Multilevel Targeting of Canine Adenovirus Type 2 (CAV2) to Expand Oncolytic Virotherapy in Canine Tumors

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
May 02, 2020

Keywords: CAV2, transductional targeting, transcriptional targeting, CRISPR/Cas9

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Abstract

Oncolytic virotherapy is a promising therapeutic approach designed to selectively eradicate cancer cells but to spare normal cells. Oncolytic adenoviruses (Ad) are an outstanding and common vehicle for efficient cancer gene therapy. Selection of appropriate intermediate animal models is a basic requirement for successful cancer virotherapy. Some unique characteristics such as inter-individual and intratumoral heterogeneity, similar genomic sequence and instability to humans, and similar environmental exposures validate the dog as a suitable intermediate animal model of cancer and other complex human diseases. Although adenoviruses are the most commonly used viral vector for gene therapy, several concerns, such as normal cell/organ toxicity, lack of suitable cell surface receptors to allow viral entry to the desired cancer cell, and activation of both innate and adaptive immune systems in patients, restrict the successful clinical application of these vectors. Hence, new approaches to improve the transductional and transcriptional targeting efficacy of Ad are required for adapting the infectivity of adenovirus in disseminated canine tumors such as multicentric lymphoma, which can serve as a model for human non-Hodgkin's lymphoma (NHL). Our goal is to explore mechanisms to target canine tumors at both the level of transduction (and infection) and transcription. Targeted modification of several viral components, such as the viral capsid, fiber knob, and the insertion of transgenes for expression, are prerequisites for conducting the necessary transductional and transcriptional targeting of adenovirus. However, the conventional approach to modify the adenoviral genome is time consuming and expensive. It is solely dependent on the presence of unique restriction enzyme sites that may or may not be present in the target site. Clustered regularly interspaced short palindromic repeat (CRISPR) along with an RNA-

guided nuclease called Cas9 (CRISPR/Cas9) is one of the most powerful tools that has been adopted for precise genome editing in various cells and organisms. Thus, we utilized *in vitro* CRISPR/Cas9-mediated editing of the canine adenovirus type 2 (CAV2) genome to promote targeted modification in the viral genome. We have conducted CRISPR/Cas9-mediated insertional mutagenesis and successfully inserted the RFP (red fluorescent protein) reporter construct into the CAV2 genome. We have also utilized the CRISPR/Cas9 system to conduct wild type gene replacement with our desired ligand in the CAV-2 genome to construct tumor-targeted vectors. Results demonstrated high efficiency and accuracy for *in vitro* CRISPR-mediated editing of the large CAV2 genome. For transcriptional targeting, several previously identified tumor up-regulated promoters, CXCR4, Survivin and TERT along with an E2F modified Canine Adenovirus 2 E1A promoter (EEE), were tested for their expression in various canine tumor cells/tissues by employing RT-qPCR and GFP reporter gene expression levels to measure endogenous and exogenous promoter activity, respectively. Endogenous expression levels measured for cTERT, cSurvivin, and cCXCR4 were low for all three, with some non-malignant and some tumor cell lines and tissues expressing the gene. Expression levels from exogenously supplied promoters were measured by both the number of cells expressing the construct and the intensity of expression in individual cells. Exogenously supplied promoters were active in more cells in all tumor lines than in normal cells, with the EEE promoter being most active, followed by cTERT. The intensity of expression varied more with cell type than with specific promoters. Ultimately, no single promoter was identified that would result in reliable expression, regardless of the tumor type. Thus, these findings imply that the three investigated promoters are unsuitable for use as lymphoma-specific promoters. In

addition, this data raises the concern that endogenous expression analysis may not accurately predict exogenous promoter activity. We hope that these approaches and findings will help us to construct oncolytic CAV-2 suitable to eliminate current obstacles for oncolytic virotherapy in multicentric canine tumors such as lymphoma. In addition, these approaches may be translated to human patients and will allow adenoviruses to be successfully utilized as gene therapy vectors for a wide range of autoimmune and other diseases beyond hematopoietic malignancies.

Acknowledgments

I would like to express my sincere gratitude to my major professor Dr. Bruce F. Smith for his continuous supervision and untiring support throughout my PhD study at Auburn University. I am grateful for getting the opportunity to work with a mentor like him. He is one of my role models and a source of inspiration. I am also grateful to my advisory committee members including Dr. Richard C. Bird, Dr. Douglas Martin, and Dr. Jey Koehler for their insightful discussion about this research work. I am also grateful to Dr. Maninder Sandey for his guidelines and support. Special gratitude is extended to my sister and co-worker Dr. Payal Agarwal for her relentless support, direction, inspiration and love. Special thanks to my co-workers Rebecca Nance, Taylor Flat, Damien Keola, Samantha Morici, Bradley Schruler, and Will Kretzschmar for their friendship and support. I am also grateful to Allison C. Bird for her sincere support with flow cytometry facilities. I am also grateful to Dr. Shamsur Rahman for providing support for statistical analysis.

I am also grateful to my family members especially to my father Abdul Muhit and to my beloved brother Abdul Mahib Tanvir for their continuous support throughout my life. I also grateful to my brother Liton Ahmed, Shipon Ahmed, Sumon Ahmed and my sisters Laizu Begum and Jennifer Akhter for their inspiration, love and support. I am also grateful to my big brothers and friends in Bangladesh (especially Nawsher Ahmed Shikder, Tanvir Ahmad Jummon, Fardous Chowdhury, Muntakim Chowdhury and Nila) as well as my friends and family in Auburn for their love, inspiration, mental and financial support.

Finally I would like to dedicate this dissertation to my beloved mother, Munira Bakul Belly and to my beloved wife Halima Begum for their inspiration, dedication,

continuous mental and physical support as well as unconditional love to turn my day of hardship into a day of smiles.

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List of Abbreviations

AA	Amino Acid
ADP	Adenoviral Death Protein
Ad	Adenoviruses
AFP	Alpha Fetoprotein Promoter
ALL	Acute Lymphoblastic Leukemia
Amp^r	Ampicillin Resistant
AML	Acute Myeloid Leukemia
BAd3	Bovine Adenovirus 3
BM	Bone Marrow
CAR	Coxsackievirus and Adenovirus Receptor
CAV2	Canine Adenovirus Type 2
Cas9	CRISPR Associated Protein 9
CDP	CCAAAT Displacement Protein
CEA	Carcino Embryonic Antigen
CLL	Chronic Lymphoblastic Leukemia
CMV	Cytomegalovirus
CPE	Cytopathic Effect
CML	Canine Melanoma
CMT	Canine Mammary Tumor
CR	Conserved Region
CRAd	Conditionally Replicating Adenovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats

Cryo-EM	Cryo-Electron Microscopy
CSC	Cancer Stem Cell
CTLs	Cytotoxic Lymphocytes
CXCR4	C-X-C Motif Chemokine Receptor 4
DK	Dog Kidney
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
dsDNA	Double Stranded DNA
DsRed	*Discosoma* species derived Red fluorescence
EEE	E2F Enhanced E1A
eGFP	Enhanced Green Fluorescent Protein
EGFR	Epidermal Growth Factor Receptor
EPCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
FBS	Fetal Bovine Serum
FDK	Fetal Dog Kidney
FF	Fiber Fibritin
FGF	Fibroblast Growth Factor
FX	Factor X
GFP	Green Fluorescent Protein
GON	Group of Nine
GOS	Group Of Six
GOI	Gene Of Interest

HAd	Human Adenovirus
hCEA	Human Carcino Embryonic Antigen
HDR	Homology Directed Repair
HEK	Human Embryonic kidney
HER	Human Epidermal Growth Factor Receptor
HIF	Hypoxia Inducible Factor
HM	Hematologic Malignancy
HR	Homologous Recombination
His	Histidine
HIV	Human Immunodeficiency Virus
Hl	Hypervariable Loop
HRE	Hypoxia Response Element
HSPG	Heparan Sulphate Proteoglycan
HSV	Herpes Simplex Virus
HSV-Tk	HSV Thymidine Kinase
HVR	Hyper Variable Region
ITR	Inverted Terminal Repeat
JAM	Junction Adhesion Molecule
KDR	Kinase Insert Domain Receptor
LPS	Lipopolysaccharide
LITR	Left Inverted Terminal Repeat
MIA	Melanoma Inhibitory Activity
MDCK	Madin Darby Canine Kidney

MHC	Major Histocompatibility Complex
MFI	Mean Fluorescence Intensity
MLP	Major Late Promoter
MLTF	Major Late Transcription Factor
MMEJ	Microhomology-Mediated End Joining
nAb	Neutralizing Antibody
NEB	New England Biolab
NCF	Normal Canine Fibroblast
NFI	Nuclear Factor I
NHEJ	Non-homologous End Joining
NM	Nuclear Membrane
OC	Osteosarcoma
OV	Oncolytic Virus
PAM	Protospacer Adjacent Motif
PAMP	Pathogen Associated Molecular Pattern
PB	Peripheral Blood
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PEG	Progression Elevated Gene
PSA	Prostate Specific Antigen
pTP	Precursor Terminal Protein
Q-PCR	Quantitative Polymerase Chain Reaction
RB	Retinoblastoma

RPMI	Roswell Park Memorial Institute
RITR	Right Inverted Terminal Repeat
RSV	Rous Sarcoma Virus
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SLIC	Sequence and Ligation Independent Cloning
scFv	Single Chain Variable Domain
sCAR	Soluble Coxsackievirus and Adenovirus Receptor
sgRNA	Synthetic Guide RNA
TAT	Transactivator of Transcription
TERT	Telomerase Reverse Transcriptase
TF	Transcription Factor
TME	Tumor Microenvironment
TNF-α	Tumor Necrosis Factor alpha
TP	Terminal Protein
TRP-1	Tyrosine Related Protein 1
TUP	Tumor Upregulated Promoter
VA	Virus Associated
VCAM	Vascular Cell Adhesion Molecule
VEGFR	Vascular Endothelial Growth Factor Receptor
VHH	Variable Heavy Chain
VSV	Vesicular Stomatitis Virus
YAP	Yeast Artificial Plasmid

CHAPTER 1

Review of Literature

1.1 Introduction

Cancer is a complex, dynamic and multistep process that, in most cases, results from multiple cumulative genetic and epigenetic changes leading to uncontrolled cell growth [1]. Cancer is considered as one of the major causes of death worldwide with an estimation for 606,520 total cancer deaths in 2020 (about 1660 cancer deaths per day) in the United States (https://cancerstatisticscenter.cancer.org/#!/). The uncontrolled growth in cancer is dictated by six unique physiological changes (also known as hallmarks of cancer) which are self-sufficiency in growth signals, lacking response to antigrowth signals provided by tumor suppressors, invasion and metastases to different tissues, evasion of cellular apoptotic mechanism, tumor angiogenesis and unlimited replication potential. Cancer cells possess the unique capability of deregulating cell growth signals that are carefully regulated in normal cells. In addition, it also possesses the capability to circumvent the negative cell regulatory signals provided by tumor suppressor genes by inducing gain- or loss-of-function mutations in these essential genes. In a healthy individual, cancer development is resisted by incorporating programed cell death by apoptosis to remove damaged or dysfunctional cells. But, cancer cells can evade apoptosis by disrupting the pro- and anti-apoptotic signaling cascades that play important roles in promoting programmed cell death in normal cells. Normal cells are able to go through a limited number of cell-growth-division cycles and once a cell reaches that cycle they enter

into a state called “senescence” where they remain viable but become non-proliferative in nature. In contrast, cancer cells possess unlimited replicative potential to resist cell senescence and become immortal.

Another important hallmark of cancer includes the invasive nature of cancer cells to spread to secondary sites, also known as metastasis, leading to local destruction and loss of normal tissue function [2]. Cancer cells can either contain multiple copies of the same cells or distinct subpopulations of tumor cells having unique characteristics within a single neoplasm, a characteristics also known as “tumor heterogeneity”. Thus, cancer is robust and functionally redundant in nature promoting its survival under adverse conditions, such as those caused by anticancer therapy, leading to recurrence of the tumor with metastatic potential fueled by the surviving subpopulations of tumor cells [3].

The classical regimen for cancer therapy includes chemotherapy, radiotherapy and immunotherapy. Each has shown promise in killing most cancer cells but failing to kill cancer stem cells (CSCs) [4]. As a result, they possess several drawbacks such as limited therapeutic efficacy, generation of drug resistance, failure to promote complete tumor regression and a plethora of treatment related serious side-effects. In addition, the responses to conventional anti-tumor therapies are also below the level of expectation for recurrent and metastatic cancer [5, 6]. Despite the remarkable improvements in cancer treatment modalities where patients show an initial response to the treatment, development of drug resistance followed by tumor recurrence are common. Thus, new alternative therapies for solid, hematologic and disseminated metastatic cancer are urgently needed

with a focus on developing tumor-selective therapeutic interventions having a large therapeutic efficacy with little or no toxicity for normal cells.

1.1.1 Oncolytic virotherapy in cancer

Gene therapy is a strategy for the removal or replacement of a defective gene with a functional one and has been established as a therapeutic strategy to treat various types of diseases such as cancer, cardiovascular, neurological, hematological, monogenic inherited and infectious diseases. By 2019, a total of 3001 clinical gene therapy trials have been initiated and a majority of these (66%) have been developed for cancer gene therapy. (Journal of Gene Medicine, http://www.abedia.com/wiley/indications.php). Cancer gene therapy incorporates several approaches such as replacement of an abnormal gene with a normal one, silencing of an overexpressed gene, insertion of genes to sensitize the cancer cells for conventional therapy, or direct killing of cancer cells by utilizing cancer immunotherapy or oncolytic viruses.

Oncolytic viruses (OVs) are self-replicating entities that possess the potential to infect, replicate and kill malignant cells with high efficiency with a single dose. Several aspects of the tumor microenvironment, including aberrant immune system subversion of anti-tumor immune responses, favor the selective growth and survival of various oncolytic viruses such as vesicular stomatitis virus, herpes simplex virus, adenovirus, etc. leading to killing of cancer cells and promotion of an antineoplastic effect [7, 8]. The replication of OVs can be regulated in a tumor selective manner facilitating the elimination of tumor cells without hampering normal cells, leading to reduced side effects. In addition, engineered OVs can promote both specific and non-specific anti-tumor immunity to promote cell

mediated immunotherapy against cancer [9]. OVs can also be engineered to express a diverse set of immunostimulatory and proapoptotic therapeutic transgenes to promote target-specific cancer cell killing [10, 11] . The tumor microenvironment is also known to promote tumor angiogenesis and OVs have been reported to promote efficient and rapid collapse tumor angiogenesis by blocking vascular growth factors. OVs have been shown to kill drug resistant CSCs, facilitating reduction in tumor recurrence. For example, Mato-Berciano *et al.* developed an oncolytic virus containing a Notch responsive element driving viral replication that showed effective killing of chemo- and radiation resistant pancreatic CSCs [12, 13].

Due to the robust potential of oncolytic virotherapy in cancer, a diverse array of virus families have been utilized for oncolytic virotherapy that include *Rhabdoviridae, Adenoviridae, Herpesviridae, Poxviridea*. These viruses have the potential to promote selective killing of various tumor cells but to spare normal cells [14]. Among all the viruses, human adenoviruses (including both replication incompetent and oncolytic adenoviruses) are the most promising vectors for gene therapy and account for 18% of nearly 3001 clinical trials that were conducted by using both viral and non-viral vectors, where most of the trails are for the treatment of cancer (Journal of Gene Medicine, http://www.abedia.com/wiley/vectors.php). Several advantages such as high titers, efficient transgene delivery and expression, lack of insertional mutagenesis, and transduction of both dividing and quiescent cells, have made adenovirus an outstanding vehicle for cancer gene therapy [15]. In addition, Ad-based therapeutic regimes have also been demonstrated safe for administration to human patients in clinical trials. However, successful application of adenovirus requires several modifications to promote viral entry

as well as viral replication followed by transgene expression in a tumor-specific manner. In addition, human adenoviruses have shown enhanced liver tropism as well as rapid viral clearance by preexisting immune responses, resulting in low therapeutic efficacy after adenoviral mediated cancer gene therapy and, thus, requiring improvement [16]. In order to circumvent immune-related drawbacks several non-human adenoviruses, such as bovine or canine adenoviruses, have been considered as alternatives due to their low or absent pathogenicity to humans and absence of preexisting humoral and cellular immune responses in humans [17]. Among, all other non-human adenoviruses, canine adenovirus type 2 (CAV2) is one of the most well-studied, as it has been used as a vaccine for hepatitis caused by CAV1, in the domestic dog. However, successful utilization of CAV2 as a gene therapy vector required genetic modification at both the transductional and transcriptional level [18].

1.2 Adenovirus structure and biology

Adenoviruses are non-enveloped viruses with a diameter of about 70-100 nanometers, belonging to the genus mastadenovirus and human adenoviruses are divided into A-G species [19, 20]. They possess a large and complex structural organization for which the complete structure has not been reported [21]. Although detailed structural studies have been developed for widely used human adenovirus type 2 (hAd2) and type 5 (hAd5), many structural features are shared by all serotypes with slight variation. The 'quasi atomic model' has been resolved by employing electron microscopy, X-ray diffraction crystallography and cryo-electron microscopy (Cryo-EM) [22-25]. Adenoviruses contain two important components, the outer capsid and the viral core (**Fig.**

1.1). The Ad virion contains a total of 12 polypeptides and 7 out of 12 of these polypeptides compose the capsid. The outer capsid creates an icosahedral symmetry and contains three major protein components, hexon, penton base, and fiber, along with four minor protein components, pIIIA, pVI, pVIII, and pIX. Hexon, the principal component of capsid and the first animal viral protein that was successfully crystalized, possesses a β-barrel structural motif, followed by a pseudo-hexagonal base containing three irregularly shaped towers extending upwards [26]. In the icosahedral network, 240 homotrimers of hexon (720 monomers) form 20 capsid facets, each of which contains 12 hexon homotrimers. In addition, groups of nine (GON) hexon trimers form the central plate of each facet of capsid whereas five peripheral hexon trimers, plus one penton base forms the GOS (Group of Six), and both GON and GOS are known as a system of tiles that describes the icosahedral architecture of adenoviruses. The Ad hexon is highly conserved among human serotypes. It contains nine hypervariable regions (HVRs) exposed on its surface, termed HVR1-HVR9, and the sequence of these varies based on serotype [27]. Conversely, the penton base forms a homo-pentamer, tulip shaped structure. Each of the five petals bears a right-handed twist surrounding the five-fold axis. The top of the base is flat in shape and contains two motifs known as integrin-interacting motifs (also known as Arg-Gly-Asp (RGD) motifs) and a hypervariable loop where the size of both motifs varies based on the serotype [28]. The RGD motif plays an important for virus internalization in the target cell via receptor mediated endocytosis. The penton base is the site of insertion of fiber. Fiber is a homotrimer that consists of a highly conserved N-terminal sequence termed the tail, that facilitates the binding of fiber to the penton base, followed by a thin triple-spiral shaped shaft region of variable length and, finally, a globular head domain, known as knob,

located at the distal end. The globular knob domain is a primary attachment site that interact with cellular receptors [29]. The shaft region also contains repeats of amino acid (AA) groups that may vary (such as 6 for hAd3 or hAd35, 22 for hAd5 and 46 for bovine Ad3) resulting in variable lengths of fiber (320 to 587 AA residues) based on the serotype or species of origin [30, 31]. The length, flexibility and trimerization potential of fiber are the key determinants for successful infection of the target cell. Adenovirus with short or monomeric fiber showed defects in viral assembly and propagation resulting in reduced infectivity [32].

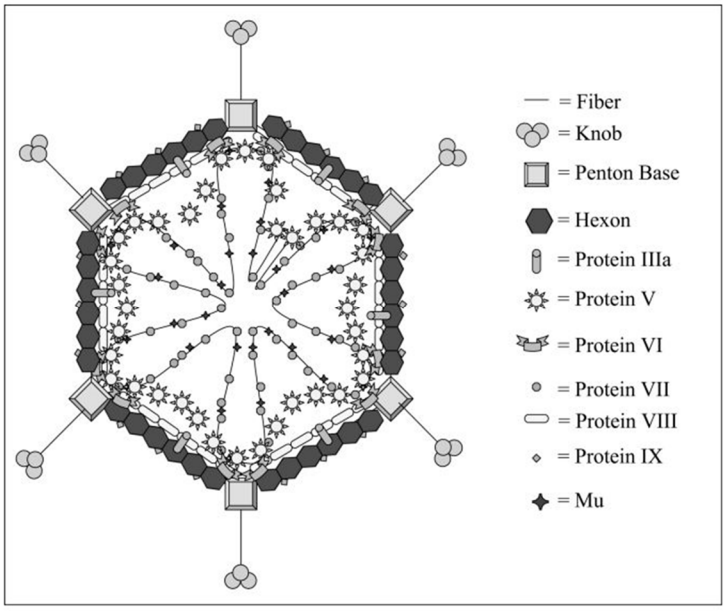

Figure 1.1: Structure of adenoviruses

Diagram showing the outer capsid and viral core (figure is from reference no. [33]**)**

Adenovirus capsid also contains minor protein components, such as proteins IX, VI and protein IIIa, that act as cement proteins to connect major structural proteins with each other as well as with the viral core and also help in virion maturation, entry, trafficking

and early gene expression [34-37]. Protein IX is located in the middle of each facet, complexed together to form four trimers with a surface exposed C-terminus that in turn helps in the stabilization of the facets on the outer surface of the capsid. [38]. In contrast, protein IIIA is located on the inner face of the capsid. A total number of 60 copies of pIIIa along with 120 copies of pVIII proteins cement the inner side of the capsid and act as linker between the hexon trimers around the three- and five-fold axes [39]. In addition, the basic core proteins, pV and pVI act to connect the capsid with the viral core. The pVII and Mu proteins act similarly to cellular histone proteins to promote condensation of DNA into nucleosome-like structures [40]. The viral core contains a linear, double-stranded DNA (dsDNA) genome with a size of 36kb. The viral genome is also bound by viral cysteine proteases and a 55kD terminal protein (TP) at both ends [26, 36, 41].

