocation toward the nucleus, the virion undergoes sequential disassembly and the viral genome is ultimately imported to the nucleus through the nuclear pore complex. Viral DNA replication begins 6–8 h postinfection and it takes 24–36 h for the virus to complete its life cycle.[16]

The viral genome is flanked by inverted terminal repeats (ITRs) of 90–140 bp that are required in *cis* for the replication of the viral genome.[17,18] The ITRs are covalently bound by terminal protein.[4] In addition to the ITRs, the packaging signal (ψ) is also required in *cis* for proper folding and packaging of the viral genome into the capsid.[19] The viral genome is divided into noncontiguous, overlapping early and late transcription regions: E1A, E1B, E2, E3, and E4 are early genes whereas L1 to L5 are late genes.[2,20] The products of early genes as well as the replication of viral DNA are prerequisites for the expression of late genes.[21]

E1A, the first transcription unit to be expressed, produces two major proteins following differential mRNA processing. These proteins are required for the transcriptional activation of other early genes (E1B, E2, E3, and E4) and also to induce an S-phase-like state in the infected cells.[22] The E1A proteins bind to retinoblastoma protein (pRb), allowing the release of E2F and ultimately forcing the infected cells to enter into S-phase.[23] Because of the crucial role of E1A in viral replication, E1A is often deleted in order to make the virus replication deficient. The two major products of the E1B transcription unit are involved in blocking host mRNA transport, promoting viral mRNA transport, and blocking E1A-induced apoptosis to prevent premature death of the infected cells.[24,25] The E1B product (E1B-55 kDa) directly binds to the p53 protein to block E1A-induced apoptosis. E1A and E1B are considered oncogenes as they have the ability, when used in combination, to transform human and rodent cells in vitro.[2,26,27]

The two transcription units in the E2 region encode proteins required for the replication of viral DNA.[28] E2a encodes the 72-kDa DNA-binding protein whereas E2b encodes the viral DNA polymerase and terminal protein precursor (pTP). The E3 region encodes at least seven proteins, most of which are involved in subversion of the host immune system to allow a more robust infection. For example, E3-gp19K blocks the presentation

of viral antigens by major histocompatibility complex (MHC) class I, thus preventing lysis of the infected cells by cytotoxic T lymphocytes.[28] The E3 region is nonessential for virus replication in vitro. At least six proteins are encoded by the E4 region. The products of the E4 region have diverse functions including facilitation of viral DNA replication, enhancement of late gene expression, and downregulation of host protein synthesis.[28] This region can also play a role in promoting the transforming ability of E1A.[29]

All the late region genes (L1–L5) are expressed from a common promoter called major late promoter. The primary major late transcript undergoes alternative splicing to produce individual transcripts. The products of late genes are mainly structural in function.[28]

1.2 Adenovirus Vectors

Different regions of the viral genome can be replaced with transgene(s) to generate mammalian gene transfer vectors. As described above, E1A-encoded proteins are crucial for the expression of both early and late viral genes and hence for replication of the virus. Deletion of the E1A region not only makes the virus replication deficient but also increases the cloning capacity of the vector. The packageable viral genome is limited in length to 105% of the wild-type genome size; thus one can insert only up to 1.8 kb in the vector without deletion of any viral sequences.[30] However, deletion of the E1 region allows insertion of transgenes up to 5.1 kb in size. Because E3-encoded proteins are nonessential for virus replication in vitro, the E3 region is often removed from Ad vectors. Deletion of E3 together with E1 can further increase the cloning capacity, accommodating insertion of foreign genes up to 8.2 kb in size.[31] Ad vectors deleted in E1, both with and without E3 deletion, are referred to as first-generation vectors.[32] First-generation vectors are the most commonly used Ad vectors for the purpose of gene therapy. In this chapter we will focus on the construction of first-generation Ad vectors.

2. Cell Lines for Propagating Adenovirus Vectors

Human Ads can undergo productive replication only in cells of primate, pig, and cotton rat origin.[33–35] Adenovirus infection of nonpermissive cells (e.g., cells from mouse, hamster, or rat other than cotton rat) results in abortive replication or occasional transformation of the cells due to rare integration of viral E1 sequences into the cellular genome.[36] Propagation of human Ad vectors is generally carried out in human cells that complement the E1 deletion in the vector. The first E1-complementing human cell line was developed by Graham and colleagues in their studies on E1-induced transformation.[37] In their landmark study, they used their novel technique of calcium phosphate coprecipitation to introduce sheared DNA from Ad5 into human embryonic kidney (HEK) cells.[38] The HEK cells transformed with sheared DNA from Ad5 were called HEK-293 cells. This cell line has been widely distributed since its isolation before 1980. According to the ISI Web of Science, the original paper describing the isolation of this cell line[37] has been cited nearly 3500 times. HEK-293 cells contain the "left end" of the Ad5 viral genome (1–4344 bp), including early region E1, integrated into chromosome 19.[39] These cells have been extensively used for the

construction and propagation of E1-deleted nonreplicating Ad vectors. Additionally, the HEK-293 cell line has been widely used for diverse transfection-related studies because of the high efficiency of transfection and high level of transgene expression. The high expression levels are thought to result from promiscuous activation of the transfected promoter by E1A; and blockage of apoptosis, induced to varying degrees by different transfection procedures, by E1B.[40] Although HEK-293 cells were long considered to be kidney epithelial cells, evidence suggests that they may have been derived from a neural cell in the complex embryonic kidney cell culture.[41]

Several investigators have attempted to stably express E1 proteins in established human cancer cell lines such as A549, for the purpose of propagating E1-deleted Ad vectors. However, limited success has been achieved with this strategy, partly because growth of established cells is not dependent on E1 expression and also because it is difficult to isolate E1-expressing cells due to E1A-mediated toxicity. Although some encouraging data have been published in generating such cells, the use of these cells for construction and/or propagation of Ad vectors has been very limited.[42,43] An advantage of HEK-293 cells is that growth of the cells is dependent on the expression of E1 and hence constant levels of E1 expression are maintained over time.

One difficulty of propagating Ad vectors is the potential for Ad sequences carried by the propagating cell line to recombine with residual E1 (or immediately downstream) sequences in the vector, regenerating a wild-type E1 region. This type of homologous recombination would give rise to replication-competent adenovirus (RCA)-contaminated vector stocks, which would be especially problematic if the wild-type virus had a growth advantage over the recombinant vector. The PER.C6 cell line was specifically established to avoid RCA contamination during the propagation of Ad vectors to produce clinical grade stocks.[42] PER.C6 cells were derived from human embryonic retina cells by transforming with a minimal E1 region of Ad5. These are discussed in more detail in a later chapter of this book. Likewise, a system based on E1-transformed amniocyte-derived primary cells has been developed for rescue and propagation of Ad vectors with no overlapping E1 sequences.[44] In this chapter, we will focus on the construction and propagation of Ad vectors in HEK-293 cells.

2.1 Propagation of Adenovirus Vectors Encoding Toxic Transgenes for Cancer Gene Therapy

Cancer is a disease caused by the accumulation of many genetic mutations that allow the cells to undergo uncontrolled division. Unlike gene augmentation therapy where the goal is to restore a defective gene, the goal of many cancer gene therapies is to kill the cancer cells. One approach to killing cancer cells through gene therapy is by delivering proapoptotic or toxic genes to cancer cells. High levels of transgene expression are usually desirable in the target cells; however, construction and propagation of Ad vectors encoding such genes are challenging as the transgene expression can induce toxicity in the packaging cells, reducing vector yield.[45] In some cases the toxicity in packaging cells is so severe that the cells die after transfection with the vector DNA resulting in total failure to obtain the viral vector. In other cases the toxicity places a strong selective pressure on the resulting viral vector to reduce or completely eliminate

transgene activity. This selective pressure may give rise to revertants or to mutations within the transgene expression cassette leading to reduction or complete ablation of transgene expression. The replicative advantage of these revertant/mutant viruses over the desired vector would reduce the feasibility of large-scale vector production.[45]

Different approaches have been proposed to address this challenge. The most common approaches involve differential regulation of transgene expression at the transcriptional level in the packaging and target cells (Figure 1). The use of tissue/tumor-specific promoters, such as the human telomerase (hTERT)[46] promoter, prostate specific antigen (PSA)[47] promoter, and carcinoembryonic antigen (CEA)[48] promoter to control the transgene, is a common strategy for achieving high levels of transgene expression in target cells with minimal expression in the packaging cells (Figure 1(A)). In our lab we have previously shown that the upstream sequence of the mammoglobin gene, a gene that is expressed at high levels in breast cancer and at very low levels in nonmammary cells, could be used to target transgene expression to breast cancer cells.[49] The use of tissue/tumor-specific promoters not only makes the construction and propagation of the vectors easier but also increases the cancer specificity and hence the overall safety of the vector. Moreover, in vivo in immune-competent animals, nonselective viral promoters such as the cytomegalovirus (CMV) immediate early promoter and the SV40 promoter are prone to silencing by TNF-α and interferon-γ; hence transgene expression is not long-lasting.[50–52] Studies comparing the duration of transgene expression driven by viral (CMV or SV40) promoters to that driven by cellular promoters have found that transgene expression lasts longer when driven by cellular promoters not only in the case of first generation Ad vectors but also in the case of helper-dependent Ad vectors, which are devoid of all viral coding sequences.[53] One drawback of this type of targeting is that tissue/tumor-specific eukaryotic promoters are usually inferior to viral promoters in terms of expression intensity.[54] Incorporation of additional elements could increase the expression intensity of the tissue/tumor-specific promoters. For instance, in our laboratory we have shown that addition of two enhancer elements upstream of the minimal mammoglobin promoter greatly increases the expression intensity of the promoter without compromising the tissue specificity.[55] Several other strategies have been used to improve the expression intensity of the tissue/tumor-specific promoter, the discussion of which is beyond the scope of this chapter. Readers are encouraged to see an excellent review on this topic by Papadakis et al.[53]

An alternative strategy to silence toxic transgenes during vector propagation is to insert a DNA sequence containing a strong transcription-terminating sequence between the promoter and the transgene (Figure 1(B)). The inserted sequence is flanked by *loxP* recognition sequences of the site-specific Cre recombinase. The presence of the inserted sequence should completely block transgene expression in the packaging cells. For therapeutic use, coinfection of target cells with another Ad vector encoding Cre causes excision of the *loxP*-flanked sequence, inducing expression of the transgene.[56] However, the requirement of an additional vector expressing the recombinase makes this system less suitable, especially for in vivo studies.