1.2.1 CAV2 structure and biology

The structure of CAV2 is very similar to that of hAd2 and hAd5. The well-defined CAV2 capsid is icosahedral in shape, harbors trimeric hexon protein with a dimension and thickness similar to those of hAd5. It also contains 12 triangular hexon proteins per facet followed by one penton base on each vertex. CAV2 hexon is shorter (908 AA vs 952 AA) than hAd5 and the N-terminal contains sequences that significantly diverge from hAd5 but it still has dimensions and structure similar to hAd5. A deletion of 24 AA after AA 144 is thought to provide a smoother shape to CAV2 hexon. In addition, there is significant sequence divergence on the exposed top of the hexon between hAd5 and CAV2 resulting in the absence of the epitopes recognized by anti-hexon antibodies widely used for the

detection of hAd5 hexon [26]. Although CAV2 penton base possesses a similar shape to hAd5 penton base, it possesses other structural differences. CAV2 penton base is smoother and shorter than that of hAd5 (477 AA vs 571 AA). Most importantly, it lacks the entire RGD loop in its hypervariable loop. However, the region directing the fiber tail-penton base interaction is conserved compared to hAds indicating similar interactions between penton base and fiber in both human and canine adenovirus.

The CAV2 fiber is homotrimeric, 330 Å long, contains 542 AA and composed of tail, shaft and knob domains. The tail consists of the first 41AA followed by 320 AA for the shaft and the last 182 AA that form the knob domain. Human Ad5 contains ~15 AA in each repeat and each of the repeats are 13 Å long resulting in a rotation of ~50 Å along the fiber axis [42, 43]. In contrast, CAV2 contains a total of 18.5 repeats in its fiber with each repeat containing a variable number of amino acids and possesses similarities with human Ad2 repeats with some exception. For example, seven repeats contain 15 AA each, three repeats contain 17 AA each with an "additional" AA inside the repeat, two repeats contain 16 AA with an "additional" AA present outside of the repeat, another two repeats contain 19 AA with "additional" AAs located inside the repeat and one repeat contains 19 AA with an "additional" AA located internally. In that case of other adenoviruses such as human Ad5 or bovine adenovirus type 3 (BAd3), irregular and long repeats have been observed near the N-terminal site of fiber that interacts with penton base to generate "bend" in this location facilitating greater access of the integrin-interacting motifs of penton base to the cell surface [30, 32, 42-44]. CAV2 fiber also contains three long repeats having 21-22 AA per repeat with an "additional" 6 or 7 AAs located between two conserved β-strand motifs. As a result, these additional AAs in the long repeat interrupt the progression of the regular

β-spiral resulting in the generation of two flexible bends providing extra flexibility to CAV2 fiber compared to hAd5 fiber [45] .

CAV2 has additional other capsid protein components such as pIX, pIIIa and pVIII. The CAV2 pIX is 105 AA long and shorter than that of hAd5 pIX which is 140 AA long. Similar to hAd5, it also consists of two domains, N- and C- terminal domains where the N-terminal domain contains some β-strands whereas the C-terminal domain contains α-helices. Both N- and C-terminal domains of pIX are connected by a linker sequence in hAd5. However, CAV2 pIX domains lack the linker region between them and are closer to each other [46]. The CAV2 pIIIa protein is 567 AA long and slightly smaller than hAd5 pIIIa which is 585 AA long but contains similar topology to that of hAd5 pIIIa. Finally, CAV2 contains a total of 120 copies of protein VIII similar to that of hAd5 [45].

1.3 Adenovirus genome and gene transcription

Adenoviral gene transcription is a highly ordered and well-orchestrated process that primarily depends on the host cell for basic macromolecular machinery and can be divided into two parts, namely, the early and late phases. The early phase represents the status of virus before DNA replication when the *E1*, *E2*, *E3* and *E4* genes are highly expressed. These genes play an important role in efficient viral infection. In contrast, the late phase occurs when viral DNA replication begins which stems from the expression of the major late promoter (MLP) and results in the expression of the late genes **(Fig 1.2).**

The *E1*, or early 1, region generates two transcripts, E1A and E1B and is expressed immediately after viral infection. The E1A and E1B products act as transactivators by regulating viral promoters for E2, E2B, major late promoter (MLP), E3, E4, and some

major cellular promoters. During the early phase of infection two major mRNAs, 12S and 13S mRNA are encoded by *E1A* followed by alternative splicing to generate 243 and 289 amino acid proteins of 26kD and 32kD, respectively. These proteins interact with several major cellular proteins, including cell cycle regulatory proteins, tumor suppressor proteins, and pathways (such as p53 dependent apoptosis) to inhibit cellular components capable of hampering viral replication. This leads to utilization of the transcriptional machinery of the infected cell for viral replication [47-50]. During Ad infection, the *E1A* gene generates two differentially expressed mRNAs (12s and 13s mRNAs) and encodes two proteins of 243 AA and 289 AA. The AA of these proteins are conserved among distantly related human adenoviruses and, are termed the conserved region (CR). E1A proteins contain three CRs (CR1, CR2 and CR3) where CR1 and CR2 domains are conserved between both proteins. The large protein contains an additional cysteine-rich 46 AA in a region called CR3. This 46 AA of CR3 along with three conserved AA residues from the N-terminus of CR2 are crucial for conducting efficient transactivation activity by E1A protein. In addition, the E1A-CR3 region of the large protein contains a DNA recognition motif called a zinc-finger motif that may help in target DNA recognition and binding [51, 52].

Viral E1A protein appropriates cellular transcription machinery to enhance viral transcription immediately after infection, inducing a pro-apoptotic environment. However, these modulations result in a crisis condition inside the infected cell and that crisis is mitigated by the products from *E1B* which are activated by E1A. The *E1B* region generates two products of 55kD and 19kD that counteract the pro-apoptotic trend initiated by E1A. The E1B 55kD protein binds and inactivates cellular p53. The E1B 19kD protein acts similarly to bind cellular Bcl2 protein, inhibiting the downstream pathways activated by

p53, and thus resisting apoptosis. Deletion of the E1B 55kD and 19kD products has been shown to enhance the oncolytic potential of adenoviruses leading to enhanced viral toxicity [53].

Mutational analysis has shown that the E2B promoter consists of a TATA box, consensus Spl-binding sequences, and other transcription factor binding regions. The TATA box plays a major role in transactivation as TATA-binding factors, like TFII, are regulated by E1A gene products [54-56]. *E2* transcription is conducted by two promoters known as the E2 early and E2 late promoters in which the E2 early promoter contains two overlapping regions. Transcription from the *E2* gene can be initiated from two regions known as the +1 site major and -26 (minor) site. The E2 early promoter also contains sites for transcription factors like E2F and activating transcription factor (ATF). ATF plays a major role in E2 early transcription from both initiation sites [51, 52].

The *E2* region, after activation by the *E1A* gene products, plays an important role in viral DNA replication by working in concert with other cellular factors including nuclear factors and octamer-binding transcription factors [57]. The E3 region expresses two proteins (19kD and 11.3kD) where the 19kD glycoprotein plays an important role in viral immune evasion. This glycoprotein interacts with the heavy chain of major histocompatibility complex (MHC) class I to block its entry into the cell, resisting antigen presentation and activation of cytotoxic lymphocytes. The E3 11.3 kD product, also known as adenoviral death protein (ADP), helps to promote viral particle release from infected cells during lysis, along with activating cell apoptosis [58-60]. The *E4* region produces at least 18 different mRNAs, driven by alternative splicing, that generate seven different polypeptides (or seven open reading frames). These products function in concert with E1B-

55kD to promote viral replication by inhibiting the cellular translation machinery [61-63]. E1A also acts to strongly induce late gene transcription by activating the MLP promoter resulting in the transcription of structural proteins. The MLP consists of a TATA box for binding TFIID and an upstream element to facilitate binding of the major late transcription factor (MLTF). Late transcription levels are increased one thousand-fold after the commencement of DNA replication to produce a single transcript that in turns undergoes alternative splicing to produce mRNAs known as the L1 family (L1-L5) and generates the structural proteins of the capsid including hexon, penton base [51, 52, 64, 65]. Apart from protein coding regions, adenoviral genome also contains protein non-coding transcripts including viral associated (VA) RNAs that fold into dsRNA to promote downregulation of cellular protein synthesis [66].

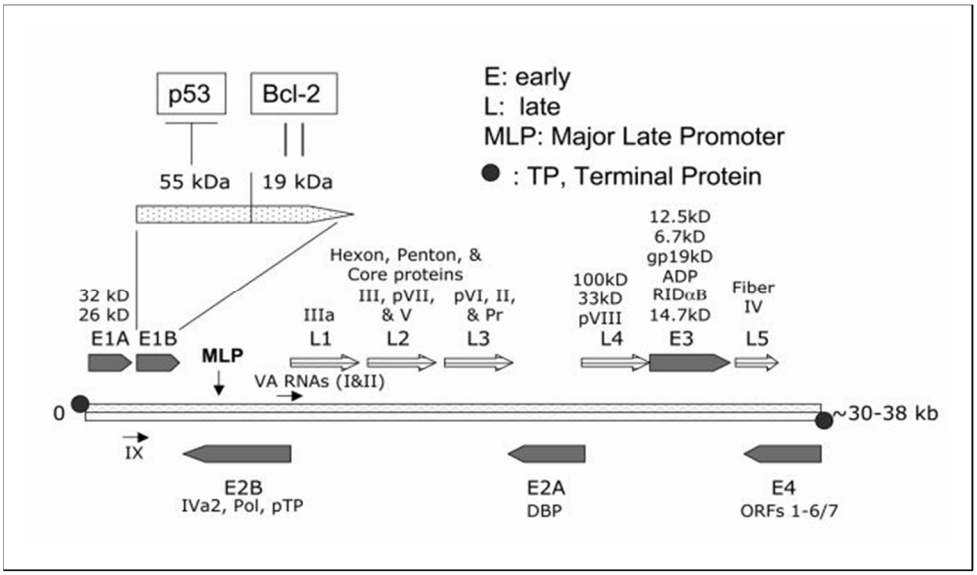

Figure 1.2: Adenovirus genome organization

Diagram showing the early and late genes and their products emanating from the Ad genome (figure is from reference no. [33]**)**

1.4 Adenovirus genome replication

The machinery required for adenoviral DNA replication is composed of an origin of replication (ori), adenovirus DNA polymerase (Ad DNA Pol), precursor of terminal protein (pTP), adenovirus DNA-binding protein (DBP), and cellular transcription factors nuclear factor I (NFI) and octamer-binding transcription factors such as Oct-1. Efficiency of DNA replication depends on the cis-acting ori sequences of Ad located within the 5' inverted terminal repeat (ITR) and composed of an essential core region and an auxiliary region that covers approximately the terminal 50bp of the viral genome. In addition, the terminal 18bp of the hAd5 viral genome, known as core origin, is required for maintaining the integrity of ori sequences. 10bp of this region is conserved in all human adenoviruses [67, 68]. The DNA-binding sites of the transcription factors nuclear factor I (NFI) and nuclear factor III (NFIII) (also known as Oct-1) are located in the auxiliary origin region of the viral genome, separated by a spacer from the core region. These proteins play important roles in proper positioning of pTP/Pol with the origin of replication (described below). The requirement for the 50bp origin may vary between different serotypes of the viruses. For example, hAd2 needs both the core and auxiliary origins whereas hAd4 only needs the core origin. Ad DNA Pol consists of the precursor terminal protein (pTP) as well as a 140 kD protein and possesses 3' to 5' exonuclease activity, like other DNA polymerases [69, 70] (**Fig. 1.3**). Adenoviral DNA replication is a very efficient process that is initiated through a mechanism termed protein-priming (**Fig. 1.4**). At the beginning of replication, early region 2 (*E2*) products interact with cellular NFI and NFII to form preinitiation complex and assemble at the origin of replication.

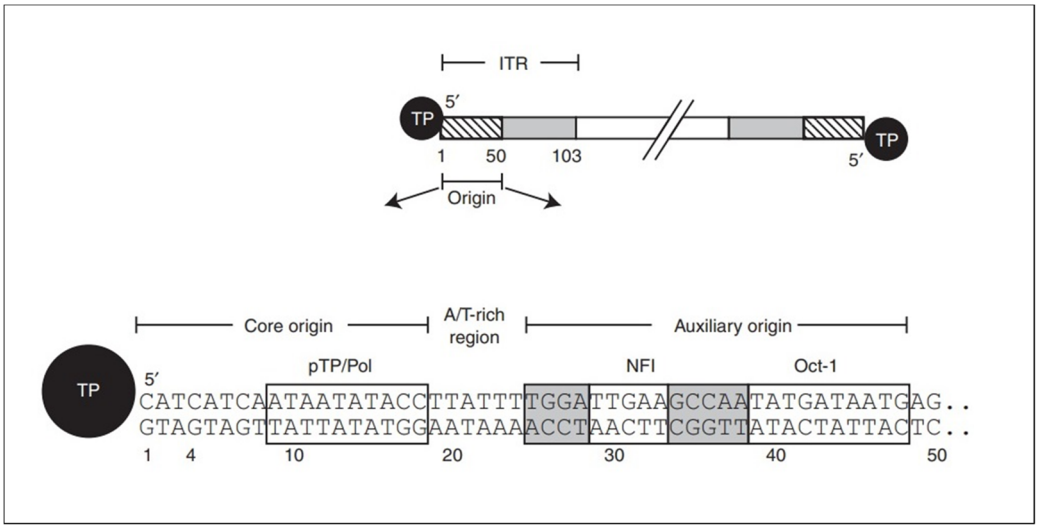

Figure 1.3: Schematic representation of adenovirus origin of replication sequence

Diagram showing essential regions of the Ad origin of replication for interaction with viral and cellular proteins. NFI= Nuclear factor I, pTP= Precursor terminal protein (figure is from reference no. [71]**)**

During the initiation stage, the duplex DNA is unwound by DBP to expose the single stranded template sequence (3′-GTAGTA-5′). After unwinding, the β-OH group of the serine (Ser580) residue of pTP covalently attaches to the α-phosphoryl group of the opposite nucleotide 4 of the template to generate pTP-C. This pTP-dCMP acts as a primer for the addition of two other nucleotides to form an intermediate complex called pTP-CAT (pTP-trinucleotide intermediate). Following this, the pTP/CAT complex jumps back by three residues and acts as a primer by providing a 3′-OH group that can be utilized by Ad DNA Pol to elongate the replication process and synthesize the new strand [72, 73]. After the movement of the pTP/CAT complex, Ad Pol dissociates from pTP, which increases the rate of replication and allows proof-reading activity by the Ad DNA polymerase. Following this, Ad DNA pol and DBP work in concert to elongate pTP/CAT

complex intermediates to generate new duplex genome as well as displacement of non-template strand. Later, new a origin of replication may be generated by the annealing of the ITRs of the displaced strand to provide a substrate for new products. At the end of DNA replication pTP is cleaved by cysteine protease to generate TP and progeny DNA that is then packaged into virions [69, 72-74] .

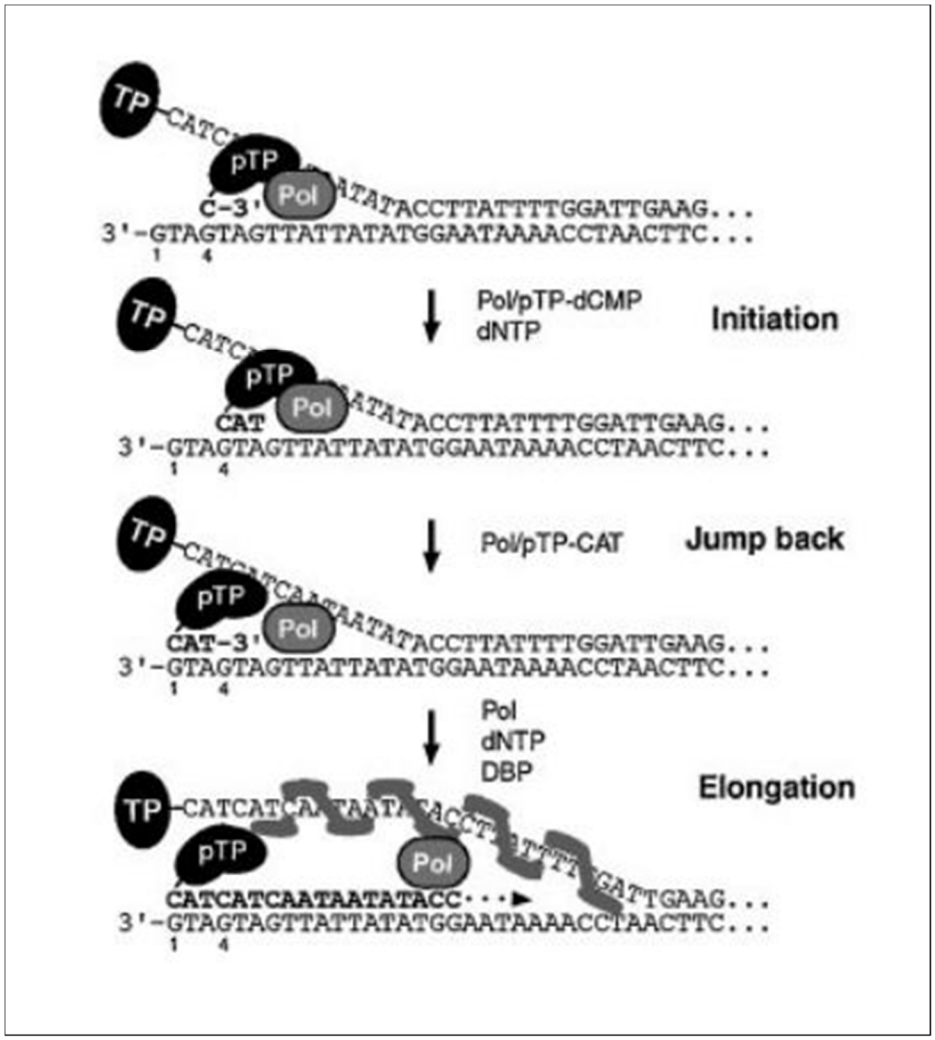

Figure 1.4: Adenovirus DNA replication

Diagram showing necessary steps of adenovirus DNA replication (figure is adapted reference no. [74])

1.5 Adenovirus genome packaging

The stable packaging capacity of adenovirus is a minimum of 75% and maximum of 105% of that of the wild type genome [75, 76]. The two ends of the adenoviral genome are composed of 100-150 bp of sequence known as inverted terminal repeats (ITRs) [77]. ITR sequences perform two key functions: i) The ITR protects the genome against degradation by cellular proteases and ii) The ITRs are recognized by TPs to facilitate genome replication. Adenoviruses contain icosahedral capsids that allow its packaging by sequential assembly mechanisms involving viral-encoded proteins IVa2, L4 33K, L4 22K, E2 72K, and L1 52/55K and IIIa, as well as cellular proteins such as chicken ovalbumin upstream promoter transcription factor COUP-TF, Oct-1 and CCAAT displacement protein (CDP) [41, 78-84] **(Fig. 1.5)**. In this process, the hexon trimers and penton capsomeres along with minor capsid proteins (pIIIa, pVI, pVIII, IX, protease and portal) and non-structural scaffold proteins, such as L1 52/55K, assemble to form an empty capsid (also called procapsids). Next, the viral genome region closest to the left end of genome (also called the packaging domain (PD) is recognized by packaging proteins (IVa2, L4 33K, L1 52/55K, and L4 22K) to form a packaging complex (PC). The PC is inserted into the empty capsid through a portal located at a unique vertex with the aid of scaffold and packaging proteins. Finally, the precursor proteins are dissociated by viral protease leading to final maturation of viral particles [85-89].