A third strategy for the construction and propagation of vectors encoding toxic genes is through the use of exogenously regulated expression systems, which have the distinct advantage of allowing pharmacological control of transgene expression

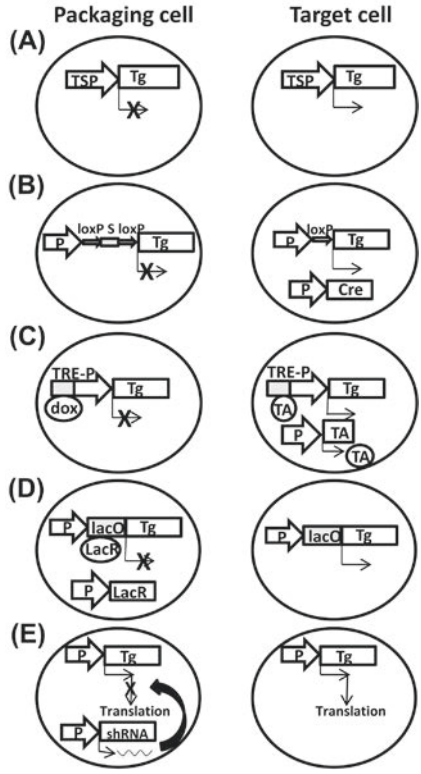

Figure 1 Strategies for rescue of Ad vectors encoding toxic transgene(s). (A) Control of transgene expression by cell-specific regulation. Tissue/tumor-specific promoters can be used to achieve low levels of transgene expression in packaging cells and high levels in the target cell type. (B) Control of transgene expression by Cre–*loxP*-mediated regulation. An exogenous sequence, containing strong transcription termination sequence(s) and flanked by *loxP* sites in direct orientation, is inserted in between the promoter and the transgene open reading frame to inhibit transcription of the transgene in packaging cells. Coinfection of target cells with this vector and another Ad vector encoding Cre recombinase causes the excision of the exogenous sequence, allowing expression of the transgene. (C) Control of transgene expression using the Tet-OFF system to silence the transgene in packaging cells. In this system, the promoter is fused to a tetracycline response element (TRE) and a transactivator is encoded either by the same vector or by a different vector. Tetracycline (or an analog such as doxycycline) prevents the transactivator from binding to the TRE, and as a result, the transgene remains silent. Transgene expression is activated in target cells in the absence of doxycycline. (D) Control of transgene expression using *lac* repressor regulation. The *lac* operator sequence, to which the *lac* repressor protein binds, is inserted in close proximity to the promoter driving transgene expression. Packaging cells are modified to express the *lac* repressor protein which suppresses transgene expression. Absence of the *lac* repressor protein in target cells allows expression of the transgene (see Section 2.1). (E) Control of transgene expression by RNA silencing. Packaging cells are modified to express an shRNA that targets the transgene transcript, preventing its expression. Absence of the shRNA in the target cells allows high-level expression of the transgene. (P, promoter; TSP, tissue/tumor-specific promoter; Tg, transgene; S, exogenous stuffer sequence with termination sequences; dox, doxycycline; TRE-P, tetracycline response element fused to promoter; lacO, *lac* operator sequence; LacR, lac repressor protein; TA, transactivator.)

both in vitro and in vivo[53] (Figure 1(C)). The tetracycline (tet) on/off system, based on the highly sensitive prokaryotic tetracycline resistance operon, is probably the most commonly used regulatable expression system. In this system, the transgene is placed under the control of a tet-response element (tet operator), and a transactivator (TA) is encoded either by the same vector or by a different vector. The TA is a fusion of a tet repressor with the activation domain of a transcription factor, such as VP16 from herpes virus. This system requires constant administration of tetracycline analogs such as doxycycline to prevent TA binding to the response element, hence the switch is "off" (tet-OFF).[53] Based on this system, Gu et al. constructed an adenoviral vector that carries the apoptotic *Bax* gene transcriptionally controlled by the tet-OFF transactivator protein, which is encoded by the same vector under the control of the hTERT promoter.[57] Expression of *Bax* can be inhibited by the addition of doxycycline, which acts by inhibiting the transactivator protein. Although propagation requires continuous administration of doxycycline to silence the transgene, no drug is needed to induce *Bax* expression in clinical applications, which is an advantage. An alternative approach is to use the (tet-ON) system,[53] in which a mutant "reverse" tet repressor binds to the response element and activates transgene transcription only in the presence of doxycycline (or other tetracycline analogs).[58] Sipo et al. used the tet-ON system to construct and propagate an Ad vector encoding the apoptotic gene FasL in which the FasL gene was driven by the tet operator fused to the CMV promoter and the reverse TA was encoded by a different Ad vector.[59]

Another commonly used regulatable expression system makes use of the prokaryotic *lac* operon repressor protein that binds to the *lac* operator sequence and suppresses gene expression (reviewed in Rubinchik et al.[45]) (Figure 1(D)). In this system, operator binding sites are placed in close proximity to the promoter driving the transgene. Binding of the *lac* repressor to the operator sequences prevents binding of RNA polymerase II to the promoter and hence represses transcription of the transgene. Packaging cell lines can be engineered to stably express the repressor protein, which would ensure that transgene expression is suppressed during virus production. However, absence of the repressor protein in target cells allows high levels of transgene expression. Zhao et al. used this system to obtain high titers of an Ad vector encoding the cytolytic HIV-1 *env* protein.[60] Matthews et al. used a related system to construct and propagate an adenoviral vector that encodes the rabies virus glycoprotein following unsuccessful attempts using standard HEK-293 cells to rescue the virus.[61]

Posttranscriptional gene silencing using HEK-293 cells stably transfected with shRNA against the transgene has also been used to grow adenoviral vectors to high titer (Figure 1(E)). Wang et al. used this strategy to produce a vector encoding hIcon, an antiangiogenic protein. Interestingly, although hIcon is not directly toxic to cells, the authors suggest that transgene silencing reduced nutrient consumption during vector propagation, thus allowing higher virus yields.[62] Alternatively, packaging cells stably expressing antiapoptotic genes have been shown to increase yields of vectors encoding apoptotic genes. Bruden et al. found that stable expression of the antiapoptotic gene *CrmA*, encoding a poxvirus serpin, in E1-complementing packaging cells such as HEK-293 or AE25, dramatically increased the yield of adenoviral vectors encoding apoptotic genes such as Fas ligand, Fas-associated protein with death domain, caspase-8, or Fas/APO1.[63]

3. Construction of First-Generation Adenoviral Vectors

3.1 Early Methods

In 1973, Graham and colleagues showed that purified DNA from Ad5 and also from simian virus 40, when coprecipitated with calcium phosphate, can be taken up by human cells, resulting in the production of infectious virus particles.[38] This observation that purified viral DNA could be infectious laid the foundation for the studies manipulating the Ad genome for the construction of recombinant Ad vectors. Early methods of modified Ad construction mainly used two approaches: (1) in vitro ligation of viral DNA following cleavage with restriction enzymes[64,65] and (2) homologous recombination between viral DNAs in cotransfected cells.[66] For the first of these approaches, Stow devised a technique that employed in vitro ligation between purified virion DNA and plasmid DNA containing the left end of the Ad genome.[67] The E1 shuttle plasmid and purified viral DNA (from the Ad5 mutant dl309 that has a unique *Xba*I site in the E1 region[68]) were both digested with *Xba*I and then ligated together in vitro. The ligation product was then used to transfect HEK-293 cells, which resulted in the production of recombinant Ad virions.[67] This study elegantly showed that infectious virus could be reconstructed using a cloned subgenomic Ad sequence to shuttle precise E1 modifications into recombinant virus. However, due to the location of the *Xba*I site, most of the E1 region is retained in the recombinant, and few other unique restrictions sites are available in the Ad genome, so this strategy is not ideal for construction of gene therapy vectors.

At about the same time, Kapoor and Chinnadurai developed a system to rescue mutations into the Ad E1 region by in vivo homologous recombination between the "left" end Ad sequences cloned into a plasmid and purified Ad virion DNA.[69] This "left end" shuttle plasmid could be easily manipulated in vitro to incorporate the desired mutations in E1. The overlapping sequence in the shuttle plasmid and the cotransfected viral DNA allowed homologous recombination to take place in HEK-293 cells, resulting in the generation of recombinant virions with alterations in E1. This system obviates the need for unique restriction enzyme sites since it does not involve ligation of two DNA molecules. However, the viral DNA must be cleaved in the left end before cotransfection in order to reduce contamination with nonrecombinant parental virus. The most commonly used sites for cleaving the viral DNA are the unique *Xba*I in Ad5 dl309 mutant and the unique *Cla*I site in the wild-type Ad5. Despite cleaving the viral DNA with these enzymes, contamination with the parental virus remains an issue. Both the *Xba*I and the *Cla*I sites are located at the very left end of the genome; hence there is a very small size difference between undigested viral DNA and viral DNA digested with *Xba*I or *Cla*I. Because of the small size difference it is difficult, using agarose gel electrophoresis, to confirm whether the digestion is complete. The undigested parental DNA generates virus more efficiently than the DNAs, which require recombination. In addition, the small fragment produced by *Xba*I or *Cla*I digestion can potentially be carried over during the transfection and the fragments may religate in the cell to generate wild-type or parental virus. This could potentially be another source of nonrecombinant virus contamination.[70,71]

Later, Mizuguchi and Kay proposed an alternate strategy to replace E1 sequences in the Ad genome with transgene expression cassettes.[72] Plasmids were constructed

containing the entire viral genome with and without the E3 region and with three unique restriction enzyme sites (I-*Ceu*I, *Swa*I, and PI-*Sce*I) in place of El. Transgene expression cassettes, flanked by an I-*Ceu*I site at one end and a PI-*Sce*I site at the other, were ligated to the genomic plasmid following digestion with these two restriction enzymes. Ligation in the presence of *Swa*I reduces the recovery of nonrecombinant parental plasmids. The modified genomic plasmid is then linearized to release viral sequences, and used to transfect HEK-293 cells to produce recombinant virus. This strategy addresses both the problems associated with lack of unique restriction enzyme sites in the viral genome and the problem associated with high levels of wild-type or parental virus contamination.[72] However, construction of each vector involves manipulation, cloning, and scale-up of separate plasmids >30 kb in size, which can be difficult in some cases.