Generally, the Ad PD is located between the *E1A* region and the left inverted terminal repeat (LITR) but can work if placed within 600bp of the right inverted terminal (RITR) for serotype F Ad such as Ad40 and 41 [90]. The PD for hAd5 is located between 220 bp to 400 bp and contains a series of A-repeats (A1 to A7 for hAd5). Ads favor polar

packaging starting from the left site to facilitate the orientation of the genome with respect to the portal and provide threading of the genome through the portal. Among the seven repeats, A1, A2, A5 and A6 play the most important role in virus packaging.

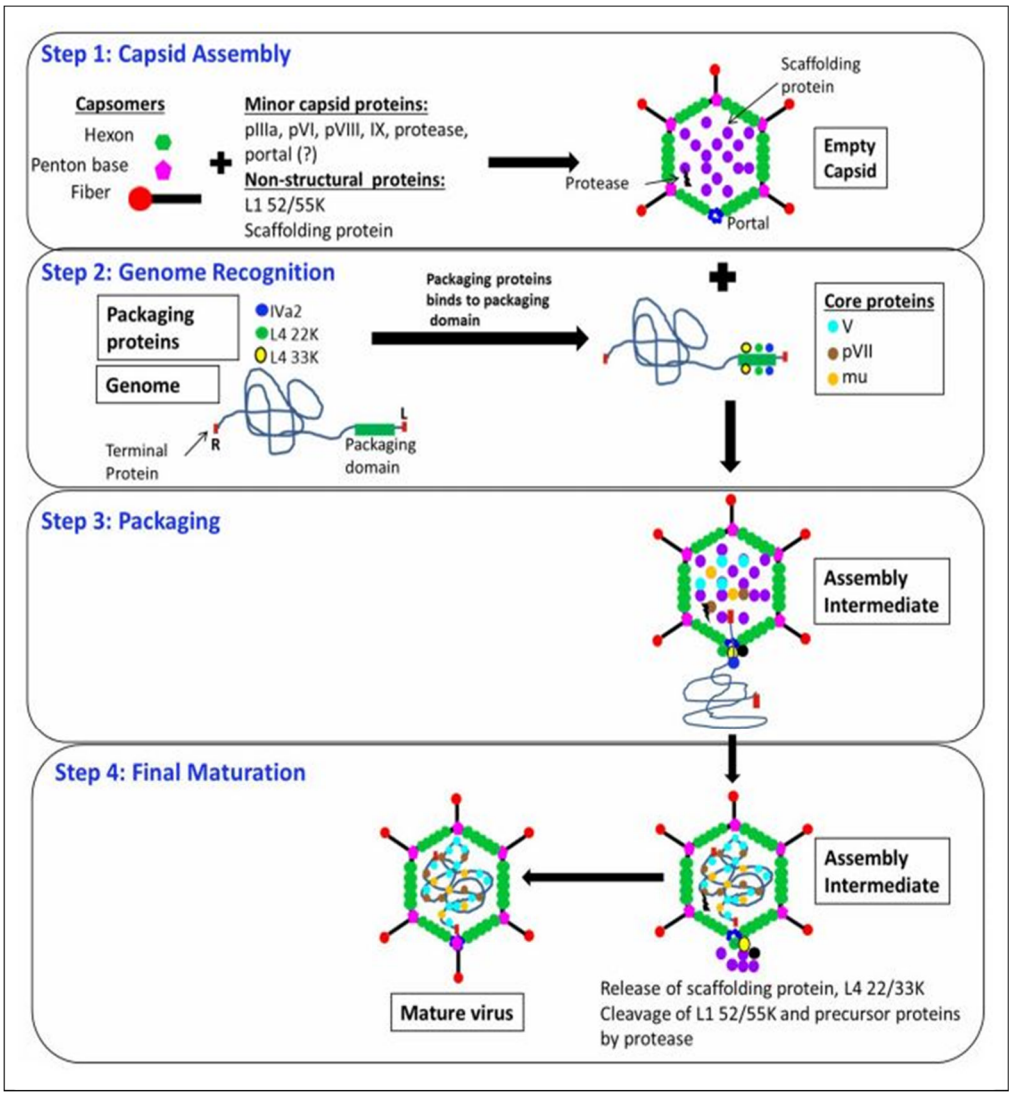

Figure 1.5: Mechanism of adenovirus DNA packaging

Diagram showing the components and necessary steps required for successful viral genome packaging (figure is from reference no. [85])

These A repeats share a bipartite consensus motif (5′ -TTTG$_{N8}$CG-3′). The 8nt spacer between TTTG and CG is non-conserved but changing (addition or deletion of bases) of the length of the spacer may drastically reduce the packaging efficiency [91-93].

The A1, A2 and A5, A6 repeats work in pairs and contain 21bp spacers (relating to two turns of the DNA helix) between them. They act as a binding site for transactivating factors ensuring the efficient formation of the packaging complex [88]. The bipartite consensus motif is reported to be conserved among adenoviruses from different species, with a minor set of variations among the A-repeats providing packaging mechanisms unique for all adenoviruses irrespective of their species of origin. However, CAV2 provides a different structure, orientation and redundant bipartite motifs. The CAV2 LITR is longer than hAd5 (180bp vs 140bp) and sequence alignment between hAd5 and CAV2 for 400bp close to LITR and 500bp close to RITR showed little similarity between those regions and hAd5. The PD of CAV2 contains twelve A-repeats (AI to AXII) similar to Ad5 in their basic structure but the spacer regions may contain a variable number of nucleotides compared to hAd5 [94].

1.6 Generation of adenoviral vectors

Adenoviral vectors that are used in gene therapy are of two types; those that are incapable of replicating, called replication-deficient adenoviruses, and those that have been modified to specifically replicate in tumor cells, called conditionally replicative adenoviral vectors or CRAds.

1.6.1 Replication-deficient adenoviral vectors

1.6.1.1 First generation adenoviral vectors

First generation adenoviral vectors were developed by replacing the *E1* and/or *E3* regions to contain a maximal transgene capacity of ±8kb. As noted above, the *E1* gene,

located at the left end of the viral genome, is essential for viral replication as it encodes proteins essential for the expression of the early and late genes. Thus, the first adenoviral vectors were E1 deleted to prevent replication. In order to make infectious viral particles, the E1 deficient genome had to be transfected into specific helper cell lines such as HEK293, HER cell line 911 or PER C6.14 where E1 was provided in *trans* [95-97]. While functional, these vectors had limited capacities for inserts (up to 5.1kb) and could recombine in the packaging cell with the cellular E1 gene to produce replication competent virus. In order to provide addition capacity for transgenes as well as reduce the production of replication competent virus, subsequent vectors were deleted in E1 and E3.

The function of the E3 region is to encode proteins to counteract host defense mechanisms. The expression of the entire E3 gene region is not essential for viral replication, and thus, does not require a complementing cell line for viral replication [98, 99] . However, expression of some E3 products, including E3-11.6k (also known as adenovirus death protein) or gp19k, are necessary to facilitate either release of the viral particles from infected cells or to reduce the host cell immune response [100-102]. Thus, up to 3.1 kb of E3 region has been deleted to allow transgene insertion [75, 103]. Since, adenovirus can package 38kb without affecting the production of infectious virus particles, deletion of both E1 and E3 regions allows the insertion of a transgene upto 8.2kb [104].

1.6.1.2 Second generation adenoviral vectors

The susceptibility of the 1st generation vectors to the host immune system, their relatively low capacity for incorporating foreign DNA, and the low efficiency in transgene expression has limited the functionality of these vectors. This has led to the development

of what are known as second generation adenoviral vectors. These have been developed with the same methodologies as first generation vectors but they have additional modifications that attempt to overcome the limitations of first generation vectors. Second generation vectors are deleted in the *E2A*, *E2B* and *E4* regions along with the *E1* and *E3* regions. Because more DNA has been deleted from the viral genome, the vector can accept larger, or multiple, foreign DNAs, typically up to 14 kb [105-107].

1.6.1.3 Third generation adenoviral vectors

Third generation adenoviral vectors are called helper dependent, gutless, gutted, or high capacity adenoviral vectors because they lack nearly all the viral genes except the ITRs and packaging regions and can accommodate up to 36 kb of foreign DNA. Because these vectors lack all functional adenoviral genes, all of the protein products necessary to produce infectious viral particles must be provided in *trans*. Since virus packaging capacity is 75-105% of the whole Ad genomes and transgenes generally do not compose more than 36kb, successful packaging leading to infectious virus particles requires use of stuffer DNA to complete the required genome size for encapsidation. While it is relatively simple to create cell lines that express *E1* or *E1* and *E3*, expression of all of the proteins needed with "gutless" adenoviruses requires the use of a second, replication competent, helper virus. Unfortunately, this helper virus would then contaminate the therapeutic virus preparation. In order to avoid this issue, the helper viruses have employed the bacteriophage cre/lox recombinase system to inactivate the helper virus by removing its packaging signal. Since it lacks all viral genes it promotes reduced innate and adaptive immune responses as well as reduced clearance by the liver. Thus, unlike first and second generation Ad vectors, gutless adenovirus vectors display reduced long-term toxicity and prolonged transgene

expression. However, using this system, still results in helper virus contamination ranging from 0.1-10% due to low recombinase activity or low endogenous expression levels of recombinase leading to inefficient excision of the helper virus packaging signal [74, 108-110].

1.6.2 Conditionally replicating adenoviruses (CRAds)

Replication incompetent viruses fail to start a new round of infection in neighboring cells including the neighboring tumor cells that would be required to start a new lytic cycle. In this regard, replication competent adenoviruses (also known as CRAds) have been developed that are capable of replicating in the target cells followed by lysing the infected cell to release the progeny virus. The new progeny virus can then infect the neighboring cells to start a new lytic cycle leading to spread thorough and elimination of tumor [111].

One of the requirements for effective gene therapy is to promote reduced normal cell toxicities. Conventional therapies such as chemotherapy, surgery, radiotherapy generate undesired side effects as these treatment approaches not only kill the cancer cells but also conduct normal cell toxicities for which adenoviral mediated gene therapy is preferable than other treatment approaches. CRAD mediated adenoviral gene therapy can restrict the drawbacks obtained by conventional therapies by reducing normal cell toxicities. There are two types of CRADs that have been reported, Type I CRADs and Type II CRADs. Type I CRADs possess genetic mutations in the genes that control replication in normal cells but are transcomplemented in tumor cells. Onyx-015 is such a CRAd. It lacks the *E1B*-55 kD gene. Since this protein normally interacts with p53, blocking the ability of the virus to replicate, the absence of E1B protein in p53 positive cells blocks

replication [112]. Viral replication can occur in p53 negative cells, which includes many cancer cells. However, this method has some limitations. Sometimes, p53-expressing cells can be permissive for Onyx-015 [113] or in some cells the Onyx-015 vector requires the *E1B*-55 kD region for proper viral replication [114]. To overcome these problems, Type II CRAds were generated by utilizing tissue specific promoters, such as the progression Elevated Gene-3 (PEG) promoter[115], prostate specific antigen promoter (PSA)[116], MUC/DF3 [117], Survivin, TERT, engineered into the viral genome to directly control the viral transcriptional factors responsible for replication, namely E1A.

1.7 Construction of adenoviruses

There are three well-established methods that are used to generated recombinant adenoviral vectors (both human and canine adenovirus). These methods are: i) bacterial homologous recombination (HR) ii) a hybrid yeast-bacteria cloning system and iii) a bacterial artificial chromosome (BAC) based system. In the bacterial based HR system, *E. coli* is co-transformed with an Ad5 genomic DNA and a restriction enzyme-linearized plasmid containing the gene of interest (GOI) and the ampicillin resistance (*Amp*^{r}) gene along with the LITR and RITR of adenovirus to promote *in vivo* HR. The resultant Amp^r colonies contain a plasmid having full length recombinant Ad genome as well as a plasmid backbone. The Ad genome is utilized by release from the plasmid by restriction enzyme digestion, to liberate viral ITRs, followed by transfection into packaging cells, to generate functional viral particles [118]. The second method among these three methods, and the most the widely used method, is based on the hybrid-yeast bacteria cloning system. In this method, two plasmids are used containing both yeast and bacterial replication elements.

One plasmid is called the shuttle plasmid that contains the gene of interest (GOI) and another plasmid contains the adenoviral genome. In order to generate recombinant Ad, both the shuttle plasmid with the GOI and the adenoviral genome plasmid are transformed into yeast to promote highly efficient recombination leading to the generation of Ad-GOI-YAP (yeast artificial plasmid). Then, the Ad-GOI-YAP is transformed into bacteria to facilitate rapid amplification of recombinant viral plasmids in bacteria. The resultant viral plasmid is then linearized to liberate viral ITRs followed by transfection into mammalian packaging cell lines to produce functional virus particles. Although these methods are well-established and being used by many researchers, the large genome size of adenovirus restricts their successful implementation and overall efficiency. These methods are multistep processes (and, thus, time consuming), labor intensive, may require multiple shuttle plasmids, are solely dependent on the availability of desired restriction enzyme sites and are dependent on homologous recombination. However, the efficiency of homologous recombination is less than 1% resulting in low yield as well as promoting non-specific insertion of the GOI or other marker genes. The homologous recombination independent method, such as Cre/lox-based system used to generate gutless adenovirus vector, may improve efficiency, however, this system incorporates an additional precaution step to prevent parental virus contamination [119, 120].

The generation of adenoviruses from species other than humans, such as CAV2, is more labor intensive and complex than that of human adenoviruses due to reduced ability to initiate CAV2 replication and poor transfectability of canine cells with large DNA fragments[121]. For example, according to Kremer *et al.,* transfection of ~10^7 cells with a transfection efficiency of 5% will routinely result in a total of <20 infectious virus particles

whereas the same approach will lead to 10^4 to 10^5 folder higher infectious particle numbers for human adenoviruses. In order to enhance the rate of virus production, attempts have been made to fuse the estrogen receptor (ER) to a yeast meganuclease called I-SceI generating a recombinant canine cell line capable of expressing the fusion protein. In the CAV2 plasmid, the I-SceI recognition sites are used to flank the viral genome to facilitate the intracellular viral genome release via 4-OH tamoxifen (an antagonist of ER) mediated nuclear translocation of ER-I-SceI after transfection of the supercoiled viral DNA into the recombinant cell line [121]. Although authors reported that this method generated high viral titers and was less time-consuming, it incorporates additional hurdles such as generation and validation of recombinant cell lines expressing the fusion protein.

1.8 Adenovirus cell entry

Adenovirus enters target cells by employing two distinct steps, attachment and internalization. First, a high affinity interaction occurs between the adenovirus knob domain and the primary cellular receptor, followed by a secondary interaction between the RGD motifs of penton base with cell surface integrins. In general, the primary receptor for adenoviruses from serotypes A, C, E and F are the coxsackievirus and adenovirus receptor (CAR), while adenoviruses from other serotypes, such as B and D, utilize other receptors including Desmoglein 2 (DSG2), heparin sulfate glucagon, Sialyted glycoproteins, and CD46 [122-126] **(Fig. 1.6)**. CAR is a 46 kD glycosylated transmembrane protein a member of the immunoglobulin superfamily. It shows different levels of expression in different organs except for brain where it is not expressed and is well-known to localize to tight junctions to promote cell-cell interactions and cell adhesion [127-129]. Low levels of CAR

expression have also been observed for some cancer cells. Low CAR expression hampers the functionality of Ad mediated cancer gene therapy [130]. Following attachment, the RGD motif on adenoviral penton base interacts with cellular integrins, such as αvβ5, αvβ3, αMβ2, αvβ1, α3β1 and α5β1, which promote the internalization of Ad via clathrin-coated vesicles [131-134].

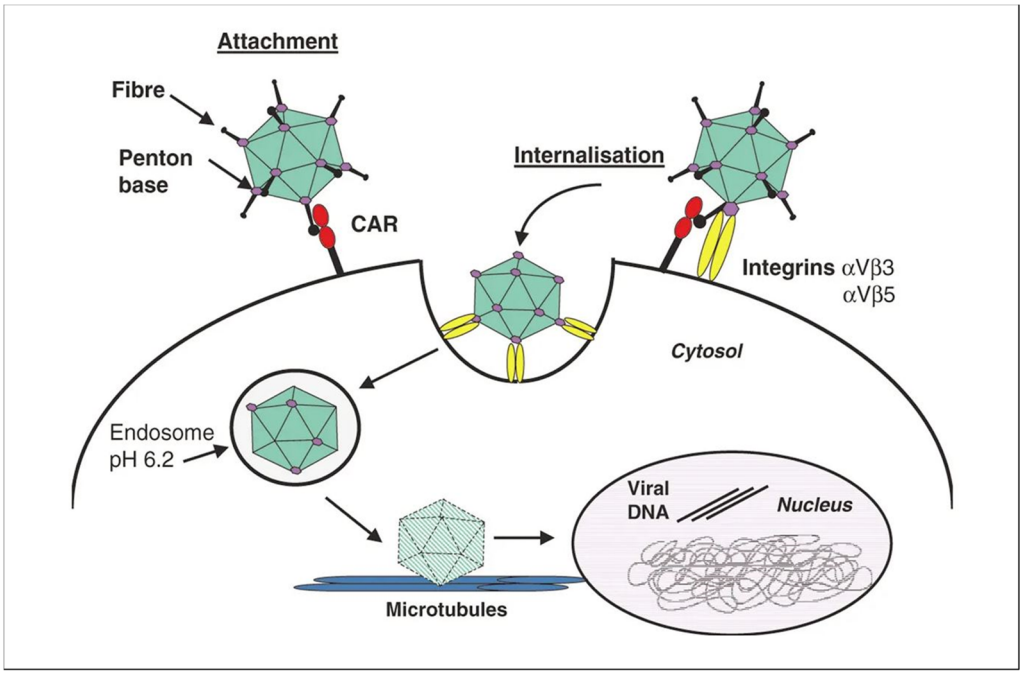

Figure 1.6: Adenovirus cell entry pathway

Diagram showing Adenovirus cell entry via cell surface coxsackievirus and adenoviral receptor (CAR) and integrins (figure is from reference no. [135]**)**

Mutation of the any of the three AA of the RGD motif showed decreased ability to bind to integrins followed by reduced infectivity [133]. Once internalized, the Ad virion is transported to an endosome via receptor-mediated endocytosis. Acidification of endosome activates viral proteases to facilitate the removal of the vertices of the virion as well as to cleave pVI to promote viral escape from the endosome [136]. The released viral particles

then utilize the cellular microtubule network to be transported to the periphery of the nuclear membrane (NM). At the NM, hexon proteins interact with nuclear pore filament CAN/Nup214 facilitating the dissembling and releasing of the adenoviral genome, along with proteins pV, pVII and mu, into the cell nucleus [137-139]. This whole process starting from viral attachment to the release of viral genome into the nucleus takes 1-2-hour [140].

Non-human adenoviruses can also utilize CAR and the integrin independent pathways to enter into the target cell. For example, bovine adenovirus 3 (BAd3) utilizes sialic acid as primary cellular receptor and porcine adenovirus 3 (PAd3) can enter into cells in a CAR- and αvβ3 or αvβ5 integrin-independent pathway [141, 142]. The cell entry pathway for CAV2 is distinct from that of hAd2/5. Early investigations showed that CAR-expressing fibroblast-like cells can successfully be infected by CAV2 indicating that CAR receptors may be sufficient for CAV2 transduction [143]. Although CAV2 lacks an RGD motif, it can mimic Ad5 transduction in some cell lines. In addition, integrin independent CAV2 transduction has been observed in some cell lines. For example, studies reported variability in CAV2 transduction capability in cells of lymphocyte origin. Previously, no wild type CAV2 transduction was reported for human B lymphoma cell lines Daudi and Ramos expressing CAR but no αvβ3/αvβ5. Interestingly, CAV2 showed enhanced transduction for the human T lymphocyte cell line, Jurkat, expressing similar levels of CAR and αvβ3/αvβ5 to Daudi and Ramos cell lines and a human monocytic leukemia line expressing little or no CAR, αvβ3 or αvβ5 integrins. Based on these findings, the authors reported that CAV2 transduction varies based on the cell lines and it may promote infection in both a CAR- and αvβ3/αvβ5- dependent and independent manner, in the latter case, by utilizing alternative cellular receptors (discussed below) [143-145].