These methods for the construction of recombinant Ads rely on the use of the viral genome either alone or in combination with a shuttle plasmid. However, the large viral DNA genome is not only time-consuming and laborious to isolate and purify, but also difficult to manipulate genetically. Some of these approaches can also lead to high levels of contamination with the nonrecombinant parental virus. It is not uncommon for the parental virus to outgrow the recombinant virus, making the rescue of the recombinant vectors further problematic. Given the potential of Ad vectors to be used in gene therapy and other purposes, an efficient method for the construction of Ad vectors with minimal wild-type or parental contamination was needed.

3.2 The Two-Plasmid Rescue System

McGrory et al. (1984) developed a two-plasmid rescue system to overcome the limitations faced by the earlier approaches of Ad vector construction.[73] The two-plasmid rescue system is based on the ability of two plasmids to undergo recombination in mammalian cells. The two-plasmid rescue system has gone through many modifications, including the switch from homologous recombination strategy to a site-specific recombination strategy, to make the method more efficient and to reduce wild-type or parental virus contamination. In addition to the two-plasmid rescue system, other methods have been developed for efficient construction of Ad vectors using bacterial systems or in vitro ligation, which are discussed in other chapters in this book. The remainder of this chapter will focus on the two-plasmid rescue system and modifications in this method for high-efficiency Ad vector construction.

3.2.1 Development of the Two-Plasmid Rescue System

A study by Berkner and Sharp in 1983 demonstrated that recombinant Ads could be produced in cotransfected HEK-293 cells by homologous recombination between cloned fragments of viral DNA.[74] Rescue of infectious Ads was dependent on cleavage of at least one of the plasmids at the junction of the ITR and the plasmid DNA, releasing the ITR. Unlike earlier methods that used purified virion DNA for the construction of Ad recombinants, this method used only noninfectious plasmids, thus avoiding the need to isolate virion DNA. Furthermore, since full-length viral DNA was not used, nonrecombinant parental virus could not be generated, which was a major problem with the earlier methods.

The linear genome of Ad is thought to replicate in a semiconservative manner, with replication starting at either end of the genome. However, Ruben et al. (1983) showed that up to 10% of Ad DNA molecules in an infected cell are joined head-to-tail due, at least in part, to the formation of covalently closed circles.[75] In purified virus stocks, viral DNA does not exist in a circular form, but circular viral DNA can be detected intracellularly before the onset of viral DNA replication.[75] This finding suggested that the full-length viral genome could be cloned and maintained as a bacterial plasmid. Subsequently, in 1984 Graham cloned the entire Ad5 genome as a plasmid (pFG140) with plasmid sequences containing the β-lactamase gene and a bacterial origin of replication inserted at the *Xba*I site at nt 1339.[76] This Ad genomic plasmid could be amplified in *Escherichia coli* in the presence of ampicillin. Moreover, this plasmid was shown to be nearly as efficient as purified virion DNA in generating infectious virus following transfection into HEK-293 cells.

Another important finding, made in 1987 by Ghosh-Choudhury et al., was the discovery that protein IX (pIX) is essential for the generation of infectious virus.[77] They constructed an Ad5 genomic plasmid that was similar to pFG140 except that it had a deletion of the gene encoding protein IX. Unlike pFG140, this plasmid was noninfectious. To confirm the essential nature of pIX, they cotransfected HEK-293 cells with the pIX-deleted genomic plasmid and a plasmid encoding the left end of the Ad genome, including the pIX gene. All viruses recovered from the cotransfection carried the pIX gene as a result of homologous recombination between the two plasmids. Later studies determined that pIX plays important roles in packaging of full-length viral genome and also in the stability of the viral icosahedrons.[77,78]

These findings laid the foundation for the development of the first two-plasmid rescue system by McGrory et al. for the construction of Ad vectors in which a transgene expression cassette replaces the E1 region.[73] In this study, the authors inserted a sequence into pFG140 to increase its size to 40 kb, which is beyond the packaging capacity of Ad. The resulting plasmid, pJM17, was noninfectious, but could serve as a template for replication in HEK-293 cells. For the second component of the system, they constructed a shuttle plasmid containing the left end of Ad5 with foreign DNA of up to 5.4 kb in place of E1. Infectious recombinant Ad vectors bearing the foreign DNA sequence were generated following cotransfection of HEK-293 cells with pJM17 and the shuttle plasmid. Since both plasmids are noninfectious, in principle, only recombinant E1-substituted vectors should be generated, resulting from in vivo homologous recombination between the overlapping Ad sequences in the genomic and the shuttle plasmids. This system was highly successful as it was able to overcome some of the limitations associated with the earlier methods of Ad vector construction, such as ease of transgene insertion and significant contamination with parental virus. However, the pJM17 genomic plasmid was able to generate a low level of infectious virus in HEK-293 cells even in the absence of a cotransfected shuttle plasmid. Infectivity of pJM17 was discovered to be due to the spontaneous deletion of sequences from the plasmid backbone resulting in reduction in size of the genomic plasmid to within the packaging constraints of Ad.[73] This posed the risk of parental virus contamination in recombinant virus preparations; hence modification of this method was needed.

In 1994, Bett et al. modified the two-plasmid rescue method by constructing an improved genomic Ad plasmid, pBHG10.[79] Like pJM17, pBHG10 contains essentially the entire Ad5 genome with some important modifications. First, it has a deletion of 3180 bp in the E1 region, removing E1A and the packaging signal (ψ) required for packaging the viral genome into the capsid. Removal of the ψ sequence renders the plasmid noninfectious. The second modification was the deletion of ~2.7 kb from the nonessential E3 region and addition of a *Pac*I restriction enzyme site in its place. Shuttle plasmids bearing the left end of viral DNA, including the ITR and packaging signal but with a deletion in E1 from 339 to 3533 bp, were also constructed. A linker containing multiple cloning sites (MCPs) was introduced in the shuttle plasmids in place of E1 to allow easy insertion of a transgene. This modified system introduced two improvements in the two-plasmid system developed by McGrory et al.[73] First, the combined E1 and E3 deletions in this system increased the cloning capacity of the resulting recombinant vectors to allow insertion of up to ~8 kb of foreign DNA. Second, one can insert foreign DNA into either the E1 or the E3 region using this system. Insertion of a transgene into the E3 region is facilitated by the unique *Pac*I site in the large pBHG10 plasmid. Insertion can be expedited by using the kanamycin-resistant pABS.4 plasmid (Microbix Biosystem Inc., Mississauga, ON, Canada). This plasmid contains a *Swa*I-flanked kanamycin resistance gene within a MCS flanked by *Pac*I sites. For E3 insertions, the transgene is first cloned into the MCS of pABS.4. The resulting plasmid is then digested with *Pac*I and the fragment bearing the transgene and the kanamycin resistance gene is then inserted into the *Pac*I site in pBHG10. The resulting large plasmid is then used to transform *E. coli* and positive clones bearing the E3 insertion are selected based on their resistance to both ampicillin and kanamycin. Finally, prior to cotransfection for vector construction, the kanamycin resistance gene is removed from the genomic plasmid by digestion with *Swa*I. The increased cloning capacity and versatility of the method as well as the absence of parental virus contamination made this version of the two-plasmid rescue system very popular for the construction of nonreplicating Ad vectors. Like other Ad vector rescue systems developed by that time, the efficiency of vector rescue was fairly low, typically requiring cotransfection of 12 to 30 60-mm dishes to ensure rescue of around 10 independent isolates of the recombinant vector.