Several studies have showed that cells of hematopoietic origin including T cells are resistant to infection by adenoviruses due to low or no expression of CAR or components of the integrin system on their surface. Hematopoietic cells were shown to be resistant to infection by adenovirus due to low expression of CAR on their surface [146]. Early work observed adenovirus infection in a large proportion of $CD34^+$ hematopoietic cells and implied that adenovirus may promote gene transduction in primitive hematopoietic cells. [147]. However, subsequent investigators observed inefficient adenoviral transduction in freshly isolated bone marrow (BM), peripheral blood (PB) cells, $CD34^+$ hematopoietic progenitors, and stem cells. Multiple studies have shown low levels of αvβ5 and αvβ3 in BM and PB cells and concluded that lack of integrin expression on the target cell surface may result in resistance to Ad transduction in all cells of the hematopoietic lineage [148-150]. These results are also supported by another study that observed high levels of adenoviral mediated transgene expression in breast cancer cells, with high numbers of Ad receptors (6-9 X $l0^3$/cell), and low levels of infection in BM and PB cells expressing low levels of Ad receptors [151]. In addition, several studies have shown that B and T lymphocytes) are resistant to infection by adenoviruses such as hAd2 and hAd5 [152-154]. Another study observed low integrin expression in human monocytes and T lymphocytes followed by reduced adenoviral infectivity in these cells. However, the authors observed enhanced adenoviral transduction in these cells when stimulated to express high level of cell surface integrins [155]. Early investigation also showed that resting B cells are harder to transduce by adenoviruses than naive T cells [156]. Recent studies utilized genetically modified Ad expressing polylysine in the knob domain to transduce murine B lymphocytes and B lymphomas. This approach showed enhanced transduction in lipopolysaccharide

(LPS)-activated splenic B cells and the B lymphoma line M12.4.1 but failed to transduce resting B cells [157]. Wattel *et al.* failed to observe adenoviral mediated efficient gene transfer in myeloma, acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL) and non-Hodgkin lymphoma (NHL) cell lines, and patient samples except for one case of myeloma and one case of CLL [158]. However, Meeker *et al.* have shown enhanced adenoviral-mediated transgene expression in anaplastic large cell lymphoma, Hodgkin's lymphoma, 2 out 7 Burkitt lymphomas, and 3 out of 5 myeloma cell lines implying potential adenovirus entry into some lymphoma subtypes [159, 160] and differential efficacy of adenoviral transduction in cells of hematopoietic origin. Moreover, Leon *et al.* have shown enhanced adenovirus transduction in hard-to-infect lymphocytes by ectopic expression of CAR in those cell lines [161]. Similarly, another study generated a CAR expressing follicular B cell lymphoma cell line and observed enhanced adenoviral mediated transgene expression indicating that CAR-expressing lymphocytes are susceptible to Ad infection [162]. O'Neil *et al.* tried to infect CD40 expressing primary canine B cell lymphoma cell and cell lines in a CAR-independent but integrin-dependent manner by employing human or canine CD40L retargeted Ad5 (Ad5-ff-hCD40L and Ad5-ff-cCD40L). However, results showed limited evidence of adenoviral transduction in both canine primary lymphoma cells and cell lines. This study observed reduced expression of αvβ3/5 integrins in the surface of the tested cell lines [163]. To validate this result, we have attempted to infect several canine hematopoietic and non-hematopoietic cancer cell lines with hAd5 expressing green fluorescent protein (hAd5-GFP). Result showed enhanced levels of hAd5 transduction in non-hematopoietic canine cancer cell lines. However, little or no hAd5 transduction was observed in hematopoietic

canine cancer cell lines. In addition, we also quantified CAR, αvβ3 and αvβ5 integrin transcript expression level in those tested cell lines. We found low levels of integrin expression but significant levels of CAR expression in the Ad-infection-resistant hematopoietic cancer cell lines [164]. These data imply that successful adenoviral infection of lymphocytes requires receptors for both attachment and internalization. CAR and integrin classically serve this purpose. One way to address this issue might be to use adenovirus from other serotypes as shown by recent study conducted by Lv *et al*. They used Ad5/35 and Ad5/11p chimeric adenoviruses, Ad5 expressing the fiber of either Ad35 or Ad11p, to study transduction efficiency in human T cells, leukemia cells and primary T cells. In addition, they used vectors containing the RGD-4c motif (a high affinity ligand for $\alpha_v\beta_3$ integrin) in either the HI-loop (HR-EG) or C-terminus (CR-EG) of Ad11p-EG. Results have shown high transduction efficiency for all vectors except HR-EG in $CD34^+$ cells, human $CD4^+$ T cells, leukemia cells, and primary human T cells, but reduced transduction in human $CD8^+$ T cells [165].

1.9 Requirement of transductional targeting of adenoviruses

Successful adenovirus 2/5 cell entry is typically CAR and integrin dependent and non-CAR expressing normal cells or cancer cells are resistant to Ad2/5 infection and gene delivery. Studies showed that cancer cells express low levels of CAR although CAR is expressed ubiquitously in normal cells [129, 166]. Thus, Ad mediated virotherapy in low-CAR expressing cancer cells may fail to treat the tumor and generate toxicities to normal cells expressing high levels of CAR. Enhancement of CAR expression in non/low-CAR expressing cells may promote elevated adenoviral transduction in those cells.

However, studies reported elevated CAR levels promote growth inhibition in some cancer cells [167]. In addition, CAR receptor is highly expressed in liver cells but differentially expressed in other tissues. This promotes Ad-mediated liver toxicities after systemic (intravenously) administration of adenovirus [168]. Initially it was thought that hepatic tropism was CAR and integrin dependent and development of CAR- and integrin-independent pathways might subvert liver toxicity. However, ablation of CAR and integrin binding in Ad capsids failed to promote liver de-targeting of Ad. Hepatic tropism is a multi-factor dependent mechanism and occurs when Ad hexon HVRs interact with Heparan Sulphate proteoglycans (HSPGs) expressed on the surface of hepatocytes. This interaction is bridged by a serine endopeptidase and a key component of human blood coagulation, known as Factor X (FX), which interacts with the hexon HVRs (Ad5 HVR5, 7 and 9) in the bloodstream [169-172]. This interaction promotes a high level of Ad sequestration in liver via hepatic Kupffer cells and also affects overall biodistribution of Ad [172, 173].

Additional studies have shown that sequences in the hexon, penton and fiber of Ad are responsible for hepatic tropism by interacting with FX. Hexon interaction with FX may also generate innate anti-Ad5 immune responses that recognize FX-bound hexon proteins [174]. Early investigations reported specific interactions between FX and HVRs of hexon resulting in liver sequestration. However, several strategies to generate genetically modified hexon have been developed that showed low or no liver sequestration. For example, Waddington *et al. s*uggested that different serotypes be utilized, as different serotypes interact with FX with different affinities. Examples include Ad35 that shows low binding to FX, and Ad26 and Ad48 that show no binding with FX resulting in low or no liver transduction. Studies reported low liver transduction with Ad-containing

mutated forms of hexon [171, 175]. For example, Alba *et al.* altered key AA residues of HVR5 and 7 to generate hexon modified Ad and reported decreased liver transduction after adenovirus mediated gene delivery [170]. Several studies also utilized other strategies such as hexon or HVR swapping between serotypes to reduce liver toxicity. Short *et al.* reported reduced liver toxicity by swapping Ad5 hexon with Ad3 (Ad5/3) hexon resulting in enhanced viral replication and tumor cell killing [176].

Apart from CAR mediated liver toxicity and innate immune response generation, Ad RGD motif interaction with cell surface integrins results in enhanced splenic transduction as well as generation of IL-1α mediated innate immune activation [177]. Deletion or mutation of the RGD motif showed reduced splenic transduction and attenuated immune response in mice [177, 178]. In addition, apart from adenoviral capsid, early Ad proteins and virus-associated non-coding RNAs (VA-I, VA-II) also promote pathogen associated molecular pattern (PAMP) recognition, followed by activation of long-lasting cellular and adaptive immune responses against Ad [179-181]. Hexon proteins are reported to contain at least three antigenic epitopes for CD4+ T cell [182, 183]. The adaptive immune response generates hexon, penton and knob specific neutralizing Abs (nAbs) that, in turn, block virus attachment, internalization or may promote rapid virus clearance [181, 184-186]. Finally, low levels of expression of CAR and integrin in cells of hematopoietic origin also hampers successful utilization of adenovirus as a suitable tool for oncolytic cancer gene therapy (as discussed in the previous section).

1.9.1 Approaches to generate tropism modified adenoviral vectors

Various approaches have been developed to generate tropism modified replication incompetent or oncolytic Ads with enhanced transduction and reduced immune stimulating capabilities. These viruses are designed to provide enhanced efficacy for virotherapy by both replication competent and incompetent vectors. In this section, a brief description of these strategies will be provided.

1.9.1.1 Peptide targeted strategies

One of the first strategies to develop CAR-independent, tropism modified, adenoviral vectors was to incorporate a targeting peptide into the fiber knob, hexon HVRs, or the C-terminus of protein pIX. Ad fiber knob consists of two anti-parallel B sheets connected by surface exposed hyper variable loops (Hl). Both the HI loop and C-terminus of the Ad knob domain showed no activity for CAR binding and were thus deemed capable of accepting targeting peptides. Initially it was thought that incorporation of peptides larger than 83 AA residues in the N-terminus and larger than 25-30 AA residues in the C terminus of knob domain would destabilize the fiber structure resulting in reduced viral yields. However, large ligands such as epidermal growth factor (EGF)-like domain heregulin-alpha (HRG) (50-55 AA in size) have been inserted in the Hl loop and successfully targeted virus to human epidermal growth factor receptor (HER) 3 HER3/ERB3 cell surface receptor. This result indicated that the HI loop can accommodate large and structured ligands [187]. In addition, another study reported the insertion of RGD-4c (Ad5-RGD-4c) peptide could be successfully inserted into the Hl loop and polylysine peptides (Ad5-pk7) have been added to the C-terminus of the knob domain. Ad-RGD-4c showed enhanced activity in alpha5beta3 expressing cells and Ad5-pk7

showed affinity for polyanion motifs such as Heparan sulfate. The vectors also showed high accessibility and flexibility of the resultant modified fiber [188]. Wu *et al.* developed a dual targeted vector called Ad5-RGD-pk7 that showed reduced activity in CAR positive cells but enhanced activity in CAR-negative tumor cells such as lung, glioma, ovarian carcinoma etc. [189]. Recently, the TAT-like peptide (transactivators of transcription of human immunodeficiency virus) was fused to the C-terminus of Ad5 fiber knob and showed therapeutic efficacy for pancreatic cancer [190]. Finally, Enhanced tropism in both CAR positive and negative cell lines was demonstrated with Ad5 displaying a peptide derived from the envelop glycoprotein of vesicular stomatitis (VSV) virus in the C-terminus region of fiber knob [191]. However, one of the limiting factors for HI loop insertions is that it requires the choice of a suitable peptide as some peptides may generate unwanted conformational changes or steric hindrance resulting in inefficient fiber trimerization and reduced infectivity of the modified virus [192].

The major structural protein "Hexon" is conserved among different Ad serotypes and each hexon contains serotype specific, non-conserved and capsid-surface exposed HVRs (HVRs1-9). These variable loops are the prime site to insert foreign peptides allowing broadening of viral tropism as well as stimulation of the immune response (if peptides are from infectious agents) as they contain an epitope for neutralizing antibodies. Several studies showed successful insertion of peptides such as RGD-4c, CKS17 (binds to tumor growth factor β), human immunodeficiency virus 1 (HIV-1) TAT etc., in the HVR5, HVR2, and HVR7 region of Ad5. In addition, studies also showed that hexon HVR insertions do not affect fiber stability, virus binding/attachment, or virus formation. These insertions do promote enhanced cellular tropism in the target cell [193-197] .

Another potential region capable of tolerating foreign peptide is the C-terminus of the pIX protein. Several studies have been conducted to insert polypeptides such as the RGD motif, pK7, leucine zippers, affibodies, and single domain antibodies in the C-terminus of the pIX protein and have demonstrated enhanced transductional efficiency [192, 198]. However, insertion of protein sequences larger than 163 AA residues in the C-terminus region of pIX protein may disrupt virus capsid assembly. In addition, high affinity binding between the ligand exposed on pIX and cell surface receptors may disrupt endosomal viral release [199, 200] .

1.9.1.2 Adapter based strategies

Another well-developed strategy to generate tropism modified adenoviral vectors is to utilize bi-specific adapters that will act as a molecular bridge. One end of the adapter contains the primary cellular receptors to Ad and will bind the Ad fiber knob and the other end contains a protein sequence capable of binding to an alternative cell surface receptor to promote CAR-independent cell entry. Anti-knob FAb antibody (a fusion of the light chain and corresponding region of the heavy chain) chemically conjugated to folate (Ad5-anti-knob FAB-folate) was the first bi-specific adapter developed and it showed enhanced transductional targeting efficiency for folate overexpressing cancer cells [201]. Several other cancer relevant targeting ligands such as fibroblast growth factor receptor (FGF2), CD40, EGF receptor, epithelial cell adhesion molecule (EPCAM), prostate specific antigen and Tag72 have been fused to the anti-knob antibody adapter to retarget Ad vectors to a wide variety of cancer cells. Bi-specific anti-knob antibody modified Ad showed enhanced tropism to the target cell and provided elevated

transgene expression [202-206]. However, generation of the FAb antibody by random chemical conjugation is a complex and time-consuming process and also generates heterologous molecules. To solve this problem, Haisma *et al.* constructed a bi-specific adapter containing single chain variable fragments (fusion of heavy and light chain variable regions) against fiber knob (scFv) fused to a scFv against various cellular receptors such as EGFR, VFGF2, EPCAM and carcinoembryonic antigen (CEA) and showed enhanced transductional targeting efficiency for the retargeted vector for those cellular receptors [207-210].

In an attempt to further improve the adapter-based strategy, Dmitriev *et al*. replaced the anti-FAB antibody with the extracellular (soluble) portion of CAR (sCAR) fused to anti-CD40 antibody, CD40 ligand (CD40L) or EGF and demonstrated high transduction and gene delivery potential. Several other adapters targeting hexon and penton base have also been developed [211]. A melanoma targeting strategy fused anti-penton monoclonal antibody to tumor necrosis factor alpha (TNFα) has also been reported [212]. Unfortunately, although adapter-based technology seems a promising technology, it may hamper gene transduction due to the weak binding of the adapter molecules to their target receptor (discussed in detail in Chapter 3) rendering adapters unstable *in vivo*.

1.9.1.3 Chimeric or Fiber mosaic adenoviruses

The goal of chimeric adenovirus design is to promote infection by incorporating CAR and integrin independent binding to the target cell. To serve this purpose, several strategies have been developed including two most utilized strategies are pseudotyping (also known as serotype switching) and xenotyping. Pseudotyped Ads

are genetically modified Ads that contain the fiber knob domain or the entire fiber from alternative serotypes that infect the same species and bind receptors other than CAR. The most commonly used serotypes for pseudotyping are from species B and D. Species D Ads utilize non-CAR receptors such as CD80, CD46 and CD80 whereas species B adenovirus utilize CD46 and sialic acid receptors to conduct target cell binding and infection [213-215]. Pseudotyped Ads have been shown to provide broader tropism and increased transduction efficiency in Ad resistant cancer cells such as prostate cancer, breast cancer, and glioma. [216-220]. Common examples of pseudotyped adenovirus use species C Ad5 with either species B Ad3 (Ad5/3) or Ad35 (Ad5/35) fiber, where Ad3 and Ad35 use Desmoglein and CD46 as primary high affinity receptors, respectively. Both Ad5/3 and Ad5/35 showed enhanced transduction efficiency, lower liver toxicity, reduced immune response resulting in higher survival rate for patients with CAR-negative melanoma, epithelial carcinoma, ovarian carcinoma, breast cancer, head and neck cancer and glioblastoma [221, 222]. Other pseudotyped Ads such as Ad5/16, Ad5/19p and Ad5/37 also showed enhanced transduction efficiency for smooth muscle and vascular endothelial cells [223]. Pseudotyped adenovirus can also be developed by swapping hexon HVRs from one serotype to other less immunogenic serotypes. Recent studies showed reduced immune response for a pseudotyped Ad5 containing Ad48 HVR (Ad5Ad48HVR) [224]. Kuhn *et al.* utilized a unique method called "Directed Evolution" to generate a pseudotyped Ad called ColoAd1 for colon cancer. In presence of stringent conditions, the authors promoted random recombination among an array of serotypes from different subgroups followed by passaging on a panel of cancer cell lines to direct the emergence of ColAd1. ColAd1 contains a capsid of Ad11p but its E2B region is a chimera from Ad3 and Ad11p such

that pTP and DNA pol regions originated from Ad3. It has been reported that the prevalence of neutralizing antibodies for Ad11 are low in comparison to other serotypes. As a result, this chimeric virus conducted cell entry via the CD46/DSG2 pathway and provided a high level of blood persistence after systemic delivery with enhanced oncolytic potential for colon cancer [225].

Another strategy to generate chimeric adenovirus is called xenotyping where wild-type fiber elements are replaced with fiber elements from non-human Ad serotypes such as fiber elements from bovine Ad3, canine Ad2, porcine Ad3 ovine Ad7, murine Ad1 or simian and fowl Ad [226, 227]. These xenotyped Ads showed enhanced transductional efficiency in human and mouse cells comparable to that of human Ads, with reduced immune responses [228, 229]. For example, Glasgow *et al.* generated xenotyped Ad5 containing CAV2 knob (Ad5Luc1-CK) and reported enhanced tropism of this xenotyped Ad5 in CAR-deficient cells. However, they also reported that the transduction of Ad5Luc1-CK in CAR-negative cell lines are highly dependent on the availability of cell surface integrins [227]. In addition, Kremer's group reported successful utilization of CAV2 for neurological diseases with enhanced cellular tropism, enhanced axonal biodistribution and long-lasting persistence with reduced immunogenicity [230].

1.9.1.4 Adenoviral complex and dual fiber mosaicism

Several advanced strategies have also been developed to further increase the transduction efficiency and diversity of the transductional properties of adenovirus vectors. In this regard, the Borovjagin group tried to insert an RGD-4c

targeted peptide in the HI loop or in both HI loop and C-terminus of the knob of either Ad5 or Ad5/3 chimeric virus. However, the results showed reduced infectivity of the modified vector containing peptide in the HI loop of Ad5/3 but not for Ad5 virus with the same HI loop insertion. In contrast, enhanced activity was observed for Ad5/3 virus containing the peptide at the C-terminus of Ad3 instead of HI loop, indicating that the HI loop of Ad3 virus was unable to accept RGD peptides [231]. Tyler *et al.* and Dobbin *et al.* showed higher infectivity in glioma, lung, prostate, breast and ovarian cell lines with tropism modified Ad5/3 vector containing an RGD motif in the C-terminus of fiber knob. However, not all Ad virus serotypes are compatible with peptide insertion in either HI loop or C terminus of the fiber knob [232, 233]. For instance, Matsui *et al.* attempted to insert a peptide in either the HI loop of Ad35 or the Ad35 fiber knob C-terminus in an Ad5/35 chimeric virus, which resulted in reduced viral infectivity and no viral progeny respectively [234].

Another strategy to enhance the broad tropism of Ad is to conduct genetic modification to incorporate multiple receptors with different receptor-binding capabilities in the same virus (known as "dual fiber mosaicism"). Takayama *et al.* constructed dual fiber mosaic virus containing both wild type Ad5 and Ad5/3 chimeric virus fibers (Ad5-Ad5/3). This dual mosaic virus demonstrated enhanced transduction efficiency in a panel of cell lines as it possessed the unique capability to utilize both CAR and DSG2 cellular receptors for cell entry [235]. Interestingly, Tsuruta *et al.* further expanded this strategy by developing a tropism modified dual mosaic Ad5 virus containing wild-type Ad5 fiber and serotype 3 Dearing reovirus, σ1 attachment protein. The σ1 attachment protein utilizes cell surface sialic acid and junction adhesion molecule 1 (JAM1) receptors [236]. This mosaic

virus was termed Ad5-5/ σ1 and showed efficient tropism in CAR negative cell lines and human ovarian carcinoma cells. In addition, this group also developed another dual mosaic virus called Ad5/3-/5/σ1 virus with Ad3 and σ1 attachment protein bearing fiber, which was capable of utilizing DSG2, sialic acid or JAM1 receptors and showed enhanced tropism for CAR negative ovarian cancer cells when compared to Ad5 vector but had reduced tropism compared to Ad5/3 vector [237].

1.9.1.5 Knobless adenoviral vector

Another strategy to improve adenoviral transduction, as well as to allow the utilization of a wide variety of targeting ligands, is to generate "knob-less" vector by replacing the entire knob domain and part of the shaft domain of Ad vector. One of the limitations for the successful utilization of this concept is the suitable replacement of the trimerization potential of the fiber shaft and knob domains that is required for proper fiber assembly and binding. In this regard, Krasnykh *et al.* utilized the trimerization potential of the highly stable "foldon" domain of T4 bacteriophage called "Fibritin" that possess overall structural similarity to the Ad5 fiber with no other non-specific receptor binding. Fibritin protein is generally found in a trimerized form in T4 bacteriophage capsid and its trimerization potential is maintained by a short, 30 AA long C-terminal region called the "foldon" domain. The foldon is stabilized by a number of hydrogen bonds and hydrophobic interactions. The trimerized fibritin protein functions to assemble the long tail fibers to promote the attachment of the fibers to the tail baseplate. In addition, this protein possesses a unique stability even after extensive amino terminal deletion or carboxy terminal insertion of AAs making it an outstanding choice for a fiber chimera.