3.2.2 Fine-Tuning of the Two-Plasmid Rescue System

A possible explanation for the low efficiency of vector rescue by the two-plasmid system developed by McGrory et al. is that homologous recombination frequencies are simply not high enough. The observation that the infectious plasmid pFG140 has a plaque-forming efficiency ~100-fold higher than that of a typical cotransfection for vector rescue supports this hypothesis. Ng et al.[80] proposed that recombination mediated by the site-specific Cre recombination system would be more efficient than homologous recombination for the rescue of recombinant Ad vectors. Therefore, they inserted *loxP* sites into pBHG10 upstream of the pIX gene and into the shuttle plasmid after the transgene expression cassette. Cotransfection of Cre-expressing HEK-293 cells with these two *loxP*-containing plasmids allowed vector rescue with

an efficiency ~30-fold higher than that mediated by homologous recombination. The efficiency of virus rescue was increased even further by replacement of the single ITR with two ITRs fused head-to-head (referred to here as an ITR junction) in the shuttle plasmid. Several reasons were proposed to explain the observed enhancement in virus rescue by replacing a single ITR with an ITR junction in the shuttle plasmid. First, in contrast to plasmids with a single ITR, plasmids containing an ITR junction should increase in copy number following cotransfection of this shuttle plasmid and the Ad genomic plasmid. The ITR junction can serve as an origin of viral DNA replication,[76] with the Ad genomic plasmid providing all the *trans*-acting viral factors essential for viral DNA replication. Recognition of ITR junction-containing plasmids as templates for the viral replication machinery results in production and amplification of linear shuttle plasmid DNA flanked by the ITRs. Since both the genomic plasmid and the shuttle plasmid contain ITR junctions, both should increase in copy number in cotransfected HEK293 cells. This increase in the pool of substrates for recombination should enhance the rescue of recombinant virus. Second, the replicating linear shuttle DNA might serve as a better substrate for recombination with linear Ad genomic DNA than the nonreplicating circular shuttle DNA. Third, recombination between a linear shuttle DNA and the Ad genomic DNA should generate a packageable, infectious genome in a single step. In contrast, generation of a packageable genome from a circular shuttle plasmid and a linear genomic DNA is likely a two-step process. In the first step the circular plasmid integrates into the linear genomic DNA at the *loxP* site. However, this is nonpackageable because the packaging signal (from the shuttle plasmid) in the recombinant DNA is far from the terminus of the molecule. Also the size of this DNA molecule exceeds the virion packaging limit. Generation of infectious DNA would require additional step(s) to eliminate extraneous sequences from the recombinant molecule. Together, the replacement of homologous recombination with Cre-mediated recombination and the replacement of a single ITR with an ITR junction in the shuttle plasmid increased the efficiency of virus rescue by ~100-fold compared to the efficiency of the earlier two-plasmid systems.[73,79]

This two-plasmid system developed by Ng et al.[80] improved the efficiency of virus rescue but virus could be rescued only in HEK-293, or other E1-complementing cells, that also express Cre. Therefore, to improve the utility of the system, a Cre expression cassette was inserted into the genomic plasmid within the plasmid backbone so that the Cre cassette is not incorporated in the final recombinant vector. This insertion eliminates the requirement for Cre-expressing cells for virus rescue. The efficiency of virus rescue obtained using this Cre-expressing genomic plasmid in parental HEK-293 cells was found to be comparable to that obtained using the previous generation of Ad genomic plasmid in Cre-expressing HEK-293 cells.[81]

The Cre/*loxP*-mediated recombination system for the generation of the Ad vectors (Figure 2) elegantly addressed most of the limitations of previously developed rescue systems. Although suitable for rescue of most Ad vectors, this system precludes the use of *loxP* sites anywhere else in the genome, for example, for the purpose of regulating transgene expression[82] or inhibiting vector packaging.[83] To circumvent this problem, Ng et al. designed an alternate site-directed recombination system based on the yeast flippase (FLP) recombinase.[84] The yeast FLP-recombinase expression cassette replaced

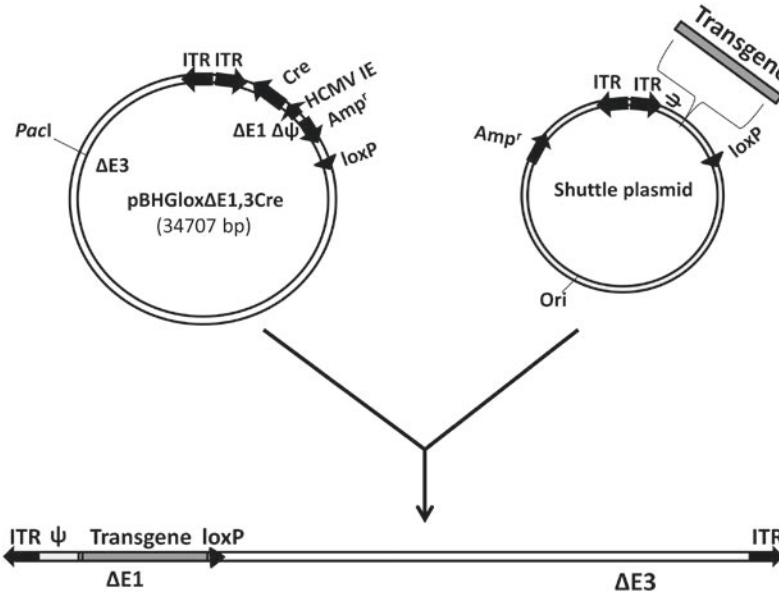

Figure 2 Two-plasmid rescue system for the construction of Ad vectors. The genomic plasmid used in this system contains most of the Ad5 genome, flanked at the "left" end by a *loxP* site and at the "right" end by two ITR sequences fused head-to-head. The plasmid backbone of the genomic plasmid contains a Cre recombinase expression cassette. The shuttle plasmid used in this system contains a transgene expression cassette flanked at the "left" end by two fused ITR sequences and a packaging signal, and at the "right" end by a *loxP* site. Cotransfection of HEK-293 cells with the two plasmids generates recombinant vectors following *loxP*-specific recombination mediated by the Cre recombinase. (ITR, inverted terminal repeat; HCMV-IE, human cytomegalovirus immediate early promoter; Amp^r, gene conferring ampicillin resistance; *Pac*I, restriction site for insertions replacing the E3 region; ψ, packaging signal.)

the Cre expression cassette in the genomic plasmid, and *frt* sites replaced the *loxP* sites in the shuttle and genomic plasmids. No significant difference was observed in the efficiency of virus rescue between the Cre-mediated and the FLP-mediated recombination systems.

4. Steps Involved in Adenovirus Vector Construction

Here we will describe the steps involved in Ad vector construction using the Cre/*loxP*-based two-plasmid rescue system developed by Ng et al.[81] Typically, a foreign expression cassette is inserted in the shuttle plasmid for rescue in the E1 region of the vector. For E3 insertions, the expression cassette is cloned directly, or via the pABS.4 transfer plasmid, into the larger (genomic) plasmid. Foreign expression cassette(s) can be inserted into the E1 and/or E3 regions in either parallel or antiparallel orientation relative to the E1 or E3 transcription units. Generally, a higher level of gene expression

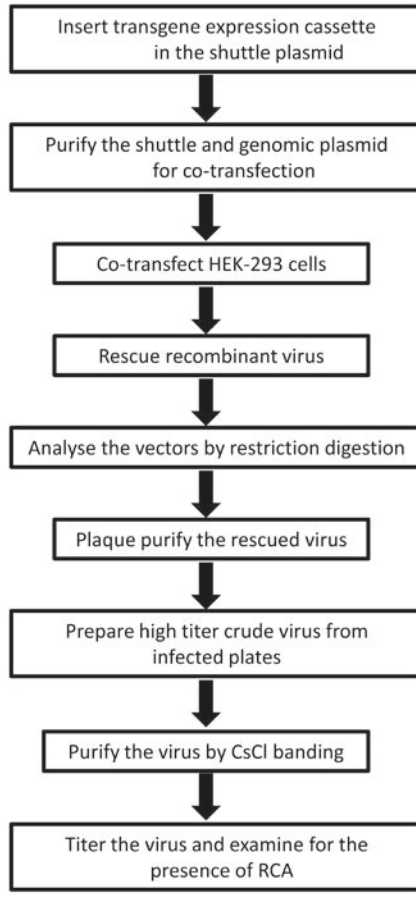

Figure 3 Flowchart for steps involved in the construction, propagation, purification, and characterization of first-generation Ad vectors.

is achieved when the transgenes are in parallel orientation to the viral transcription units they replace; however, the overall expression levels also depend on the type of promoter and sequence of the insert itself.[31] The following sections describe methods to rescue, purify, and titer the recombinant Ad vectors (Figure 3).

4.1 Preparation of Adenovirus Genomic and Shuttle Plasmid DNA for Cotransfection

Based on the size of transgene and desired application of the vector to be generated, the genomic plasmid can be selected from a variety of plasmids available from Microbix Biosystem Inc. (Mississauga, ON, Canada). The genomic plasmids pBHGloxΔE1Cre and pBHGfrtΔE1FLP (formerly designated as pBHGloxE3Cre and pBHGfrtE3FLP) retain an intact E3 region, and thus have a reduced cloning capacity compared to the E3-deleted plasmids. Foreign expression cassettes of up to 5 kb can be rescued

into vectors using these plasmids owing to the size constraints of Ad. Genomic plasmids (pBHGloxΔE1,3Cre and pBHGfrtΔE1,3FLP) have deletions of 2653 bp in the E3 region, allowing the rescue of up to 8 kb foreign sequence in the vector. Although these plasmids offer the highest cloning capacity, the vectors generated from them have slightly reduced growth (around twofold) compared to vectors that retain more of the E3 region.[31] The unique *Pac*I site, as in the original pBHG10-based system, can be used to insert a transgene cassette in the E3-deleted region of pBHGloxΔE1,3Cre or pBHGfrtΔE1,3FLP if the transgene is desired in the E3 region of the recombinant vector. The desired genomic plasmid is amplified in *E. coli* and purified for cotransfection. High-speed plasmid purification kits (e.g., from Qiagen) may be adequate for small quantities of plasmid DNA; however, for large quantities of genomic plasmid DNA, CsCl gradient purification procedures may be preferable.

Shuttle plasmids for the construction of Ad vectors based on Cre- or FLP-mediated recombination are available from Microbix Biosystem Inc. The E1 insertion plasmids pDC311 and pDC312 allow rescue of transgene cassettes into the vector via Cre-mediated recombination; pDC511 and pDC512 allow vector rescue via FLP-mediated recombination. The transgene cassette, including promoter, transgene, and polyadenylation (poly(A)) signal, is inserted into the pUC-based shuttle plasmid at the MCS. To further simplify cloning, shuttle plasmids are available that contain the immediate early promoter of murine cytomegalovirus (MCMV) and the poly(A) sequence from SV40. The MCMV promoter drives high-level expression in most cell types of both human and murine origin, in contrast to the HCMV promoter that is less active in murine tissues.[85] pDC315 and pDC316 are based on the Cre recombination system, and pDC515 and pDC516 are based on the FLP recombination system. The choice of shuttle plasmid depends on which MCS orientation is most convenient for transgene insertion, which site-specific recombination system is desired for vector rescue, and whether high levels of transgene expression are required for the intended application. Prior to use in cotransfections, the shuttle plasmid containing the transgene cassette should be amplified in *E. coli* and purified using standard plasmid purification kits (e.g., from Qiagen).

4.2 Cotransfection of HEK-293 Cells with Genomic and Shuttle Plasmid

Cotransfection of HEK-293 cells with the two plasmids gives rise to recombinant Ad that are observed as plaques on the cell monolayer. Individual plaques are then isolated for expansion of the vector; therefore it is desirable to have plaques that are well separated from each other. The number of plaques formed depends on many factors, including the transfection efficiency, state of cells, quality of plasmid DNA, and the amount of plasmid DNA used. In the Cre- or FLP-mediated recombination system, an average of ~40 plaques are formed per 60-mm dish of HEK-293 cells transfected with 2 μg of shuttle plasmid and 2 μg of genomic plasmid. The infectious Ad genomic plasmid pFG140 can be used as a control for transfection efficiency and plaque formation. Under optimal conditions, pFG140 should yield up to ~100 plaques per 0.5 μg DNA. It is a good practice to transfect with a range of plasmid concentrations to obtain a high number of plaques without risk of cross-contamination between the plaques.

To prepare for cotransfection, low passage (<p40) HEK-293 cells are grown in 60-mm dishes using complete MEMF11 supplemented with 10% FBS. Cultures at ~70–80% confluency are best for cotransfection. In general, a nearly confluent 150-mm dish of HEK-293 cells can be split into eight 60-mm dishes, which would be ready for transfection the next day. For the construction of one recombinant vector, generally 16 cultures in 60-mm dishes are prepared which are enough to perform the cotransfections in quadruplicate using three different concentrations of experimental plasmids and one concentration of pFG140. The next day, 1 h prior to cotransfection, the medium in each of the 60-mm dishes is replaced with freshly prepared medium. To prepare the DNA, 0.08 mg salmon sperm DNA, used as carrier DNA, is added to 8 ml of HEPES-buffered saline and then vortexed for 1 min to shear the DNA. The sheared salmon sperm is divided among four polystyrene tubes labeled A, B, C, and D. Each of these tubes is sufficient for cotransfection of four dishes of HEK-293 cells. To tubes A, B, and C, add 2, 8, and 20 µg, respectively, of both shuttle and genomic plasmids. To the control tube D add 2 µg of the infectious plasmid pFG140. The tubes are mixed well, and then 0.1 ml 2.5 M $CaCl_2$ is added to each tube dropwise with gentle mixing. Finally, 0.5 ml of the resulting suspension from each tube is added dropwise to the medium in separate 60-mm dishes (four dishes per tube). One day later, the medium is replaced with an agarose overlay (0.5% in MEMF11). Plaques (round turbid areas in the transparent monolayer) are generally visible within a week post cotransfection, and can be isolated between day 10 and day 14. Well-isolated plaques are collected by repeated stabs through the agarose at the site of the plaque using a sterile cotton-plugged Pasteur pipette or a 1 ml pipet tip. The agarose pieces from each plaque isolate are transferred to a vial containing 0.5 ml phosphate buffered saline (PBS) supplemented with Mg^{2+} and Ca^{2+} (PBS++) and 10% glycerol and then stored at –80 °C. Isolation of 5 to 20 plaques for further analysis is recommended.

4.3 Analysis of the Rescued Recombinant Adenovirus Vectors

The vector isolates obtained by picking plaques are amplified in HEK-293 cells to verify recombinants by analysis of viral DNA and to generate seed stocks for subsequent vector production. Briefly, HEK-293 cells are grown to near confluence in 60-mm dishes. (Note: It takes a longer time for complete cytopathic effect (CPE) to appear if the cells are too confluent or older.) The virus plaque suspensions are freeze–thawed three times, and then half of each suspension is used to infect separate 60-mm dishes of cells. The reader is discouraged from amplifying pFG140-based virus at the same time as nonreplicating vectors due to the risk of cross-contamination and potential growth advantage of pFG140-based virus. Cultures are incubated until most of the cells are rounded up and detached from the dish (complete CPE). Semiadherent cells are collected by gentle pipetting and combined with nonadherent cells. (Note: If infections are harvested too soon, it will be difficult to observe vector DNA bands above the background of cellular DNA when the DNA is analyzed by gel electrophoresis.) Approximately 3.5 ml of cell suspension from each dish is transferred to a vial containing 0.5 ml sterile glycerol and stored at −80 °C for use as a vector seed stock. The remaining 1.5 ml of cell suspension is centrifuged briefly in a microfuge tube to pellet the cells. The supernatant is aspirated, leaving behind 0.1 ml in each tube to aid in

resuspending the pellet. A solution of Pronase–sodium dodecyl sulfate (SDS) is added to the infected cells, and viral and cellular proteins are degraded by overnight digestion. Viral DNA is purified from the lysate by ethanol precipitation and resuspended in 50 µl buffer.

For the analysis of viral DNA, 5 µl of the DNA from the infected cells is digested with *Hind*III and then the resulting fragments are separated by agarose gel electrophoresis and stained with ethidium bromide. Viral DNA bands should be clearly visible under UV light, above a background smear of cellular DNA. *Hind*III digested wild-type Ad DNA can be run alongside that of the recombinant vector for the purpose of comparison. It should be noted that *Hind*III digestion of human DNA produces a band at 1.8 kb. To further verify the candidate recombinant Ad vector, the extracted DNA can be digested with other restriction enzymes and analyzed by agarose gel electrophoresis. When using the AdMax kit (Microbix Biosystem Inc.), virtually all the plaques obtained should be correct. However, it is good laboratory practice to carry out at least one round of plaque purification to ensure that all the recombinants in a high-titer vector stock have the same genome, having descended from a single infectious virus particle.

4.4 Plaque Purification of Recombinant Adenovirus Vector

Plaque assays are commonly used both to purify and to determine the titer of adenovirus vectors. In both cases, confluent HEK-293 cells in 60-mm dishes are infected with virus stock serially diluted in PBS++, at a range of 10^{-2} to 10^{-6} for partially purified virus or 10^{-5} to 10^{-10} for highly purified and concentrated virus. An agarose overlay is applied to the cell monolayer after infecting the monolayer with the virus. The agarose overlay immobilizes viruses and prevents cross-contamination among plaques. Plaques should be visible by day 4 postinfection. At day 10 postinfection, well-isolated plaques are collected and correct recombinants verified by DNA analysis as described in Section 4.3.

4.5 Preparation of High-Titer Virus Stock (Crude Lysate)

High-titer Ad virus stocks can be prepared by concentrating infected HEK-293 cells as the virus is not released from the cells until very late in infection when the cell lyses. For preparing high-titer stocks, cells can be infected either in monolayer (HEK-293) or suspension culture (HEK-293N3S). Suspension culture is more amenable for large-scale vector production, due to the ease of infected cell collection. However, complete CPE, used frequently to determine the appropriate time to harvest the infected cells, is easier to visualize in monolayer cultures. In this section, we will describe the protocol for achieving high-titer virus stock from both monolayer and suspension cultures.

4.5.1 Preparation of High-Titer Crude Virus Stocks from Monolayer Culture

It is desirable to infect the cells at a multiplicity of infection (MOI) of 1–10 pfu/cell. However, as a close approximation, we generally dilute an infected cell lysate (such

as that generated in Section 4.3) 1:8 in PBS++ and use 1 ml to infect each of eight 150-mm dishes of near confluent HEK-293 cells. Cultures should be examined every day for the appearance of CPE. When most of the cells are rounded up (but not all detached), scrape the adherent cells and combine with the cells in suspension. Pellet the infected cells, and combine pellets from all eight plates, resuspending in 8 ml of PBS++ with 10% glycerol. Aliquot and store at –80 °C. Perform three freeze–thaw cycles to release the virus prior to use for infection or further amplification. To purify virus by CsCl banding (see Section 4.6 below), this crude lysate should be used for one further round of amplification in forty 150-mm dishes, and the final infected cell pellet resuspended in 15 ml 0.1 M Tris, pH 8, and stored at −80 °C.

4.5.2 *Preparation of High-Titer Crude Virus Stocks from Suspension Culture*

Suspension cultures of HEK-293N3S,[86] a derivative of HEK-293 cells, can be used for large-scale preparation of Ad vectors. HEK-293N3S cells are most conveniently maintained as a semiadherent monolayer culture until expansion is desired. Three 150-mm dishes of nearly confluent HEK-293N3S cells are sufficient to establish a 500-ml suspension culture in Joklik's modified MEM supplemented with 10% horse serum. This suspension culture can be expanded by diluting 1:2 or 1:3 when the cell density reaches ~5×10^5 cells/ml. A 4 l culture is generally sufficient to prepare enough vector for CsCl gradient purification. To infect HEK-293N3S cells, the culture is centrifuged and resuspended in 0.1 vol of fresh medium, and then inoculated with virus (MOI of 1–20 pfu/cell). After gentle stirring for 1 h, the culture is brought to its original volume with fresh medium and incubation continued. Unlike infections in monolayer cultures, CPE in infected suspension cultures cannot be simply visualized under a microscope. In order to determine the optimal time for harvesting the infected cells, a small sample is taken from the suspension culture daily and examined for the presence of inclusion bodies by orcein staining. Late in infection, inclusion bodies appear as densely stained nuclear structures that result from the accumulation of a large amount of viral products. Uninfected cells should be used as a negative control for staining. When the inclusion bodies are visible in 80–90% cells (usually at day 3, depending on the MOI used), cells are harvested by centrifugation and resuspended in 20 ml PBS++ supplemented with 10% glycerol and stored at −80 °C. For purification by CsCl banding, the final infected cell pellet should be resuspended in 15 ml 0.1 M Tris, pH 8, and stored at −80 °C.