The fibritin trimerization domain was added after the tail and two AA repeats of the Ad5 shaft domain of fiber followed by a 6 histidine (6-his) motif at the C terminus. This fiber-fibritin-6 his chimera showed superior transductional efficiency and enhanced gene expression when targeted to cells expressing an artificial 6-his binding receptor. This strategy demonstrated the feasibility of incorporating specific receptor binding peptides, protein ligands, or, most importantly, single chain antibodies at the C-terminus of fibritin via a short linker [238] Matsui *et al.* further expanded this strategy by inserting synthetic monobodies (does not contain disulphide bond) specific for epidermal growth factor (EGFR) or vascular endothelial growth factor receptor 2 (VEGFR2) in the C-terminus for the fiber-fibritin chimera. The resultant fiber modified virus showed enhanced cellular binding and transfection efficiency for EGFR/ VGFR2 positive cells with reduced normal cell toxicity and tissue damage [239]. In contrast to using synthetic monobodies, Kaliberov *et al.* demonstrated the feasibility of single domain variable heavy chain antibody (VHH) derived from camelids by incorporating anti-human carcinoembryonic antigen (hCEA) VHHs in the C-terminus of an Ad5-fiber-fibritin chimera [240]. Camelid antibodies only possess two variable heavy chains (VHH, linked by a disulphide bond) but no light chains and show unique folding properties and enhanced cytosolic stability, even in a reducing cytosolic/nuclear environment. VHHs have a high compatibility with phage display bio-panning, are easy to produce and are less expensive than double chain antibodies or their derivatives. In addition, due to their highly stable nature and unique structural features they allow target cell binding, biosynthesis, assembly and release [241]. These tropism modified Ad5 showed enhanced cell binding and transductional efficiency for CEA-expressing cancer cells with no change observed in

either the trimerization potential of the Ad5 fiber, or antigen recognition potential for the VHH antibody [240]. Kim *et al.* utilized this strategy to incorporate a glioma specific peptide at the C-terminus of a fiber-fibritin chimera and generated highly efficient, transductionally targeted Ad5 with no infection observed in normal human astrocytes, neuronal stem cells, lung carcinoma, ovarian carcinoma or breast carcinoma cells [242]. Recently, Kim *et al.* successfully targeted IL13Rα2 expressing cancer cells by incorporating the variable region of a single chain antibody (scFv) for IL13Rα2 in the C-terminus of an Ad5 fiber-fibritin chimera. The IL13Rα2 targeted Ad5 showed enhanced binding to IL13Rα2 on glioma cells but showed no binding to IL13Rα1 receptor, which is widely expressed by normal cells [243].

Other "de-knobbing" strategies have been developed by incorporating large or small peptides in knobless fiber peptides. Magnussen *et al.* incorporated an external trimerization domain followed by an integrin-binding RGD motif and successfully targeted integrin expressing cells [244]. Further progress was shown by the Belousova group when they incorporated HER2/Neu specific affibody (small peptide) into the HI loop to generate knobless fiber to target HER2/Neu expressing prostate cancer cells. Results showed the potential of affibodies to promote the function of knobless fiber as well as HER2/Neu retargeted virus to accomplish gene delivery to HER2/Neu expressing prostate cancer cells [245, 246]. Finally, further expansion of this strategy was conducted by the Izumi group fusing CD40 ligand in the C terminus of an Ad5-fiber-fibritin chimera which promoted CD40-specific adenoviral mediated gene delivery *in vivo* in mice [247].

1.10 Transcriptional targeting of oncolytic viruses

1.10.1 Tumor upregulated promoters

Extensive cancer genome analysis via proteomics, metagenomics and transcriptomics have revealed that aberrant gene regulation is a hallmark of cancer. It is an outcome of recurrent somatic mutations in the genes required for maintaining normal cellular functions. Aberrant gene regulation affects every component of normal transcriptional control, promoting uncontrolled cellular growth, where genes are either "switched on" (gene activation) or "switched off" (gene silencing). In molecular terms, these genetic alterations incorporate modifications in several key components of the gene regulatory network such as chromatin remodeling, transcriptional control, RNA stability, translational control, as well as protein structure, stability, and function. In normal cells, transcription is a dynamic process regulated by a complex network of tissue-specific transcription factors (TFs) collaborating for gene activation and gene silencing. TFs conduct gene regulation by binding cooperatively to specific *cis* and *trans* gene regulatory sequences of target genes, such as promoters, enhancers, super-enhancers (clusters of enhancers) and chromatic regulators. By regulating gene expression they control the cell cycle, cell growth and development, specialized cell functions, and cell-type specificity (master TFs) and, finally, cell death [248]. In cancer, mutations in TFs may result in changes in TF structure and function, promoting altered binding of TFs to gene regulatory sequences. This facilitates uncontrolled expression of specific genes leading to conversion of proto-oncogenes into oncogenes or silencing of essential genes such as tumor suppressor genes. Alternatively, somatic mutations in gene regulatory sequences can alter the binding potential of TFs promoting aberrant transcription and expression, again leading to uncontrolled cell growth. The promoter is defined as the upstream regulatory unit (stretches

of DNA sequences) of the gene. It is both position and direction dependent. The promoter is located immediately 5' of the transcription start site. The promoter facilitates binding of RNA polymerase for RNA transcription and possess cell/tissue-specific regulatory functions [249]. Studies have shown that TFs can activate promoters by directly binding to consensus sites within the promoter or by interacting with enhancer regions to recruit co-activators, modulators or repressors. [250]. Promoters that show enhanced transcriptional activity specifically in tumor cells but show little or no activity in normal cells are known as tumor upregulated promoters (TUPs) [251]. TUPs are considered outstanding candidates for cancer gene therapy to promote cancer specific expression of therapeutic genes [252-254].

Harrington *et al*. divided TUPs into subgroups based on their characteristics, functionality, and purpose of use [255]. The first of these groups are cancer-specific promoters that show enhanced expression in tumor cells with little or no activity in normal cells. This type of promoter is hard to find and only one human gene, telomerase, can be included in this group. 90% of human cancers express high levels of telomerase with reduced expression in normal cells [256-258]. Telomerase gene expression is driven by the catalytic protein subunit telomerase reverse transcriptase (TERT) which also showed high level of expression in embryonic stem cells, progressively dividing cells, and cancer cells with low expression in normal cells [259].

The second group consists of promoters that are oncofoetal in origin and show enhanced expression in certain types of tumors. Examples of such promoters include carcinoembryonic antigen (CEA) for adenocarcinomas, α-fetoprotein promotor for

hepatocellular carcinomas, erB2/HER2 for breast and pancreatic tumors, MUC1 glycoprotein for breast and cholangiocarcinomas and osteocalcin (OC) for osteogenic sarcomas [260-263]. Included in this group are cell-cycle related proliferation associated genes that show enhanced expression during cancer progression and can be considered as tumor type-upregulated promoters such as E2F response promoters (E2F-1), cell cycle related promoters such as cyclin A or cdc25c, and insulin-like growth factor 1 (IGF-1) [264]. The functionality of E2F promoters are dependent the activity of retinoblastoma (RB) tumor suppressor gene. RB represses E2F transcription factors and in turn regulates uncontrolled cell growth. Tumor cells are RB-defective showing enhanced activity of E2F-regualted promoters [263].

The third type of TUPs are promoters that show elevated expression based on tumor microenvironment (TME) or physiology. TME is the environment around tumors that consists of cellular elements such as fibroblasts, neuroendocrine cells, adipose cells, immune inflammatory cells, blood and lymphatic vessel networks along with an extracellular matrix that, together, govern tumor evolution, resulting in advanced malignancies by promoting tumor angiogenesis (uncontrolled proliferation of endothelial cells) and immune tolerance [265]. Thus, TME promotes activation of specific genes and those genes possess the potential to be used as TUPs. One of the key factors that enhance specific gene upregulation in TME is the presence of hypoxia. Hypoxia plays an important role in gene upregulation by recruiting hypoxia-inducible factor-1 (HIF-1). HIF-1 is capable of binding to the hypoxia response elements (HREs) of a variety of hypoxia responsive TUPs such as vascular endothelial growth factor (VEGF), erythropoietin, and phosphoglycerate kinase 1 which are important in hematoma, high-grade glioma and

fibrosarcoma, respectively [266, 267]. There are other TME-induced TUPs such as endothelial specific kinase inserts domain receptor (KDR/FIK-1), E selectin, and vascular endothelial cell adhesion molecule 1 (VCAM-1) that show elevated expression in tumor endothelium [268, 269]. Glucose responsive promoters are induced in response to low glucose, hypoxia, acidic pH, and show upregulated expression in solid tumors. Examples include glucose regulated protein 78 (GRP78) in breast cancer and hexokinase II in lung cancer [270, 271].

The fourth type of TUPs are those promoters that can be regulated exogenously such as radiation, heat, and drug-inducible promoters. Ionizing radiation facilitates the transcription upregulation of several cellular gene promoters such as *Egr*-1 and radiation-responsive elements CARrG, Waf-1, and cIAP2 [272, 273]. Heat-inducible promoters such as heat shock protein 70 (HSP70B) or stress-inducible promoters such as Gadd152 showed enhanced tumor activity after hyperthermia treatment [274, 275]. Drug inducible promoters provide advantages over other promoters as they are strong and can be regulated exogenously. Examples include tetracycline, rapamycin, or tamoxifen-inducible estrogen-responsive promoters that show enhanced activity in cancers such as breast, colon, melanoma, glioma, and possess the potential to be used as TUPs to drive tumor specific therapeutic gene expression [276-279].

1.10.2 Application of TUPs to therapeutic viruses

The use of TUPs adds another layer of safety and protection on top of transductional targeting (discussed in section 1.9) by regulating the expression of therapeutic transgenes, and restricting replication of oncolytic viruses to tumors. While these functions can be

served by universal promoters such as the retroviral long terminal repeat (LTR), the cytomegalovirus immediate early promoter (CMV) or the rous sarcoma virus (RSV) promoter, providing both high levels of expression and transcriptional activity in a broad range of host cells, they also provide non-specific expression of the transgene due to their indiscriminate pattern of expression.

TUPs can play an important role in developing "transcriptionally targeted" viruses capable of restricting their functionality to malignant cells [280]. Transcriptional targeting allows regulation of therapeutic gene expression to ensure that the transgene expression level is regulated according to therapeutic need. Therapeutic genes, introduced into cancer cells under the control of suitable TUPs, limit transgene expression to malignant cells and reduce collateral expression in normal cells, hence reduced normal cell toxicities [281] . For example, PSA, MUC1, and CEA promoter regions have been used to selectively drive expression of the herpes simplex virus-1 thymidine kinase (HSV-Tk) gene in PSA^+, $MUC1^+$ or CEA^+ cells in both *in vitro and in vivo*. Additional suicide gene therapy Ads have been created by driving tumor necrosis factor alpha (TNF-α), purine nucleoside phosphorylase, and cytosine deaminase under the control of TUPs [282, 283] .

Adenoviruses can be engineered into conditionally replicative adenoviruses (CRAds) by putting the essential genes of replication, such as E1A or E1B, under the control of a TUP. The E1 region contains E1A, E1B-19KD, and E1B-55k genes that play key roles in regulating viral replication. CRAds can replicate inside tumor cells, generate progeny, induce transgene expression, and subsequently, induce tumor cell death. That cytopathic effect will release CRAds that will infect additional tumor cells. CRAds fail to

replicate inside normal cells leading to normal cell survival [284, 285]. Several TUPs such as AFP, PSA, and MUC1 have been used in place of the adenoviral E1A promoter to drive Ad replication in a tumor specific manner *in vitro,* for cancers such as liver, breast, and prostate. CRAds did not show any transcriptional leakiness [33]. Other conditionally replicative Ads have been generated by placing E1A under the control of promoters such as human TERT (hTERT), HRE, EGR-1, E-selectin, cyclooxygenase -2 (COX2), and E2F to promote oncolytic virotherapy.

Another approach to control tumor specific activity is to control both *E1A* and *E4*. Banerjee *et al.* developed this CRAd by placing both the *E1A* and *E4* regions under the control of the tyrosinase promoter (containing a tandem enhancer and core promoter region of human tyrosinase) that showed enhanced and targeted activity for human melanomas [286]. Transcriptionally targeted adenovirus was also developed by utilizing inducible promoters capable of taking advantages of cancer defects, TME, or that can be induced exogenously. Alcoceba *et al.* developed a dual-responsive CRAd by placing *E1A* under the control of a minimal dual-specificity promoter capable of responding to estrogen and hypoxia as well as putting the *E4* region under the control of the E2F-1 promoter. This virus showed enhanced breast cancer specificity [287].

Transcriptional targeting has also been conducted to generate conditionally replicating CAV2. Hemminki *et al.* developed a canine osteosarcoma targeted oncolytic CAV2 by placing the CAV2-*E1A* under the control of the osteocalcin promoter, which is overexpressed in osteosarcoma. This virus showed enhanced osteosarcoma-based activity *in vitro* and in murine xenotransplants [288]. In addition, Laborda *et al.* generated a CAV2

based CRAd by modifying the E1A promoter to drive *E1A* in E2F enriched cells and using the major late promoter/IIIA protein splice acceptor to drive hyaluronidase expression. Hyaluronan (HA) is a structural component of extracellular matrix, that shows enhanced expression in cancer and degradation with hyaluronidase enzyme could facilitate the spread of anticancer agents to the malignant core [289]. This dually targeted CAV2 showed enhanced antitumor efficacy along with increased spread of the CRAd to tumor cells such as adenoma, osteosarcoma, mastocitoma, hepatic carcinoma [290].

TUPs can also perform a dual role by controlling both viral replication and therapeutic gene expression in the same vector to enhance the full potential of oncolytic virotherapy. Several tumor upregulated promoters such as alpha fetoprotein promotor for liver and prostate specific antigen (PSA) promoter for prostate cancer have been used [249] [291-293]. However, in many cases, the TUPs are too weak to generate sufficient levels of transgene expression and thus, require additional modification to convert them into strong promoters. There are two general strategies to convert a weak TUP into a strong one, i) induction of a positive feedback loop and ii) Cre recombinase/loxP mediated induction. In the first process, a weak tissue specific promoter containing a LexA binding site upstream is used to express both desired transgene and a gene encoding a VP16-LexA fusion protein. Once the weak promoter starts transcription of the gene, VP16-LexA fusion protein is produced that, in turn, binds to the LexA binding site, leading to transactivation enhanced activation of the weak promoter [269]. In the second case, a plasmid was generated that contained a therapeutic transgene separated from a universal promoter by a “stop” cassette bracketed by two loxP sites. Co-transfection of the target cells with both the transgene containing plasmid and a second plasmid encoding a Cre recombinase gene driven by a

promoter that removes the stop cassette by the Cre/loxP system and places the transgene under the control of a universal promoter leading to enhanced tumor-specific transgene expression [294].

CHAPTER 2

In vitro Functional Genetic Modification in Canine Adenovirus Type 2 Genome by CRISPR/Cas9

2.1 Introduction

Oncolytic virotherapy is a promising therapeutic approach for patients with relapsed and refractory cancers. Oncolytic viruses (OVs) utilize tumor-upregulated promoters to facilitate selective eradication of cancer cells/tissues while sparing normal cells. Payloads that attack tumors or enhance anti-tumor immune responses may also be incorporated into OVs As a result, this approach provides a multidimensional therapeutic opportunity to modulate the immunosuppressive tumor microenvironment and stimulate host anti-tumor immune responses as well as targeted killing of cancer cells [295, 296]. Suitable viral vectors are an essential tool for promoting successful oncolytic virotherapy. A broad spectrum of DNA and RNA viruses, such as retroviruses, adenoviruses and adeno associated viruses (AAV), have been engineered to perform as oncolytic viruses and have been used in clinical trials for cancer treatment [297]. Conditionally replicative recombinant Adenoviral (CRAd) vectors are currently the most common vehicle for oncolytic virotherapy for cancer. Adenoviruses are also commonly used for other gene therapy applications and account for 18.6% of nearly 3001 clinical trials that were conducted by using both viral and non-viral vectors as of 2019 (http://www.abedia.com/wiley/vectors.php). Advantages of adenoviral vectors include genetic stability, well-defined biology, little or no insertional mutagenesis, the ability to accommodate large inserts, and high titers [298]. Apart from human adenovirus, non-human adenoviruses have gaining significant attention in clinical applications to bypass

potential clinical disadvantages provided by human adenoviruses, including pre-existing immunity to the virus. Canine adenovirus type 2 (CAV2), a non-human adenovirus derived from the dog, can serve as a vector for both canine and human applications and, in human patients, may bypass the disadvantages of pre-existing humoral immunity and stimulation of memory cytotoxic lymphocytes (CTLs) [299, 300].

However, construction of adenovirus with conventional methods is challenging due to their large genome size (~32kb). Currently, the genetic modification of adenovirus is dependent on the availability of suitable restriction enzyme sites. However, restriction enzyme-based cloning methods are limited by the availability of such suitable, unique, restriction enzyme sites [301]. Several commercial backbone plasmids for human adenovirus 5 are available to overcome some of the difficulties. However, the subsequent steps for adenoviral vector construction are complex, requiring laborious multistep methods, have low efficiency, are time consuming, and in some cases, are contaminated with parental adenoviral vectors [298]. Genetic modification of CAV2 is similarly complex, time consuming and inefficient, both because it has a similar genome size to Ad5, and due to the lack of commercial or lab-based shuttle plasmids and viral backbones [298].

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein (Cas9) is a recently developed genetic editing system that was adapted from a microbial immune response. CRISPR-Cas9 has been used to conduct precise genomic modification of key genes in a variety of model organisms [152, 302-304]. This system allows precise genome editing by utilizing an RNA guided nuclease, Cas9, to make double stranded DNA cuts. It is independent of restriction enzyme digestion and is quick

and cost-effective [152]. This technique has been used to efficiently promote precise genome editing in large DNA viruses such as Ad5 and HSV1 [305, 306].

However, the feasibility of this technique has not been tested for its potential to promote genetic modification in CAV2. In this respect, we investigated the potential for CRISPR-Cas9 to precisely cut the CAV2 genome creating a site suitable for insertion of new material in the CAV2 genome (**Fig. 2.1**). The viruses produced were then assessed for their ability to reproduce and serve as CRAds.

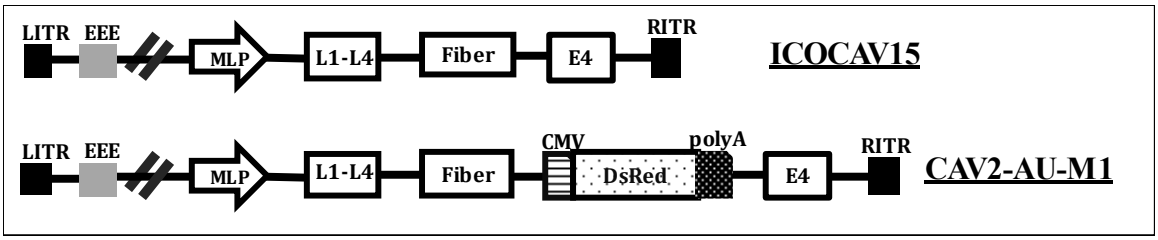

Figure 2.1: Schematic representation of recombinant CAV2 genomes

ICOCAV15, a parental CAV2 virus, was used in this study for modification. CAV2-AU-M1 is the recombinant virus generated in this study by inserting CMV-DsRed between the fiber and E4 regions of ICOCAV15 via CRISPR/Cas9 technology. LITR = Left Inverted Terminal Repeat, RITR= Right Inverted Terminal Repeat, EEE= E2-F Modified E1A promoter, MLP = Major Late promoter, L1-L4 = Late transcript 1 to 4, CMV= Cytomegalovirus immediate early promoter. cIL2= Canine Interleukin 2, cIL2RasdAb= Canine Interleukin 2 Receptor alpha Single Domain Antibody.

2.2 Materials and methods

Cell culture:

Canine mammary tumor (CMT28), fetal kidney (FDK), osteosarcoma (D17), kidney (DKCre) cell lines, and normal canine fibroblasts (NCF) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Corning) with penicillin (100 IU/ml, Corning), streptomycin (100 ug/ml, Corning), amphotericin B (0.5ug/ml, Corning), and 10% FBS (fetal bovine serum, Sigma) All cells were grown at 37^0C and 5% CO_2 and were validated to be of canine origin by species specific PCR [307].