4.6 *Purification of High-Titer Adenovirus Vector by CsCl Banding*

CsCl gradient purification is commonly used to purify and to concentrate adenovirus. Although crude virus stocks can be used for some in vitro experiments, the virus must be purified for other experiments, particularly in vivo work. The CsCl banding described here can be used for the purification of crude lysate from a 4 l suspension culture (HEK-293N3S) or thirty to forty 150-mm dishes of monolayer cultures (HEK-293), which have similar virus yields. Infection and collection of crude lysates

from suspension and monolayer cultures are described in Section 4.5. The infected cell lysate is subjected to three freeze–thaw cycles, and then sodium deoxycholate is added to a final concentration of 3.75%. After 30 min at room temperature, the solution should be highly viscous, and all virus particles should be released from the cells. The lysate is digested with DNase I to reduce viscosity, and then clarified by centrifugation. The supernatant is carefully layered over a three-stage CsCl step gradient prepared by layering CsCl solutions at densities of 1.25d and 1.35d (each at about half the volume of the lysate) over a 1.5d cushion of CsCl solution. Spin at 20,000*g* for 1 h. Collect the virus band at the interface between 1.35d and 1.25d, pool all tubes from the same virus preparation, and recentrifuge overnight. The virus, visible as a turbid band, should be collected in the smallest possible volume and then dialyzed against 10 mM Tris, pH 8, or desalted by column chromatography (e.g., PD-10) and glycerol added to a final concentration of 10%. Depending on the application, other storage buffers may also be appropriate.[87] Store the purified virus in small aliquots at −80 °C.

4.7 Characterization of Adenovirus Vectors

After preparation of the viral vector, the DNA structure should be confirmed, the titer of virus particles and infectious units should be determined, transgene expression should be ascertained, and the stock must be tested for the presence of RCA.

The identity of the recombinant vector can be verified by restriction enzyme analysis as described in Section 4.3, using 0.025 ml purified virus as starting material instead of infected cells. The vector preparation can be titrated using the classical plaque assay or using commercially available kits. For titration, plaque assays (described in Section 4.4) should be carried out with a broad range of virus dilutions (10^{-4} to 10^{-10}). Plaques are usually counted 10 days postinfection. Alternatively, virus titers can be determined using the Adeno-X™ rapid titer kit (Clontech, Cat. No. 632250), which detects the viral hexon protein within infected cells. This assay has the advantage of being significantly faster (~48 h) than the plaque assay (~10 days). Determination of viral particle concentration and the test for RCA are described below.

4.7.1 Determination of Particles to Plaque-Forming Units Ratio

In addition to determining the concentration of infectious vector (pfu/ml), it is necessary to determine the concentration of virus particles, including noninfectious particles, especially if the vector is to be used in humans. In fact, the FDA recommends that patient doses be calculated on the basis of virus particles rather than the infectious particles.[88] This recommendation is based on two important facts. First, the determination of virus particle is based on physical measurement, and hence is more precise than the determination of infectious particles. Second, a primary toxicity of Ad vectors is from the innate immune response directed against the viral coat, which is dependent on particle number and largely independent of transgene expression. There are many methods to determine the concentration of virus particles such as anion exchange high-performance liquid chromatography (HPLC), measurement of virion DNA using

a DNA-binding dye (e.g., PicoGreen), reverse-phase HPLC analysis of viral protein components, and spectrophotometric analysis after solubilizing the vector.[88] To determine the concentration of virus particles by spectrophotometric analysis, purified virus is diluted in Tris–EDTA buffer supplemented with 0.1% SDS and heated to 56 °C for 10 min, and the OD_{260} is determined using a UV spectrophotometer. Based on the extinction coefficient of wild-type Ad as determined by Maizel et al.,[89] the concentration of viral particles is calculated as follows:

$$\text{Particles/ml} = (OD_{260})\ (\text{dilution factor})\ (1.1 \times 10^{12}).$$

The particle:pfu ratio is between 20:1 and 80:1 for most Ad vector preparations.

4.7.2 Replication-Competent Adenovirus Assay

HEK-293 cells,[39] for many years the only cell line that would support growth of E1-deleted Ad vectors, are transformed with the left end of Ad5 (viral nucleotide sequence 1–4344) that includes the E1 region.[39] During the propagation of Ad vectors in HEK-293 cells, viral sequences in the HEK-293 cells may recombine with viral sequences in the vector, producing E1-positive RCA. Although the frequency with which the recombination occurs is not known, RCA is likely to replicate faster than many E1-deleted vectors in HEK-293 cells. Therefore, prolonged propagation of the vector may increase the proportion of RCA in the vector preparation, and should be avoided. To minimize RCA contamination, one should scale up vector production from a single plaque to large-scale culture in as few steps as possible. RCA contamination is considered a safety issue especially if the vector will be used clinically. The FDA recommends that there be no more than one RCA in 10^9 infectious adenovirus virions in a clinical stock.[88] Several different approaches have been developed for the detection of RCA, including Southern blot hybridization, quantitative polymerase chain reaction, and biological assay.[88]

The biological assay used for RCA detection in our lab is based on the induction of CPE in the non-E1-complementing A549 cell line following infection with the test vector. Infection with Ad, even in the absence of RCA, will frequently result in death of the initially infected A549 monolayer due to toxicity of viral proteins in the inoculum. Therefore, the RCA test is carried out in two stages. In the first stage, 150-mm dishes of A549 cells are infected with 10^6, 10^7, or 10^8 pfu vector (one dish per virus amount). Greater amounts of vector can be tested if detection of RCA at higher sensitivity is required. One week after infection, or sooner if most of the culture shows CPE, the infected cultures (monolayer plus medium) are harvested. The harvested cultures are taken through three freeze–thaw cycles, and then 1 ml of each lysate is used to infect a fresh dish of A549 cells. This second round of infection is observed for 3 weeks, replacing medium every 5 days. Any signs of CPE in this second stage would indicate the presence of RCA. If no CPE is observed then the original inoculum of vector used to generate the lysate would have been free from RCA. If CPE is observed in any plate, then viral DNA can be recovered from that plate and analyzed by agarose gel electrophoresis after digestion with appropriate restriction enzymes for further confirmation (as described in Section 4.3).

5. High-Efficiency Construction of Adenovirus Vectors for Generating Adenovirus-Based cDNA Expression Libraries

The human genome project revealed the presence of an estimated 25,000 genes and a much larger number of proteins encoded by these genes.[90] The functions of the majority of these gene products are unknown. It is important to identify the activities of the genes, especially those whose products play a role in human disease, as these genes could be potential targets for therapy. However, identification of the full spectrum of a gene's function is very difficult partly due to complex interactions of the gene product with other proteins and factors that vary depending on the specific cell type or developmental stage under study. Thus, there is a need for a highly efficient mammalian expression system that would facilitate cloning and direct determination of gene function on a genomic scale in cell-based assays.

The ability of Ad to efficiently transduce a wide variety of cell types including primary cells makes it an ideal vector for cDNA delivery in functional assays. Other desirable features for an expression library vector are that the method of construction should yield only recombinant vectors (with minimal wild-type or parental vector contamination) and the method should produce a large number of clones.[91] Although several methods of Ad vector construction, including the two-plasmid rescue systems discussed in earlier sections, generate recombinant vectors that are free from contamination with parental virus, the efficiency of vector rescue with these methods is not sufficient for generating a cDNA expression library of high complexity. Fewer than 100 plaques per microgram of vector DNA are obtained at best, using the conventional methods of Ad vector construction.[91]

Mammalian cells are speculated to contain ~10^5 mRNA species; thus, at least 10^6 independent clones must be produced for adequate representation of all transcripts in the cDNA library. Construction of such a complex population of recombinant Ad vectors by conventional methods would not be feasible. The low efficiency of transgene rescue following transfection with a plasmid-derived vector genome is thought to be partly due to low infectivity of cloned viral DNA. It has been found that plasmid-derived viral DNA is ~1000-fold less efficient than virion DNA in producing infectious virus. This huge difference is due to the absence of terminal protein (TP) in the cloned viral genome. In the virion, TP is bound covalently to both ends of the linear Ad genome and plays an important role in enhancing infectivity and template efficiency for viral DNA replication.[92,93] Miyake et al. developed an efficient method for construction of Ad vectors that employs viral DNA termini complexed to TP as a substrate in the generation of recombinants.[94] In this study, they inserted the transgene at a unique site in the full-length viral genome carried in a cosmid backbone. The cosmid vector was used to cotransfect HEK-293 cells together with TP-bound virion DNA cleaved at several sites with a restriction enzyme to reduce recovery of nonrecombinant virions. The use of DNA–TP complexes greatly increased the efficiency of vector generation: several hundred plaques were formed per microgram of viral DNA–TP complex. However, only a fraction of the resulting clones were the desired

recombinants, suggesting that fragmentation of the Ad-TP donor genome is not sufficient to prevent its contamination of the recombinant pool.

In order to more easily identify and recover recombinant clones, reporter genes can be either incorporated in the parental viral genome, such that it is lost following transgene rescue and can be used for negative selection, or incorporated in the shuttle plasmid, such that it is rescued along with the transgene and can be used for positive selection. Schaack et al. employed the *E. coli LacZ* gene for positive selection of Ad clones expressing their gene of interest.[70] The use of reporter genes for the selection of positive clones is useful but such screening would be time- and reagent-consuming if making a library of several thousand clones. This selection system is further hampered by the fact that often recombinant vectors have a growth disadvantage relative to the parental virus, which makes the isolation of recombinant clones from a library difficult unless the clones are positively selected for growth.

Elahi et al. (2002) developed a positive selection system that should be compatible with generation of a large number of recombinant Ad clones.[91] In this study they made use of the essential late Ad protease (PS) gene. Ad deleted of the PS gene can undergo only one round of DNA replication in HEK-293 cells. For this strategy, the authors made a shuttle plasmid bearing the left end of Ad and containing, in place of E1, a bicistronic expression cassette incorporating the PS gene and the transgene. HEK-293 cells were transfected with this shuttle plasmid following infection with a PS-deleted full-length Ad. Because the PS-deleted viral genome cannot go beyond one round of replication, only recombinant vectors that have acquired the PS gene can result in productive infection. With this interesting and scalable system, virtually all of the recovered viruses are recombinant vectors with a diversity predicted to be as high as one million clones.

Hatanaka et al. (2003) developed a Cre–*loxP*-based recombination system for the generation of an Ad cDNA expression library.[95] Unlike all the strategies discussed above, the recombination event in this method takes place in vitro. First, a cDNA library was constructed in an Ad shuttle plasmid background, with a *loxP* site just downstream of the cDNA cassette. A pool of linearized shuttle plasmids was added in vitro to a complex of TP and viral DNA deleted of the left end and the mixture was treated with Cre recombinase. The resulting recombinant DNA was then used to transfect HEK-293 cells to obtain a library of infectious vectors. Using this system the authors were able to isolate cDNA for CD2 (present at a frequency of less than 1 in 3000 T cell transcripts) from human T cells.

All the methods discussed above provided evidence that construction of Ad cDNA expression libraries is feasible. However, one common drawback in all the approaches for creating Ad-based cDNA expression libraries is that they are technically demanding and time-consuming. In 2006, Hillgenberg et al. modified the previously developed Cre–*loxP*-based Ad construction methods to generate ~10^6 independent clones of recombinant Ad[96] in a short time. In this system the shuttle plasmid carries the viral 5′ ITR, complete viral packaging signal, the cDNA expression cassette, and a single *loxP* site. A mixture of shuttle plasmids is used to transfect HEK-293 cells expressing Cre recombinase that have been infected with donor Ad attenuated by partial deletion of the packaging signal, which is flanked by *loxP* sites. Site-specific recombination

causes excision of the packaging signal from the donor virus rendering it completely nonpackageable. A second recombination between the *loxP* site in shuttle plasmid and the *loxP* site in the donor virus rescues the transgene and packaging signal, resulting in an infectious recombinant vector. Individual clones are then identified and purified by plaque assay. The residual donor viruses are counterselected during the amplification of recombinant vectors because of their impaired growth. This rapid, efficient, and elegant construction system should prove very useful for the production of cDNA expression libraries of sufficient complexity for identification of gene function in cell-based assays.

6. Conclusion

As described in this chapter, the two-plasmid rescue system using mammalian cells, particularly HEK-293, is one of the earliest and most commonly used methods for the construction of Ad vectors. The two-plasmid rescue system depends on recombination between a shuttle and a genomic plasmid. Replacement of homologous recombination with site-specific recombination as a means to rescue the transgene into the vector has greatly increased the efficiency of recombinant virus production. With advancements made in the construction process, as discussed in the last section of this chapter, it is now feasible to construct Ad-based human cDNA libraries in a short time.

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References

1. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* 1953;**84**:570–3.
2. Shenk T. Adenoviridae: the viruses and their replication. In: Fields BN, Knipe DM, Howley PM, editors. *Virology*. 3rd ed. Philadelphia: Lippincott-Raven Publishers; 1996. p. 2111–48.
3. Wadell G, Hammarskjold ML, Winberg G, Varsanyi TM, Sundell G. Genetic variability of adenoviruses. *Ann NY Acad Sci* 1980;**354**:16–42.
4. McConnell MJ, Imperiale MJ. Biology of adenovirus and its use as a vector for gene therapy. *Hum Gene Ther* 2004;**15**:1022–33.
5. Berget SM, Moore C, Sharp PA. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc Natl Acad Sci USA* 1977;**74**:3171–5.
6. Lewis Jr AM, Baum SG, Prigge KO, Rowe WP. Occurrence of adenovirus-SV40 hybrids among monkey kidney cell adapted strains of adenovirus. *Proc Soc Exp Biol Med* 1966;**122**:214–8.

7. Pierce WE, Rosenbaum MJ, Edwards EA, Peckinpaugh RO, Jackson GG. Live and inactivated adenovirus vaccines for the prevention of acute respiratory illness in naval recruits. *Am J Epidemiol* 1968;**87**:237–46.
8. Lewis Jr AM, Rowe WP. Isolation of two plaque variants from the adenovirus type 2-simian virus 40 hybrid population which differ in their efficiency in yielding simian virus 40. *J Virol* 1970;**5**:413–20.
9. Sadeghi H, Hitt MM. Transcriptionally targeted adenovirus vectors. *Curr Gene Ther* 2005;**5**:411–27.
10. *J Genet Med* 2014. Accessed at:www.wiley.co.uk/genmed/clinical.
11. Douglas JT. Adenoviral vectors for gene therapy. *Mol Biotechnol* 2007;**36**:71–80.
12. Defer C, Belin MT, Caillet-Boudin ML, Boulanger P. Human adenovirus-host cell interactions: comparative study with members of subgroups B and C. *J Virol* 1990;**64**:3661–73.
13. Wang K, Guan T, Cheresh DA, Nemerow GR. Regulation of adenovirus membrane penetration by the cytoplasmic tail of integrin beta5. *J Virol* 2000;**74**:2731–9.
14. Ambriovic-Ristov A, Gabrilovac J, Cimbora-Zovko T, Osmak M. Increased adenoviral transduction efficacy in human laryngeal carcinoma cells resistant to cisplatin is associated with increased expression of integrin alphavbeta3 and coxsackie adenovirus receptor. *Int J Cancer J Int Cancer* 2004;**110**:660–7.
15. Kelkar SA, Pfister KK, Crystal RG, Leopold PL. Cytoplasmic dynein mediates adenovirus binding to microtubules. *J Virol* 2004;**78**:10122–32.
16. Liu H, Naismith JH, Hay RT. Adenovirus DNA replication. *Curr Top Microbiol Immunol* 2003;**272**:131–64.
17. Wolfson J, Dressler D. Adenovirus-2 DNA contains an inverted terminal repetition. *Proc Natl Acad Sci USA* 1972;**69**:3054–7.
18. Garon CF, Berry KW, Rose JA. A unique form of terminal redundancy in adenovirus DNA molecules. *Proc Natl Acad Sci USA* 1972;**69**:2391–5.
19. Ostapchuk P, Hearing P. Minimal cis-acting elements required for adenovirus genome packaging. *J Virol* 2003;**77**:5127–35.
20. Horwitz MS. Adenoviridae: the viruses and their replication. In: Fields BN, Knipe DM, et al., editors. *Virology*. 2nd ed. New York: Raven Press; 1990. p. 1679–721.
21. Thomas GP, Mathews MB. DNA replication and the early to late transition in adenovirus infection. *Cell* 1980;**22**:523–33.
22. Moran E, Mathews MB. Multiple functional domains in the adenovirus E1A gene. *Cell* 1987;**48**:177–8.
23. Akusjarvi G, Pettersson Ulf, Roberts Richard J. Structure and function of the adenovirus-2 genome. *Adenovirus DNA Viral Genome Expr* 1986:53–96.
24. Pilder S, Moore M, Logan J, Shenk T. The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol Cell Biol* 1986;**6**:470–6.
25. Moran E. Interaction of adenoviral proteins with pRB and p53. *FASEB J Off Publ Fed Am Soc Exp Biol* 1993;**7**:880–5.
26. Graham FL. Transformation by and oncogenicity of human adenoviruses. In: Ginsberg HS, editor. *The adenoviruses*. New York (NY): Plenum Press; 1984. p. 339–98.
27. Green M, Wold WS, Brackmann KH, Cartas MA. Identification of families of overlapping polypeptides coded by early "transforming" gene region 1 of human adenovirus type 2. *Virology* 1979;**97**:275–86.
28. Jared D, Evans PH. Adenovirus replication. In: Curiel DT, editor. *Adenoviral vectors for gene therapy*. (USA): Academic Press; 2002. p. 39–70.
29. Moore M, Horikoshi N, Shenk T. Oncogenic potential of the adenovirus E4orf6 protein. *Proc Natl Acad Sci USA* 1996;**93**:11295–301.

30. Bett AJ, Prevec L, Graham FL. Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* 1993;**67**:5911–21.
31. Ng P, Graham FL. Adenoviral vector construction I: mammalian systems. In: Curiel DT, Douglas JT, editors. *Adenoviral vectors for gene therapy*. 1st ed. San Diego (California): Academic Press; 2002. p. 71–104.
32. Danthinne X, Imperiale MJ. Production of first generation adenovirus vectors: a review. *Gene Ther* 2000;**7**:1707–14.
33. Lubeck MD, Davis AR, Chengalvala M, Natuk RJ, Morin JE, Molnar-Kimber K, et al. Immunogenicity and efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus. *Proc Natl Acad Sci USA* 1989;**86**:6763–7.
34. Torres JM, Alonso C, Ortega A, Mittal S, Graham F, Enjuanes L. Tropism of human adenovirus type 5-based vectors in swine and their ability to protect against transmissible gastroenteritis coronavirus. *J Virol* 1996;**70**:3770–80.
35. Pacini DL, Dubovi EJ, Clyde Jr WA. A new animal model for human respiratory tract disease due to adenovirus. *J Infect Dis* 1984;**150**:92–7.
36. Trentin JJ, Yabe Y, Taylor G. The quest for human cancer viruses. *Science* 1962;**137**:835–41.
37. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol* 1977;**36**:59–74.
38. Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973;**52**:456–67.
39. Louis N, Evelegh C, Graham FL. Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. *Virology* 1997;**233**:423–9.
40. Berk AJ. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene* 2005;**24**:7673–85.
41. Shaw G, Morse S, Ararat M, Graham FL. Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *FASEB J Off Publ Fed Am Soc Exp Biol* 2002;**16**:869–71.
42. Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, et al. New helper cells and matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses. *Hum Gene Ther* 1998;**9**:1909–17.
43. Imler JL, Chartier C, Dreyer D, Dieterle A, Sainte-Marie M, Faure T, et al. Novel complementation cell lines derived from human lung carcinoma A549 cells support the growth of E1-deleted adenovirus vectors. *Gene Ther* 1996;**3**:75–84.
44. Schiedner G, Hertel S, Kochanek S. Efficient transformation of primary human amniocytes by E1 functions of Ad5: generation of new cell lines for adenoviral vector production. *Hum Gene Ther* 2000;**11**:2105–16.
45. Rubinchik S, Norris JS, Dong JY. Construction, purification and characterization of adenovirus vectors expressing apoptosis-inducing transgenes. *Methods Enzym* 2002;**346**:529–47.
46. Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. *Eur J Cancer* 1997;**33**:787–91.
47. Gotoh A, Ko SC, Shirakawa T, Cheon J, Kao C, Miyamoto T, et al. Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J Urol* 1998;**160**:220–9.
48. Schrewe H, Thompson J, Bona M, Hefta LJ, Maruya A, Hassauer M, et al. Cloning of the complete gene for carcinoembryonic antigen: analysis of its promoter indicates a region conveying cell type-specific expression. *Mol Cell Biol* 1990;**10**:2738–48.
49. Shi CX, Long MA, Liu L, Graham FL, Gauldie J, Hitt MM. The human SCGB2A2 (mammaglobin-1) promoter/enhancer in a helper-dependent adenovirus vector directs high levels of transgene expression in mammary carcinoma cells but not in normal nonmammary cells. *Mol Ther J Am Soc Gene Ther* 2004;**10**:758–67.