***In vitro* synthesis of sgRNA**

The synthetic guide RNA (sgRNA) sequences were designed using the online CRISPR Design Tool (http://cripsr.mit.edu) which selected 20bp guide RNA sequences followed by the PAM sequence "NGG". After selection, sgRNAs were checked for off-target binding aligning the sgRNA sequence against the CAV2 genome vector (pICOAV15) using DNASTAR software. The sgRNA target, GCGGTAAGGCTGCCGCCTTC, located at 28441bp to 28460bp of pICOCAV15, followed by the PAM sequence AGG, located at 28461bp to 28463bp, was identified (**Table 2.1**). This was predicted to generate a double stranded cut at base 28457bp. An oligonucleotide containing the T7 promoter sequence, target sgRNA sequence and sgRNA scaffold, were designed online using the "sgRNA designer" tool (NEB). The oligonucleotide was commercially synthesized (Eurofin MWG Operon) and used as a

template for sgRNA synthesis. In order to synthesize sgRNA, *in vitro* transcription was conducted using an EnGen® sgRNA Synthesis Kit (catalog no. E3322, NEB) according to the manufacturer's protocol. Briefly, 5uM of template DNA oligo was mixed with the required volume of buffer and enzyme mix followed by one-hour incubation at 37^0C. After synthesis, sgRNA was quickly transferred on ice and treated with DNase for 15 mins at 37^0C to promote enzymatic degradation of the template oligo nucleotide followed by purification using the MEGAclear™ Transcription Clean-up kit (catalog no. AM1908, Thermo Scientific™) according to the manufacturer's protocol.

Table 2.1: sgRNA oligos, sequence and location

Oligo	Location	PAM (5′to 3′)	Sequence (5′to 3′)
sgRNA oligo for Insertion of CMV-DsRed insert	Top strand (28441bp to 28460bp)	28461bp to 28463bp	TTCTAATACGACTCACTATAGCGG TAAGGCTGCCGCCTTCGTTTTAGA GCTAGA
Violet= T7 promoter, Orange= Added G's, Red= sgRNA sequence and Green= RNA scaffold, PAM= Prospacer Adjacent Motif			

Linearization of CAV2 genome vector using sgRNA and Cas9 single and double digestion

pICOCAV15, a plasmid containing a modified oncolytic canine adenovirus derived from CAV2 (gift of Dr. Ramon Alemany, Translational Research Laboratory, IDIBELL-Institut Català d'Oncologia, Barcelona, Spain) was used as a the substrate for CRISPR-Cas9 cleavage [290]. For *in vitro* Cas9 mediated single digestion of the pICOCAV15 genome vector, 3.5ug pICOCAV15, 3.5ug Cas9 (New England Biolabs, catalog no. M0386) and 7ug sgRNA were incubated in buffer 3.1 (NEB) at 37^0C overnight. The

reaction was terminated by incubating the tubes at 65^0C for 10 minutes to inactivate Cas9 nuclease. In order to remove the sgRNA, the reaction was treated with 0.2mg/ml RNAse A (catalog no. EN0531, Thermo Scientific™) and incubated at 37^0C for 15 mins to 1 hour based the amount of sgRNA used. The reaction mixture was then treated with SDS (1%) and 1mg/ml proteinase K (Thermo Scientific™) and incubated at 55^0C for 30 mins. Finally, the Cas9 digested vector was recovered by phenol/chloroform extraction and verified by gel electrophoresis.

Production of insert and homology directed repair (HDR)

The HDR insert, consisting of CMV-DsRed insert flanking the homologous region at both 5' and 3' end, was amplified by overhang PCR using pPrime-CMV-DsRed plasmid (Addgene), that is a 3rd generation lentiviral transfer vector (Empty backbone) with a CMV promoter controlling DsRed expression, as template (**Table 2.2**).

Table 2.2: PCR, sequencing and Taqman qPCR primers

Purpose	Primers	Sequence (5'to 3')
Overhang PCR	Forward Primer	GTCTTAACCTTTACCTATGTAGGCGAAAATCAATAAAACCAGAAAAAA ATAAGTAGTAATCAATTACGGGGTCATTAG
	Reverse Primer	GAACTGTTTACAGAGTAACTTTTCCTGAAGGCGGCAGCCTTACCGCTC GCGTGTATGAAAAATAAAGCTTTTAAACCGAGTGAGAGACACAAAAA ATTC
Colony PCR	Forward Primer	CATCTCCCGCTGTGGACTAG
	Reverse Primer	GACACCTGTAGTGCTTCAC
Taqman qPCR	Forward Primer	CTCCCGCTGTGGACTAGACAG
	Reverse Primer	AACAGGGTACCTCCGCTTAGTC
	Probe	[6-FAM] GCTGTGACTGCCGCGGAGACTGTTTCT[Tamra-Q]

Overhang PCR assays were performed using 0.5ng of template DNA and Phusion Hot start II high fidelity PCR master mix (Thermo Scientific™, catalog# F-565S) according to manufacturer's instruction. Thermocycling conditions were: 1 cycle at 98^0 C for 30 seconds, 5 cycles of 98^0C for 10 seconds, 58^0C for 15 seconds (annealing), 72^0C for

1 min, and 40 cycles of 98^0C for 10 seconds, 62^0C for 15 seconds (annealing), 72^0C for 1 min (extension) and 1 cycle for 72^0C for 5 min (final extension). The PCR product was then gel purified by agarose gel electrophoresis in 1.2% Agarose TBE gel and extracted using the GeneJET Gel Extraction Kit (Thermo Scientific™, catalog# K0691).

Homology directed repair of Cas9 digested vector was conducted according to the protocol described in NEBuilder® HiFi DNA Assembly Kit (NEB, catalog no. E5520). Briefly, Cas9 digested vector and HDR insert were combined at a DNA molar ratio of 1:4 respectively and a total of 0.2 picomoles were incubated in the assembly buffer for 30 mins at 50^0C in a thermocycler.

Transformation and positive colony selection

Following homologous recombination, 5 ul of the assembled product were transformed into 5 alpha (NEB, catalog# C2987) competent cells according to the manufacturer's instruction and grown on LB agar with 100ug/ul carbenicillin. Positive clones were then selected by colony PCR with forward primers located upstream and reverse located downstream of the insert **(Table 2.2)**. Briefly, each colony was picked with a sterile toothpick and dipped into 40ul of nuclease free water (NFW), vortexed to mix properly and 5ul from the mixture was then added to the subsequent PCR reaction. The PCR assay was performed using GoTaq® Green Master Mix (Promega Corporation, catalog# M7122). Thermocycling conditions were: 1 cycle at 95^0 C for 2 mins, 40 cycles of 95^0 C for 30 seconds, 61^0 C for 15 seconds (annealing), 72^0C for 1 min 15 seconds (extension) and 1cycle for 72^0 C for 5 mins (final extension). Positive colonies providing the desired amplicon in colony PCR were then used for further analysis. Preparation of plasmid DNA was carried out using QIAprep spin Mini prep kit (Qiagen, catalog # 27106).

The insert was validated by sequencing (Eurofins MWG Operon) followed by a larger scale preparation of positive plasmid DNA by using ZymoPURE™ II Plasmid Midiprep Kit (Zymo Research Corporation, catalog# D4200).

Restriction digestion and transfection

Linearized recombinant CAV2 vector was constructed by double digesting with NotI-HF restriction endonuclease (NEB, catalog# R3189S). Briefly, 100ug of CAV2 vector was mixed with required volume of cut-smart buffer and 200 unit of NotI-HF, incubated 37^0 C for 3hrs followed by gel purification with Zymoclean™ Large Fragment DNA Recovery Kit (Zymo Research Corporation, catalog# D4045). Linearized recombinant CAV2 vector was then used to transfect DKCre cells by using Lipofectamine P3000 transfection reagent (Thermo Scientific™, catalog# L3000015). The day before transfection, 2.5×10^5 adherent DKCre cells were seeded in a 12-well plate. On the day of transfection, cells were washed with 1 ml of 1X PBS and refed with DMEM containing 5 % FBS without antibiotics or fungizone. For transfection, 1.5ug of digested DNA was mixed with 6ul of P3000 and added to 69ul of Opti-MEM media to bring the volume to 75ul. This was mixed with 12ul of Lipofectamine reagent diluted in 63ul of Opti-MEM media (Thermo Scientific™) and incubated for 20 mins at room temperature. 150ul of transfection mix was then added to the respective wells dropwise in DMEM +5% FBS with no antibiotics and antibiotics-containing medium and incubated at 37^0C. After 24hrs, the media was changed to fresh DMEM containing 5 % FBS without antibiotics and fungizone and incubated at 37^0C for 6 days.

Virus rescue, infection and mass production of virus

Seven days post-transfection, transfected DKCre cells were collected by scraping the cells off of the 12-well plate in the media that was already present. The cells were centrifuged to form a pellet and the cell pellet was resuspended in 1X PBS. The virus was then freed from the cells by three freeze-thaw cycles, alternating between liquid nitrogen and 37^0C, and then centrifuged to remove cellular debris. Half of the cleared lysate was incubated with a fresh monolayer of DKCre cells and collected 48-72hr post-infection, when cytopathic effect was observed. Production was then scaled up via amplification in 6-well plates, T25 flasks and, finally, in multiple T175 flasks, showing complete cytopathic effect 48hr post-infection. The viruses were then purified using an Iodixanol-Gradient as described by Marta Giménez-Alejandre et al [308].

For infection, 3×10^5 cells of DKCre, CMT28, FDK, D17 and NCF were plated in 12-well plates in triplicate one day prior to virus infections. Cells were washed with 1X PBS (Phosphate Buffered Saline), and infected with 100ul of DMEM/RPMI (2% FBS), containing the virus with different MOI viz 0, 1, 10, 100. Cells were monitored at 24, 48, 72, and 96hr post infection for red fluorescence using an inverted fluorescent microscope (EVOS FL Cell Imaging System). After 48h, infected adherent cells were harvested by trypsinization and suspended cells were harvested by centrifugation. Cells were washed twice with PBS. The cells were resuspended in PBS and 0.1% BSA, and analyzed for DsRed expression by flow cytometry (Accuri C6).

Virus quantification

ICOCAV15 and CAV2-AU-M1 genome copy number were measured by absolute quantitative Taqman PCR using a standard curve method. Forward primer, reverse primer

and probe were designed to target the unique fiber region of CAV2 (**Table 2.2**). A standard sample of viral plasmid DNA for the standard curve was generated using the plasmids pICOCAV15 and pCAV2-AU-M1 for ICOCAV15 and CAV2-AU-M1, respectively. One hundred-fold serial dilution were then conducted to obtain 10^8, 10^6, 10^4 and 10^2 plasmid copy number per 5ul. Standard curves for each plasmid were constructed by plotting Ct values versus the log of the starting sample amount using Bio-Rad CFX^{TM} Manager Software (Bio-Rad). Absolute quantification was performed using 0.5uM each of forward primer, reverse primer, and probe. The reactions were amplified with Sso advanced Universal Probes supermix (Bio-Rad, catalog# 1725281) according to the manufacturer's instruction. Thermocycling conditions were: 1 cycle at 95^0 C for 3 min, 40 cycles of 95^0 C for 10 sec, 60^0 C for 30 sec.

Virus infectious particle number (VP) for CAV2-AU-M1 was quantified by using serial dilution followed by fluorescent plaque assay. Briefly, the day before infection, 3×10^5 cells adherent DKCre cells were seeded in a 12-well plate. Then, cells were infected in triplicate with viruses having MOI ranging from 0.000001 to 1. Red fluorescent cell were counted, 24 hours post infection, by eye using an inverted fluorescence microscopy.

Crystal violet cell killing assay

Cells in 12-well plates were infected with ICOCAV15 and CAV2-AU-M1 for 24hr at 37°C with multiplicity of infection (MOIs) of 0, 1, 10 and 100. Cells were then washed once and incubated at 37°C in 2% FBS medium. On day 4 crystal violet staining was performed as described [309]. Briefly, After 4 day, cells were washed once with 1X PBS and fixed with 1ml of freshly prepared 4% paraformaldehyde in each well and incubated

at room temperature for 10 min. Paraformaldehyde was then removed and 1.5 ml of crystal violet was added in each well and incubated for 10 min at room temperature. After 10 min, the wells were washed with water.

2.3 Results

To demonstrate the ability of CRISPR-Cas9 to create a single break in the CAV2 genome and subsequently allow insertion of additional sequence, a CMV promoter driven DsRed (red fluorescent protein from *Discosoma sp.*) [310] construct was inserted between the fiber and E4 region of the plasmid pICOCAV15 (~36kb) (CAV-AU-M1) (**Fig. 2.1)**.

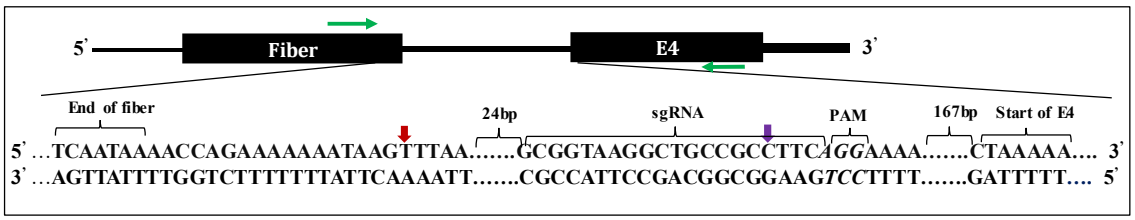

Figure 2.2: Schematic representation of the sequence and location of sgRNA in CAV2 vector genome

The sgRNA is located between the fiber and E4 region followed by the PAM sequence (italics). The red arrow represents the insertion site for CMV-DsRed. The violet arrow represents the position of the Cas9 cut site and green arrows represent the locations of colony PCR and sequencing primers. PAM= Protospacer adjacent motif.

The pICOCAV15 contains a complete CAV2 genome and plasmid backbone with an ampicillin resistance gene and origin of replication. Based on the location of the fiber and E4 viral genes, it was determined that the CMV-DsRed construct should be inserted at position 28412bp of pICOCAV15. A 20 bp sgRNA,

GCGGTAAGGCTGCCGCCTTC followed by the PAM sequence AGG was identified that would create a double stranded break at position 28457bp **(Fig. 2.2)**.

An oligonucleotide was synthesized that contained the reverse compliment of the target, additional RNA sequence for the common sgRNA scaffold and the T7 promoter. The sgRNA was synthesized by T7 polymerase. The synthesized sgRNA was validated by TBE-urea gel electrophoresis (**Fig. 2.3A**) and DNase and RNase treatment (**Fig. 2.3B**).

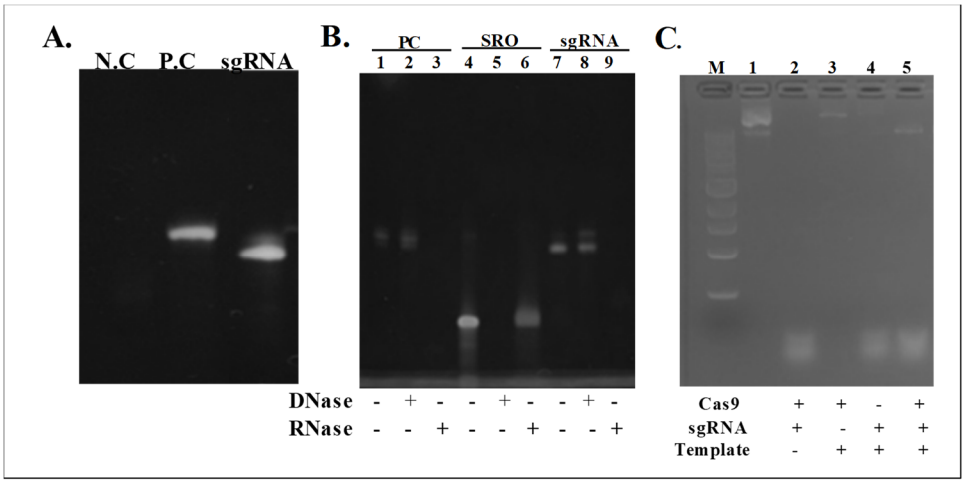

Figure 2.3: sgRNA production, validation and Cas9 mediated digestion

A) TBE Urea gel electrophoresis showing the presence of sgRNA. N.C= Negative control; P.C=positive control; sgRNA=synthetic guide RNA. B) Validation of sgRNA by DNase and RNase treatment. PC= positive kit control; SRO = sgRNA template oligo. C) Cas9 mediated digestion of CAV2 vector. M = 1Kb extend DNA ladder (NEB). 1= Uncut-pICOCAV15.

The sgRNA was shown to be absent after RNase treatment but remained present after DNase treatment. DNase treatment of the template oligo resulted in degradation but the template remained after RNase treatment. Once the sgRNA was synthesized and

validated, it was used to conduct Cas9/sgRNA-mediated digestion of the viral genome plasmid. Digestion of the plasmid resulted in a linear DNA of approximately 36 kb, which was visualized by agarose gel electrophoresis, demonstrating the expected band (**Fig. 2.3C**).

Homology directed repair (HDR) was used to insert the CMV-DsRed insert into the linearized CAV2 vector in a process known as seamless cloning. The HDR insert was designed to contain 53 bp and 75 bp overlaps with pICOCAV15 in the left and right arms, respectively (**Fig. 2.4**).

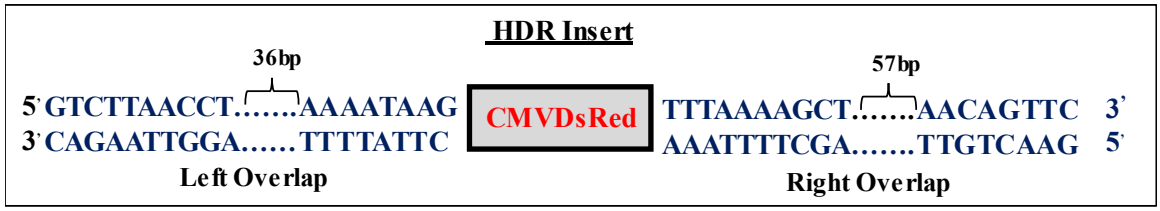

Figure 2.4: Schematic representation of HDR insert sequence.

Diagram showing left and right overlap encompassing the homology directed repair (HDR) insert. The left and right overlap are 53bp and 75bp respectively.

The Cas9/sgRNA digested, linearized CAV2 vector and CMV-DsRed were incubated with Hifi DNA assembly master mix, followed by transformation into high efficiency competent cells. After 18 hrs of growth, 20 clones were randomly selected and colony PCR was performed. Results showed that 8 out of 20 clones were positive for the desired CMV-DsRed insert (**Fig. 2.5A**). DNA sequencing confirmed the presence of the

insert construct in the desired location without any additional alterations at the junction point (**Fig. 2.5B and 2.5C**).

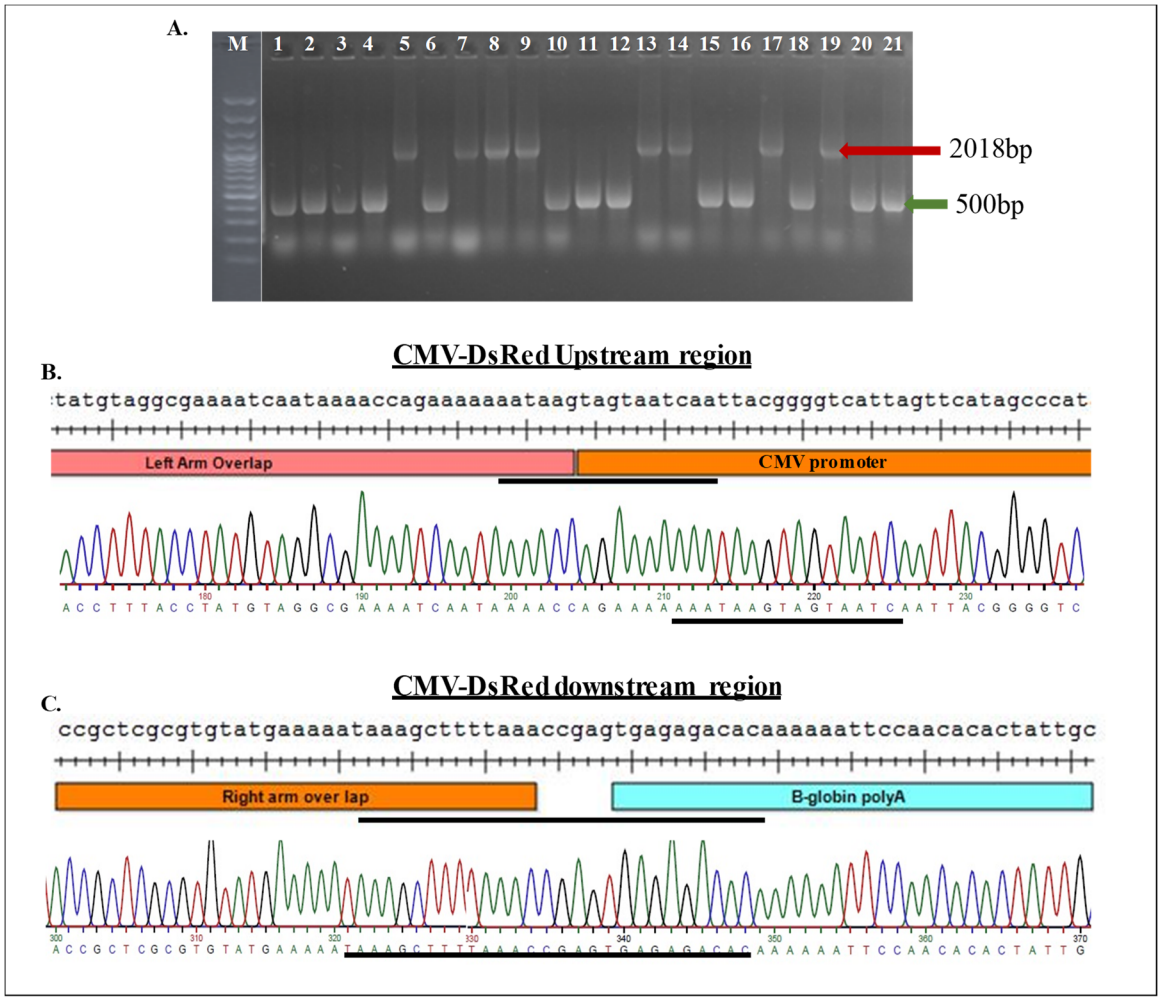

Figure 2.5: Diagram showing efficiency of CRISPR/Cas9 mediated insertional mutagenesis of CAV2

A) Colony PCR amplification of the target region. M = Gene ruler100bp plus DNA ladder (ThermoFisher scientific, catalog #SM0321). Lanes 1-20= Clones, 21= Unmodified pICOCAV15. CMV-DsRed Insert= 2018 bp (red arrow). Region without insert = 500 bp (green arrow). (B) and (C) Sequencing analysis for insertional mutagenesis in recombinant vector. B) Top: Partial sequence for the junction point

connecting viral left arm overlap with CMV promoter region. Bottom: Panel includes the chromatogram showing no insertional mutagenesis in the junction point. C) Top: Panel showing partial sequence for the junction point connecting the B-globin polyadenylation (polyA) signal sequence with viral right arm overlap. Bottom: Panel includes the chromatogram showing no insertional mutagenesis in the junction point. Black line represent the junction point- potential site for insertional mutagenesis. Note that (C) is reversed with respect to the virus, as the sequence was derived from the non-coding strand.