50. Acsadi G, O'Hagan D, Lochmüller H, Prescott S, Larochelle N, Nalbantoglu J, et al. Interferons impair early transgene expression by adenovirus-mediated gene transfer in muscle cells. *J Mol Med Berl* 1998;**76**:442–50.
51. Harms JS, Splitter GA. Interferon-gamma inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter. *Hum Gene Ther* 1995;**6**:1291–7.
52. Qin L, Ding Y, Pahud DR, Chang E, Imperiale MJ, Bromberg JS. Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression. *Hum Gene Ther* 1997;**8**:2019–29.
53. Papadakis ED, Nicklin SA, Baker AH, White SJ. Promoters and control elements: designing expression cassettes for gene therapy. *Curr Gene Ther* 2004;**4**:89–113.
54. Nettelbeck DM, Jerome V, Muller R. A strategy for enhancing the transcriptional activity of weak cell type-specific promoters. *Gene Ther* 1998;**5**:1656–64.
55. Shi CX, Graham FL, Hitt MM. A convenient plasmid system for construction of helper-dependent adenoviral vectors and its application for analysis of the breast-cancer-specific mammaglobin promoter. *J Gene Med* 2006;**8**:442–51.
56. Okuyama T, Fujino M, Li XK, Funeshima N, Kosuga M, Saito I, et al. Efficient Fas-ligand gene expression in rodent liver after intravenous injection of a recombinant adenovirus by the use of a Cre-mediated switching system. *Gene Ther* 1998;**5**:1047–53.
57. Gu J, Zhang L, Huang X, Lin T, Yin M, Xu K, et al. A novel single tetracycline-regulative adenoviral vector for tumor-specific Bax gene expression and cell killing in vitro and in vivo. *Oncogene* 2002;**21**:4757–64.
58. Rendahl KG, Quiroz D, Ladner M, Coyne M, Seltzer J, Manning WC, et al. Tightly regulated long-term erythropoietin expression in vivo using tet-inducible recombinant adeno-associated viral vectors. *Hum Gene Ther* 2002;**13**:335–42.
59. Sipo I, Hurtado Picó A, Wang X, Eberle J, Petersen I, Weger S, et al. An improved Tet-On regulatable FasL-adenovirus vector system for lung cancer therapy. *J Mol Med Berl* 2006;**84**:215–25.
60. Zhao C, Crews CJ, Derdeyn CA, Blackwell JL. Lac-regulated system for generating adenovirus 5 vaccine vectors expressing cytolytic human immunodeficiency virus 1 genes. *J Virol Methods* 2009;**160**:101–10.
61. Matthews DA, Cummings D, Evelegh C, Graham FL, Prevec L. Development and use of a 293 cell line expressing lac repressor for the rescue of recombinant adenoviruses expressing high levels of rabies virus glycoprotein. *J Gen Virol* 1999;**80**(Pt 2):345–53.
62. Wang L, Qi X, Shen R, Sun Y, Tuveson DA. An shRNA silencing a non-toxic transgene reduces nutrient consumption and increases production of adenoviral vectors in a novel packaging cell. *J Cell Physiol* 2009;**219**:365–71.
63. Bruder JT, Appiah A, Kirkman 3rd WM, Chen P, Tian J, Reddy D, et al. Improved production of adenovirus vectors expressing apoptotic transgenes. *Hum Gene Ther* 2000;**11**:139–49.
64. Carlock LR, Jones NC. Transformation-defective mutant of adenovirus type 5 containing a single altered E1a mRNA species. *J Virol* 1981;**40**:657–64.
65. Solnick D. An adenovirus mutant defective in splicing RNA from early region 1A. *Nature* 1981;**291**:508–10.
66. Chinnadurai G, Chinnadurai S, Brusca J. Physical mapping of a large-plaque mutation of adenovirus type 2. *J Virol* 1979;**32**:623–8.
67. Stow ND. Cloning of a DNA fragment from the left-hand terminus of the adenovirus type 2 genome and its use in site-directed mutagenesis. *J Virol* 1981;**37**:171–80.
68. Jones N, Shenk T. Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 1979;**17**:683–9.

69. Kapoor QS, Chinnadurai G. Method for introducing site-specific mutations into adenovirus 2 genome: construction of a small deletion mutant in VA-RNAI gene. *Proc Natl Acad Sci USA* 1981;**78**:2184–8.
70. Schaack J, Langer S, Guo X. Efficient selection of recombinant adenoviruses by vectors that express beta-galactosidase. *J Virol* 1995;**69**:3920–3.
71. Munz PL, Young CS. End-joining of DNA fragments in adenovirus transfection of human cells. *Virology* 1991;**183**:160–9.
72. Mizuguchi H, Kay MA. Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum Gene Ther* 1998;**9**:2577–83.
73. McGrory WJ, Bautista DS, Graham FL. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 1988;**163**:614–7.
74. Berkner KL, Sharp PA. Generation of adenovirus by transfection of plasmids. *Nucleic Acids Res* 1983;**11**:6003–20.
75. Ruben M, Bacchetti S, Graham F. Covalently closed circles of adenovirus 5 DNA. *Nature* 1983;**301**:172–4.
76. Graham FL. Covalently closed circles of human adenovirus DNA are infectious. *EMBO J* 1984;**3**:2917–22.
77. Ghosh-Choudhury G, Haj-Ahmad Y, Graham FL. Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *EMBO J* 1987;**6**:1733–9.
78. Rosa-Calatrava M, Grave L, Puvion-Dutilleul F, Chatton B, Kedinger C. Functional analysis of adenovirus protein IX identifies domains involved in capsid stability, transcriptional activity, and nuclear reorganization. *J Virol* 2001;**75**:7131–41.
79. Bett AJ, Haddara W, Prevec L, Graham FL. An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci USA* 1994;**91**:8802–6.
80. Ng P, Parks RJ, Cummings DT, Evelegh CM, Sankar U, Graham FL. A high-efficiency Cre/loxP-based system for construction of adenoviral vectors. *Hum Gene Ther* 1999;**10**:2667–72.
81. Ng P, Parks RJ, Cummings DT, Evelegh CM, Graham FL. An enhanced system for construction of adenoviral vectors by the two-plasmid rescue method. *Hum Gene Ther* 2000;**11**:693–9.
82. Anton M, Graham FL. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J Virol* 1995;**69**:4600–6.
83. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci USA* 1996;**93**:13565–70.
84. Ng P, Cummings DT, Evelegh CM, Graham FL. Yeast recombinase FLP functions effectively in human cells for construction of adenovirus vectors. *BioTechniques* 2000;**29**:524–6. 528.
85. Addison CL, Hitt M, Kunsken D, Graham FL. Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. *J Gen Virol* 1997;**78**(Pt 7):1653–61.
86. Graham FL. Growth of 293 cells in suspension culture. *J Gen Virol* 1987;**68**(Pt 3):937–40.
87. Croyle MA, Cheng X, Wilson JM. Development of formulations that enhance physical stability of viral vectors for gene therapy. *Gene Ther* 2001;**8**:1281–90.
88. Hutchins B, Sajjadi N, Seaver S, Shepherd A, Bauer SR, Simek S, et al. Working toward an adenoviral vector testing standard. *Mol Ther J Am Soc Gene Ther* 2000;**2**:532–4.
89. Maizel Jr JV, White DO, Scharff MD. The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 1968;**36**:115–25.

90. National Human Genome Research Institute, NIH. 2012. Accessed at: http://www.genome.gov/12011238.
91. Elahi SM, Oualikene W, Naghdi L, O'Connor-McCourt M, Massie B. Adenovirus-based libraries: efficient generation of recombinant adenoviruses by positive selection with the adenovirus protease. *Gene Ther* 2002;**9**:1238–46.
92. Stillman BW. The replication of adenovirus DNA with purified proteins. *Cell* 1983;**35**:7–9.
93. Pronk R, van der Vliet PC. The adenovirus terminal protein influences binding of replication proteins and changes the origin structure. *Nucleic Acids Res* 1993;**21**:2293–300.
94. Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, et al. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci USA* 1996;**93**:1320–4.
95. Hatanaka K, Ohnami S, Yoshida K, Miura Y, Aoyagi K, Sasaki H, et al. A simple and efficient method for constructing an adenoviral cDNA expression library. *Mol Ther J Am Soc Gene Ther* 2003;**8**:158–66.
96. Hillgenberg M, Hofmann C, Stadler H, Loser P. High-efficiency system for the construction of adenovirus vectors and its application to the generation of representative adenovirus-based cDNA expression libraries. *J Virol* 2006;**80**:5435–50.