Production of functional CAV2-AU-M1 virus from CRISPR/Cas9 modified vector

The feasibility of making infectious virus particles from the CRISPR/Cas9 modified viral genome was evaluated.

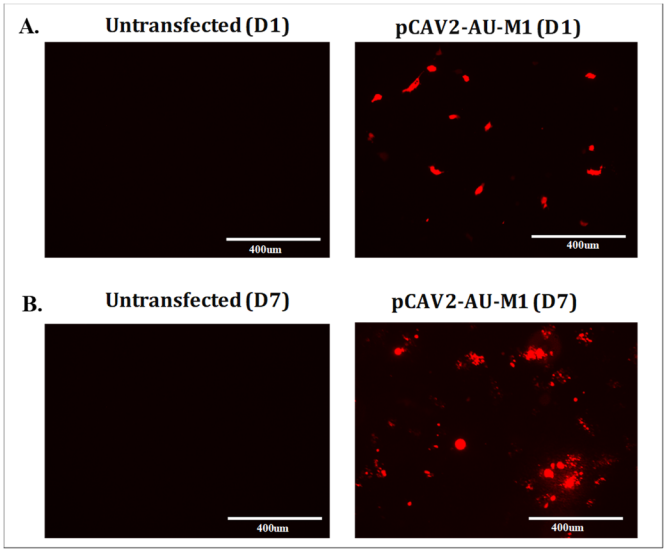

Figure 2.6: DsRed Expression by DKCre cells transfected with pCAV2-AU-M1

DKCre- cells were transfected with Not-I double digested and linearized recombinant pCAV2-AU-M1 vector. DsRed expression in cells was captured by

inverted fluorescence microscopy at A) Day 1 (D1) and B) Day 7 (D7) from the day of transfection. All fluorescent images were taken at 10X magnification.

Digestion of the CAV2-AU-M1 genome vector with NotI restriction enzyme resulted in linearized viral genome, free of plasmid sequences, with the left and right inverted terminal repeats (ITRs) of the viral genome exposed. Linearized CAV2-AU-M1 vector was then used to transfect freshly plated DKCre packaging cells. 24hrs post-transfection, red fluorescence was observed in approximately 30% of cells, indicating that the CMV-DsRed construct was functional (**Fig. 2.6A**). Seven days post transfection, red fluorescence was observed to have spread to additional cells indicating the presence and production of functional recombinant CAV2-AU-M1 virus (**Fig. 2.6B**).

To further evaluate the infectious properties of the CRISPR/Cas9 modified recombinant CAV2-AU-M1, the virus was liberated from the transfected cells by repeated freeze/thaw cycles.

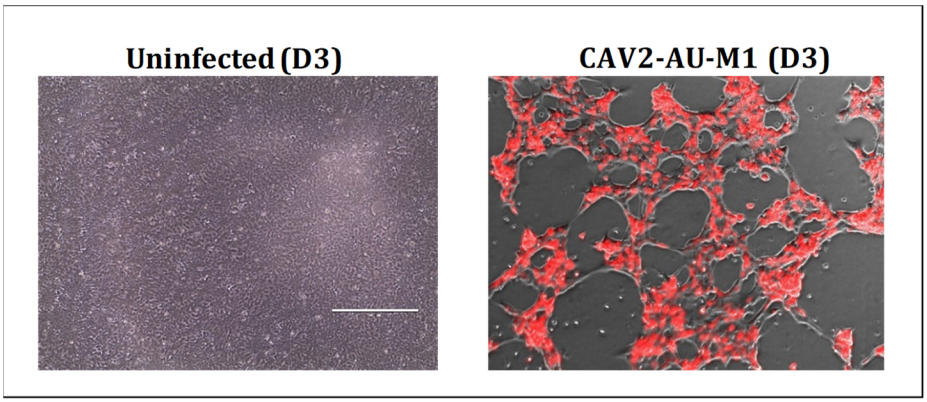

Figure 2.7: Evaluation of infectious properties of CRISPR/Cas9 modified CAV2-AU-M1

DKCre- cells were infected pCAV2-AU-M1 transfected cell lysate. DsRed expression in cells were captured by inverted fluorescence microscopy 72hr post

infection with inverted fluorescent microscopy. All fluorescent images were taken at 10X magnification.

The cell lysates were added to freshly plated DKCre cells. Red fluorescence was observed after 24hr. Red fluorescence and viral cytopathic effect (CPE) were observed 72hr post-infection (**Fig. 2.7**) confirming the production of infectious virus particles. Additional infection and expansion of the virus produced similar results.

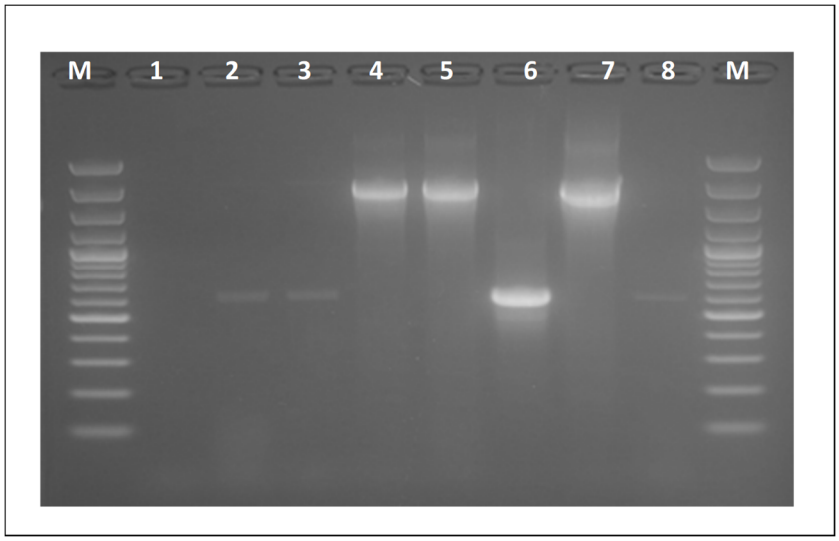

Figure 2.8: PCR confirmation for the absence of parental virus (ICOCAV15) contamination

1= No template control; 2= Uninfected media; 3= Uninfected Lysate; 4 =CAV2-AU-M1 infected Media 5= CAV2-AU-M1 infected Lysate; 6= pICOCAV15 plasmid; 7= pCAV2-AU-M1; 8= DKCre DNA. M = Gene ruler100bp plus DNA ladder. CMV-DsRed Insert= 2018bp. Region without insert = ~500bp.

To demonstrate that the recombinant virus was free of unmodified virus that might act as a helper virus, PCR with primers flanking the CMV-DsRed insert fragment (2018bp) was performed. Unmodified virus without the CMV-DsRed insert (ICOCAV15) produced a 611bp amplicon while CAV2-AU-M1 would produce a ~2000 bp amplicon. Both the cell culture media and cell lysate from infected cells showed the 2018 bp band, and did not

show the 611 bp band **(Fig 2.8, lanes 4 and 5**) indicating that the infected samples only contain recombinant virus.

Functional characterization of recombinant CAV2-AU-M1 virus

Our next goal was to evaluate the transductional and infectious potential of recombinant CAV2-AU-M1 as well as compare its functionality with that of unmodified virus (ICOVAV15). To achieve this goal, we infected canine normal cells/cell lines and cancerous canine cell lines including normal canine fibroblast (NCF), D17 (canine osteosarcoma cell line), CMT28 (canine mammary tumor cell line 28) and FDK (fetal dog kidney cell line) with either recombinant CAV2-AU-M1 or ICOCAV15 in triplicate at multiplicities of infection (MOI) of 0, 1, 10 and 100. The transductional properties of the recombinant virus were determined by observing expression of red fluorescence **(Fig. 2.9**).

A significant amount of red fluorescent as well as cytopathic effect was seen in the infected cells starting from 24hr post-infection and peaking at 72hr post-infection. The negative control cell line, NCF, showed three red fluorescent cells 72hr post infection with the highest MOI (MOI 100) but no red fluorescence observed with MOI 1 and 10. DKCre fiber, D17 (osteosarcoma cell line) and FDK showed around 100%, 20% and 80% transduction, respectively, as early as 24hr post infection with an MOI of 100 and around 70%, 10% and 50% , respectively, as early as 24hr post infection at an MOI of 10. These cell lines also showed little transductional activity at an MOI of 1. Canine mammary tumor cell line, CMT28, showed around 5% transductional activity at an MOI of 100, two red fluorescent cells at an MOI of 10 and no transduction at an MOI of 1. For MOI 100, CMT28 cells showed low levels of transductional activity compared to DKCre, D17 and FDK cell lines **(Fig. 2.9**).

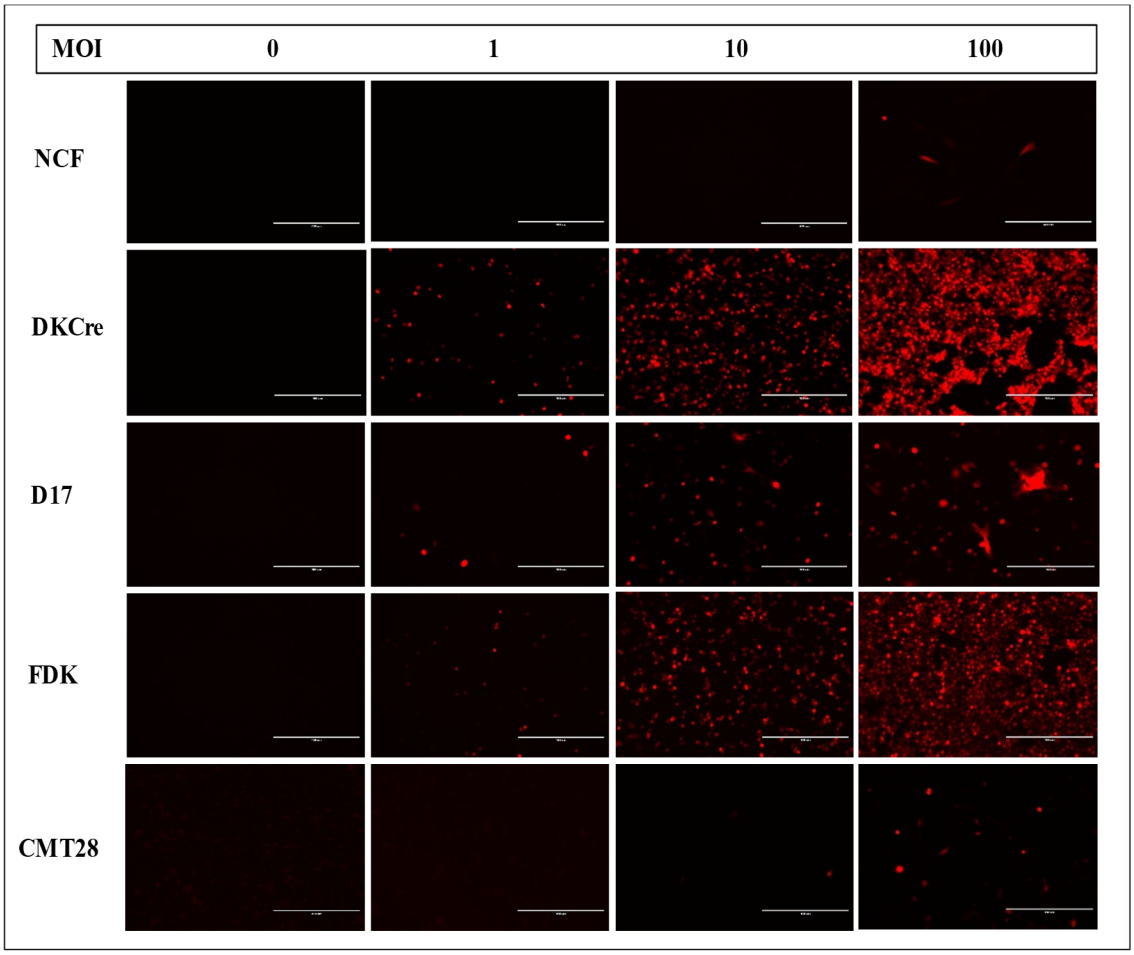

Figure 2.9: Analysis of transductional properties of CRISPR/Cas9 modified CAV2-AU-M1 in various canine normal and cancer cell lines

Cells were transduced by CAV2-AU-M1 at three different multiplicities of infection (MOI 1, 10, and 100 virus particles per cell). DsRed expression in cells was analyzed by inverted fluorescent microscopy 48hr after infection. All fluorescent images were taken at 10X magnification. NCF= Normal Canine Fibroblast, DKCre = Dog Kidney Cell line, D17= Canine Osteosarcoma Cell line, FDK= Fetal Dog Kidney Cell line, CMT28= Canine Mammary Tumor Cell line 28.

A crystal violet staining assay was used to further validate the cytopathic properties of the recombinant virus (**Fig. 2.10A and B**). Crystal violet stains live cells as dead cells typically detach from the substrate as part of the assay and are washed away. If there is no

killing, a dark purple well with a confluent lawn of cells will be observed after crystal violet staining. In contrast, cell killing by virus will generate wells with reduced to no stain (clear) after crystal violet staining.

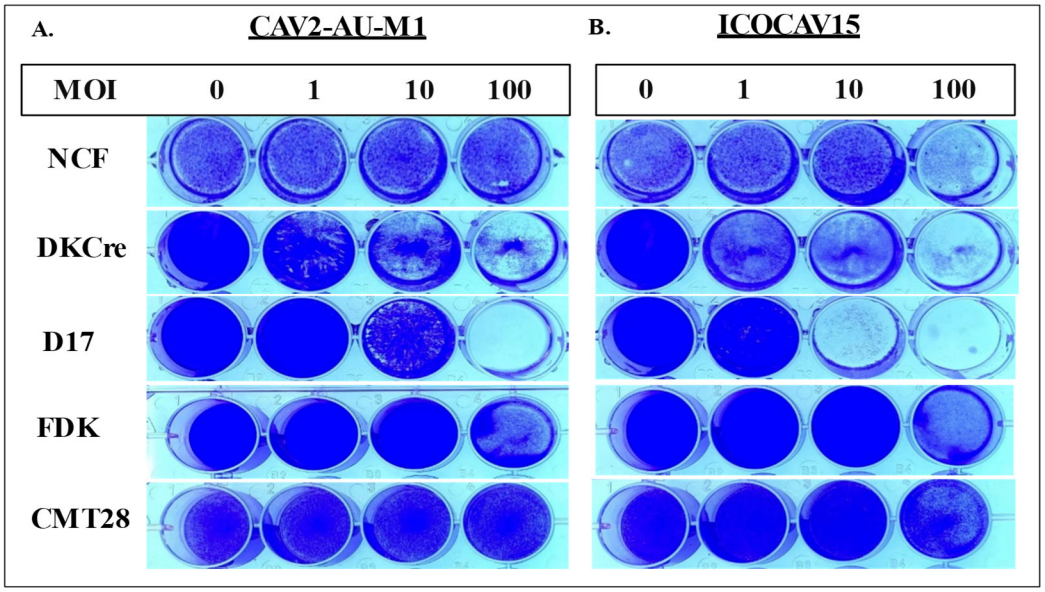

Figure 2.10: Crystal violet killing assay

Cells were transduced by either A) CAV2-AU-M1 or B) ICOCAV15 at three different multiplicities of infection (MOI 1, 10, and 100 virus particles per cell). A crystal violet killing assay was performed to detect attached cells four days post infection when complete cell killing was visually evident. NCF= Normal Canine Fibroblast, DKCre = Dog Kidney Cell line, D17= Canine Osteosarcoma Cell line, FDK= Fetal Dog Kidney Cell line, CMT28= Canine Mammary Tumor Cell line 28.

The positive control parental virus, ICOCAV15, killed all cells in D17 at MOIs of 10 and 100 and no cells at an MOI of 1. In contrast, ICOCAV15 killed all cells in DKCre at MOI of 100, some cells at MOI of 10 and some cells at an MOI of 1. In NCF, light stain at an MOI of 0 indicated that cells were not as confluent as other cell lines such as DKCre. However, ICOCAV15 showed some patchy cell killing in NCF, FDK and CMT28 at the

highest MOI of 100 (**Fig. 2.10B**) and no cell killing was observed for these cell lines at MOIs of 10 and 1. Similar levels of functionality were seen for the recombinant virus. CAV2-AU-M1 also killed all cells in DKCre and D17 and showed low levels of killing in FDK at an MOI of 100 but showed no killing of cells at MOIs 10 and 1. In addition, CAV2-AU-M1 showed no cell killing for CMT28 and NCF at MOIs of 100, 10 and 1 (**Fig. 2.10A**).

2.4 Discussion

The CRISPR/Cas9 system facilitates site-specific cleavage of target DNA to generate double stranded breaks (DSBs). The resultant DSBs are then repaired either *in vitro* or *in vivo* by non-homologous end joining (NHEJ), homology directed repair (HDR), or Microhomology-mediated end joining (MMEJ). These repair mechanisms can be exploited to generate insertions, deletions, or can promote the replacement of a sequence with another sequence by HDR [311-313]. Previously, molecular cloning techniques for adenoviral genome modification were solely dependent on the availability of suitable and unique restriction enzyme sites. These procedures may restrict the feasibility of conducting desired mutations in a large vector (<10kb) that possesses either multiple cut sites for a desired restriction enzyme or lacks unique restriction sites. To overcome the limitations of restriction enzyme-based cloning methods, several other cloning techniques have been evaluated, such as quick and clean cloning, sequence and ligation-independent cloning (SLIC), Gateway cloning and Gibson assembly [314-317]. Among those, Gateway cloning and Gibson assembly techniques are widely used to promote direct and seamless cloning. However, Gateway cloning has several limitations including the requirement of specific vectors and requires linearization of vector by inverse PCR. Linearization of large cloning vectors is not only time consuming but also complex due to the presence of high GC

content, repeats, and long sequences in large vectors such as adenoviral or baculoviral vectors. Alternative approaches include insertion of alternative recombination sites such as attR1 and attr2 sites followed by Gateway cloning, but this technique requires a specific vector as well as complex and time-consuming modification of the existing construct. This modification may also generate undesired insertion or deletion mutations. In contrast, Gibson assembly (also known as isothermal cloning) uses the feasibility of 5'-exonuclease, DNA-polymerase and DNA-ligase to ligate the desired single or multiple inserts, with 20-40bp homology to the vector, into linearized vector in one isothermal reaction (https://www.addgene.org/mol-bio-reference/cloning/). Studies have shown that Gibson assembly, following CRISPR/Cas9 mediated digestion of target vector, could be successfully used to promote insertion, deletion or replacement of desired fragments in large vectors (<10kb) including the Ad5 genome [301, 306].

The standard method to generate recombinant adenoviral vectors including CAV2 is dependent on the availability of suitable restriction enzyme sites and includes insertion of desired fragments into a smaller shuttle vector followed by transfer of the cloned insert into a large vector via homologous recombination [318]. This process is complex, time-consuming, may provide undesired mutations in the insertion site, and can be difficult to use in the modification of non-human adenovirus (such as CAV2) due to the lack of suitable shuttle plasmids. In this regard, we evaluated the feasibility of CRISPR/Cas9 mediated digestion in combination with Gibson assembly to successfully modify the CAV2 vector followed by successful production infectious virus particle.

In this study, the sgRNAs were designed and synthesized *in vitro* and utilized to successfully conduct CRISPR-Cas9 mediated linearization of the target vector. Isothermal cloning provided efficient generation of positive clones where the cloning efficiency was ~40% for single digestion (**Fig. 2.5A**). In addition, this study also showed that *in vitro* CRISPR/Cas9 mediated technique to modify large genome of CAV2 was very accurate as the junction point was perfectly conserved with no insertion or deletion occurring. No other changes were detected at any other location including inserts or recombinant vector. CRIPSR/Cas9 has the potential to promote off-target effects resulting in cleavage of non-target sites that may limit its application in large complex genomes [319, 320]. However, in this study, no off-target cleavage has been observed during digestion at either single sites or multiple sites. The presence of off-target cuts may result either in multiple bands after Cas9 digestion or the presence of the insert in an alternative location. However, these results have not been observed in this study indicating the efficiency of this technique for the modification of large vectors.

Prior to this application of CRISPR-cas9 technology, construction of recombinant CAV2 vectors has been a time-consuming process that requires more than a month[318]. In this study, positive clones have been obtained as early as 7 days and the highest spread of virus (by observing red fluorescence) was observed 7 days post-transfection indicating that this is the optimum time to rescue the virus. This results in a total production time of approximately 2 weeks. However, observation of red fluorescence did not, a *priori*, indicate the production of functional virus particles as the DsRed construct was driven by the CMV promoter whose expression is independent of virus production. Production of functional virus particles will promote an increase in DsRed expression and the number of

cells expressing DsRed would increase over time. To further test the functionality of the modified virus, viral suspensions used to infect various normal and cancerous canine cell lines. The CRISPR/Cas9 modified CAV2 provided significant levels of transduction as well as CPE in DKCre, D17, FDK and CMT28 in a manner similar to that of the parental virus, ICOCAV15. The slight differences observed between CAV-AU-M1 and the parental virus may be related to the actual calculation of viral particles rather than differences functionality. The infection experiments with CAV-AU-M1 used virus concentrations determined by absolute quantification by Taqman qPCR (data not shown). However, absolute quantification provides the viral genome copy number rather than the number of infectious virus particles. Thus, to determine the number of infectious virus particles, serial dilution followed fluorescent plaque assay, was conducted with DKCre cells which are infected by this virus with high efficiency (**as shown in Fig. 2.9**). Calculations indicate that three red cells should be observed with an MOI of 0.0001, however, three red cells were counted with an MOI of 0.001 an the other dilutions supported this result. Thus, concentration of infectious virus particles was 10-fold less than that of the concentration obtained from absolute quantification.

2.5 Conclusions

Our goal was to utilize CRISPR/Cas9 *In vitro* to generate recombinant CAV2. This study is the first attempt to utilize the feasibility of *in vitro* CRISPR/Cas9 in combination with Gibson assembly to conduct genetic modification in large CAV2 genome vector. Results showed that the choice of insertion site as well as the technique adopted provided a unique mechanism to generate functional, recombinant CAV2 virus in a rapid and less-complex manner. This technique can successfully be used to directly, and seamlessly,

manipulate the CAV2 virus to generate recombinant oncolytic viruses to promote the feasibility of oncolytic virotherapy.

CHAPTER 3

Towards Effective and Selective Targeting of Lymphocytes with Genetically Modified Adenoviruses

3.1 Introduction

Hematologic malignancies (HM) refer to a very large and diverse set of genetic diseases that originate in the blood cells of the bone marrow or lymph system. Based on the cell lineage from which cancer is originated, it can be broadly classified into two groups. These are myeloid cancers such as acute myeloid leukemia, chronic myeloid leukemia and lymphoid cancer such as non-Hodgkin and Hodgkin lymphomas, and chronic lymphoblastic leukemia [321, 322]. An estimated 176,200 new cases were diagnosed for various types of hematologic malignancies and an estimated 56,770 people died from these diseases in 2019 (https://www.lls.org/facts-and-statistics/facts-and-statistics-overview/facts-and-statistics). The classical regimen for the treatment of HM includes chemotherapy with antiproliferaitve drugs such as DNA alkylating agents, antimetabolites, anthracyclines, radiotherapy, and treatment with monoclonal antibodies. These treatments show enhanced levels of initial remission in a high percentage of patients [323-325]. However, several studies have reported high levels of relapse and of tumors that are refractory to treatment in patients, as well as normal hematopoietic cell toxicities, creating major challenges to achieving durable success with these conventional therapies [323].

Oncolytic virotherapy is considered as an ideal tool for hematological malignancies as it can promote selective killing of malignant hematopoietic cells while sparing normal

hematopoietic cells [326]. However, current vector systems, including lentivirus and retrovirus, possess several vector-related issues, such as insertional mutagenesis, inefficient transduction, and low viral titers. These issues restrict the exploitation of the full potential of oncolytic virotherapy [327]. Adenoviruses are attractive alternative vector systems to use for oncolytic virotherapies due to their high transduction efficiencies in both dividing and quiescent cells, low risk for insertional mutagenesis, and robust gene expression [328, 329]. These benefits, combined with the lack of suitable medical treatment methods to treat hematologic malignancies, as well as the systemic nature of these malignancies, make adenoviral mediated gene therapy approaches to cure hematologic malignancies attractive. As with all vector systems, tropism can be an issue. Most adenoviruses have broad tissue tropism, however most HMs appear to be resistant to adenovirus infection. For this reason, it is necessary to identify approaches to modify adenoviral tropism to allow infection of HMs.

Adenovirus infection is typically a two-step process. Initial binding of virus fiber knob to the cell surface coxsackie adenovirus receptor (CAR) is followed by secondary binding of an RGD motif in the Penton protein with $\alpha_v\beta_{3/5}$ integrins on the cell surface. Both steps are prerequisites for successful viral internalization into the target cell [330]. However, cells of lymphocyte origin are poorly infected by adenovirus due to the paucity of both CAR [331] and cell surface integrins in the surface of blood cells [163, 164]. Despite multiple strategies that have been developed to generate CAR-independent fiber-retargeted adenoviruses the efficiency and successes rate for these approaches are limited [332, 333]. Moreover, current model systems, such as mouse, are inefficient and may not reflect the true dynamics of adenovirus dissemination, infection, and replication in human

patients [334-336]. Thus, it is essential to design new targeting approaches to facilitate efficient transduction and replication of adenoviruses in lymphocytes, and to do so in model systems that accurately represent the biological activities of these viruses in human patients.

In this regard, we have developed canine adenovirus 2 (CAV2) as a translantional model system for human adenovirus, including the very similar human adenovirus 5 (Ad5). Using this model system, CAV2 vectors are being developed that will bypass both CAR and integrin requirements to infect cells of lymphocyte origin by targeting the cell surface Interleukin 2 receptor (IL2R). IL2R is a receptor complex that consists of three subunits; IL2Rα, IL2Rβ and Il2RϒΥ. These cooperatively facilitate the binding of its cognate ligand, Interleukin 2 (IL2). Generally, expression of the IL2 receptor complex is T-cell specific. It is highly expressed in $CD4^+$ and $CD8^+$ T cells and works to promote T cell differentiation when activated by interacting with IL2 [337, 338]. IL2Rα is the key determinant for IL2 mediated T cell activation as this subunit is the first to interact with IL2. The IL2/IL2Rα complex then recruits the β and ϒ subunit to promote direct internalization of IL2 by receptor mediated endocytosis [339] . Thus, we hypothesize that IL2/IL2Rα retargeted CAV2 will both bind to the receptor and be internalized into malignant lymphocytes, providing both adenoviral entry functions in a single interaction [339-341]. Our hypothesis is supported by murine studies that showed enhanced adenovirus transduction in murine lymphocytes by utilizing IL2-IL2R signaling (9). Thus, we endeavored to generate modified CAV2 vectors using interleukin 2 and/or single domain camelid antibodies (sdAb) to IL2R. These targeting moieties were cloned as fusion proteins with CAV2 fiber

and phage T4 fibritin where fibritin will retain the trimerization potential of the fiber shaft region.

Conventional antibodies are complex in nature and consist of heavy and light chains. In addition, they possess an N-linked oligosaccharide attached to its second heavy-chain constant region (CH2) that plays important role in promoting antibody mediated effector functions. In contrast, the antibody produced by camelids, including Bactrian and Dromedary camels, and the South American camelids, llamas, guanacos, vicuñas and alpacas possess single-domain variable heavy chains (VHHs), lack light chains and are also devoid of CH1 regions responsible for interacting with light chains and, to a lesser extent, VH domains, in conventional antibodies [342]. Interestingly, even though they lack light chain, their VHH domain is capable of binding to antigen independent of domain pairing [241, 343]. Camelid VHH antibodies possess several advantages over conventional antibodies that make them an outstanding candidates for use in biotechnological applications including cancer gene therapy [344]. Camelid VHH antibodies are relatively easy to produce including the ease of handling and immunization of the camelids. In addition, due to the single domain nature, the VHH libraries retain full functional diversity compared to conventional antibodies requiring reshuffling of light and heavy chain domains during library preparation. Moreover, VHHs are highly stable, provide high solubility, and facilitates easy production of bivalent or multivalent compounds [241, 345]. Several studies have shown successful modification of Ad tropism by genetically incorporating antibody ligands. However, conventional antibodies failed to withstand cytosolic adenovirus capsid synthesis and assembly resulting in loss of binding and, thus, are biologically incompatible to be used as a component of fiber protein. In contrast,

several advantages of camelid VHHs, including their high cytosolic stability, compatibility with phage display biopanning selection and ease to manipulate and produce, have made them outstanding candidates for use in adenovirus fiber retargeting [240, 346, 347].

CRISPR/Cas9 promote precise genome editing in large DNA viruses such as Ad5 and HSV1. In addition, this method also promote efficient insertion of desired transgenes in CAV2 (as shown in Chapter 2) in a less-complex, less-time consuming manner. In addition, they do not cause any undesirable insertional mutagenesis in the insertion site. However, the feasibility of this technique has not been tested for its potential to promote gene replacement in CAV2.

In this respect, we evaluated the feasibility of CRISPR/Cas9 mediated digestion in combination with Gibson assembly to successfully replace the wild type fiber of CAV2 with our desired construct, to generate fiber retargeted adenovirus. In summary, our overall goal was to generate CRISPR/Cas9 modified CAV2 vectors using interleukin 2 and/or single domain camelid antibodies (sdAb) to IL2R. These targeting moieties, cloned as fusion proteins with phage T4 fibritin and the basal portion of CAV2 fiber will generate Fiber-Fibritin-IL2/anti-IL2RsdAb (FF-IL2, FF-anti-IL2R-sdAb) chimeras (also termed as CAV-AU-M2 and CAV2-AU-M3 respectively). (**Fig 3.1**).

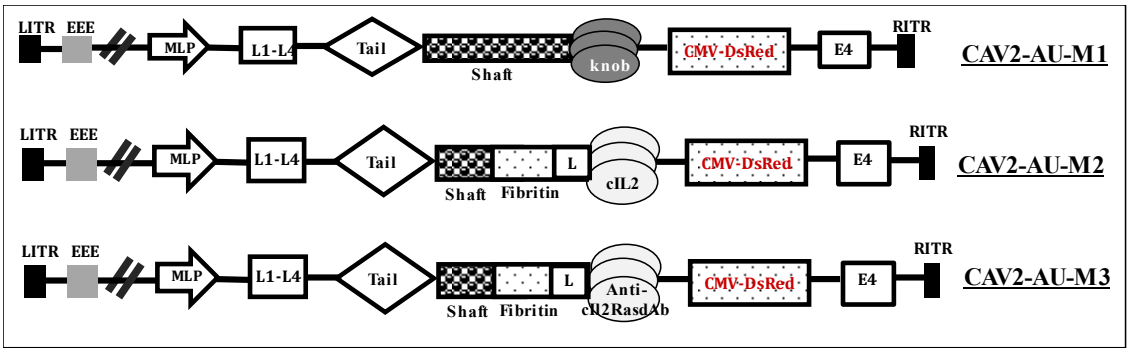

Figure 3.1: Schematic representation of recombinant CAV2 genomes

CAV2-AU-M1 is the canine adenovirus containing wild type fiber and a red fluorescence gene under the control of CMV promoter. CAV2-AU-M2 is the modified version of CAV2-AU-M1 containing the fiber-fibritin-IL2 (FFIL2). LITR = Left Inverted Terminal Repeat, RITR= Right Inverted Terminal Repeat, EEE= E2-F Modified E1A promoter, MLP = Major Late promoter, L1-L4 = Late transcript 1 to 4, CMV= Cytomegalovirus immediate early promoter. cIL2= Canine Interleukin 2, cIL2RasdAb= Canine Interleukin 2 Receptor alpha Single Domain Antibody.

3.2 Materials and methods

Cell culture

Canine Kidney (DKCre) and Human embryonic kidney cell line 293 (HEK293) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Corning) with penicillin (100 IU/ml, Corning), streptomycin (100 ug/ml, Corning), amphotericin B (0.5ug/ml, Corning), and 10% FBS (fetal bovine serum, Sigma). DKCre cell line contains an expression cassette for bacteriophage *Cre* recombinase and is a derivative of Dog kidney (DK cells)/E1-28 cells that contain the CAV2-*E1* region stably integrated into the genome where the *E1A* region under the control of CMV promoter and the *E1B* region under the control of its own promoter [348] [94]. DKCre cells expressing wild type CAV2 fiber (DKCre-WT-F) (a

kind gift from Dr. Maninder Sandey, Department of Pathobiology, Auburn University) were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Corning) with penicillin (100 IU/ml, Corning), streptomycin (100 ug/ml, Corning), hygromycin (450ug/ml), amphotericin B (0.5ug/ml, Corning), 10% FBS (fetal bovine serum, Sigma). Canine B cell lymphoma line 17-71 [349] and CLBL-1[350], canine T cell lymphoma line CL-1 [351] (a kind gift from Dr. Steven Suter, North Carolina State University) and canine peripheral T cell lymphoma line OSW [352] (gift from Dr. William C. Kisseberth, The Ohio State University) were cultured in RPMI (Roswell Park Memorial Institute medium, Corning) with penicillin (100 IU/ml, Corning), streptomycin (100 ug/ml, Corning), amphotericin B (0.5ug/ml, Corning) and 10% FBS (Sigma). All cells were grown at 37°C and 5% CO_2 and were validated to be of canine origin by species-specific PCR [307].

Total RNA isolation and RT-PCR

Total RNA was isolated from cell lines (CMT28, OSW and 17-71) and primary cells (PBMCs), using Tri-reagent (Molecular Research Center, Inc.). All cells were homogenized in 1 ml Tri-reagent. RNA was isolated from the aqueous phase by isopropanol precipitation. RNA concentration was determined by absorbance at 260 nm. cIL2Rα gene expression was measured by reverse transcriptase PCR (RT-PCR). cDNA from 1ug of RNA was synthesized using MyTaq^{TM} One –Step RT-PCR kit (Bioline, Catalog # BIO-65048) with cIL2Rα specific forward and reverse primer (0.5uM each) in a volume of 20ul (**Table 3.1**). Thermocycling conditions were: 10 minutes at 50^0 C, then 1 cycle of 95^0 C for 5 minutes, 40 cycles of 95^0 C for 10 seconds, 50^0C for 30 seconds, 72^0 C for 45 seconds on a Bio-Rad T100^{TM} Thermal cycler. Agarose gel electrophoreses was conducted by using 1.2% agarose mixed in 1X TAE buffer as well as GelRed® Nucleic

Acid Gel Stain (Biotium, Catalog # 41002). Primer specificity was validated by sequencing the PCR product (Eurofins MWG, Operon).

Table 3.1: IL2R RT-PCR Primers

Purpose	Primers	Sequence (5' to 3')
IL2R primers for RT-PCR	Forward Primer	CTTTCTCTTCACTGCCCTCAAG
	Reverse Primer	CTTCTTGGCTTCTTACCACTCTTG
Primers were designed using NCBI Primer blast. Primers are based on canine sequences.		

Similarity Analysis among Canine, Human and Mouse IL2

Canine IL2 (NP_001003305.1), human IL2 (NP_000577) and mouse IL2 (AAD25891) protein sequences, and canine IL2R (NP_001003211.2), human IL2R (NP_000408.1) and mouse IL2R (NP_032393.3) protein sequences were obtained from NCBI databases. The sequences were then aligned using the Jotun Hein Method in DNASTAR software.

Infection of canine lymphoma cell lines with Ad5GL-sCAR-mIL2

The Ad5GL virus, which encodes GFP (Green Fluorescence Protein) and Luciferase under control of the cytomegalovirus (CMV) immediate early promotor alone or complexed with sCAR-mIL2 adapter (a gift from Dr. David T. Curiel, St Louis, MO) was used for infection. Ad5G/L virus vector particle number was calculated by measuring optical density at 260 nm [353]. Virus infections were done at three different multiplicities of infection (MOI): 10, 100, and 1000 virus particles/cell. 5ng and 10ng of sCAR-mIL2 were incubated with MOI of 10, 100 and 1000 virus particles in separate tubes in triplicate and incubated for 30 mins at 37^0C. The sCAR-mIL2/Ad5 complexes were then transferred to the cells containing DMEM+2% FBS and incubated for 1 h at 37^0C. After one hour virus was removed and medium was replaced with 500ul of DMEM/RPMI (10% FBS). Cells

were monitored at 24, 48, and 72 hours post-infection for green fluorescence using an inverted fluorescent microscope (EVOS FL Cell Imaging System).

Infection of canine lymphoma cell lines with CAV2-AU-M1

The CAV2-AU-M1 virus, which encodes DsRed under the control of the cytomegalovirus (CMV) immediate early promotor, was used to infect canine lymphoma cell lines OSW, 17-71, CL1 and CLBL1 and DKCre cells (positive control) at multiplicities of infection (MOI) of 0, 1, 10, and 100 virus particles/cell. 3×10^5 cells of adherent DKCre cells were plated in 12-well plates one day prior to virus infections. 4×10^5 cells of 17–71, OSW, CL-1, and CLBL-1 cell lines were plated the same day of infection. Cells were washed with 1X PBS (Phosphate Buffered Saline), and infected with 100ul of DMEM/RPMI (2% FBS), containing the virus with the required MOI. Cells were monitored for 48hr post infection for red fluorescence using an inverted fluorescent microscope (EVOS FL Cell Imaging System). After 48hr, infected adherent cells were harvested by trypsinization and suspended cells were harvested by centrifugation. Cells were washed twice with PBS. The cells were resuspended in PBS and 0.1% BSA, and analyzed for DsRed expression by flow cytometry (CytoFLEX Flow cytometer).

***In vitro* synthesis of sgRNA**

The synthetic guide RNA (sgRNA) sequences were designed using the online CRISPR Design Tool (http://cripsr.mit.edu) which selected 20bp guide RNA sequences followed by the PAM sequence "NGG". After selection, sgRNAs were checked for off target binding by alignment against the CAV2 genome vector (pICOAV15) using DNASTAR software. The two sgRNAs target, 5'-CGGCCTCATCACAAATACCG-3'

located in top strand at 26981bp to 27000bp followed by the PAM sequence AGG located at 27001bp to 27003bp and 5' CCTAAAGGTAAGGGTGTAAG-3' located in bottom strand at 28310bp to 28291bp followed by the PAM sequence TGG located at 28290bp to 28288bp, were identified so that they would generate a double stranded cut at base 26997bp and 28457bp respectively (**Table 3.2**). A target specific oligo, containing the T7 promoter sequence, target sgRNA sequence and sgRNA scaffold, were designed online using the "sgRNA designer" tool (NEB). The target oligo was synthesized commercially (Eurofin MWG Operon) and used as a template for sgRNA synthesis. In order to synthesize sgRNA, *in vitro* transcription was conducted using an EnGen® sgRNA Synthesis Kit (catalog no. E3322, NEB) according to the manufacturer's protocol. Briefly, 5uM of target DNA oligo was mixed with the required volume of buffer and enzyme mix followed by one hour incubation at 37^0C. After synthesis, sgRNA was quickly transferred on ice and treated with DNase for 15 mins at 37^0C to promote enzymatic degradation of template oligo nucleotide followed by purification using a MEGAclear™ Transcription Clean-up kit (catalog no. AM1908, Thermo Scientific™) according to the manufacturer's protocol.

Table 3.2: sgRNA oligos

Oligo	**Location**	**PAM 5/to 3/**	**Sequence 5/to 3/**
sgRNA upstream	Top Strand (26981bp to 27000bp)	27001bp to 27003bp	**TTCTAATACGACTCACTATAGCGGCC TCATCACAAATACCGGTTTTAGAGCT AGA**
sgRNA downstream	Bottom Strand (28310bp to 28291bp)	28290bp to 28288bp	**TTCTAATACGACTCACTATAGCCTAA AGGTAAGGGTGTAAGGTTTTAGAGCT AGA**
Violet= T7 promoter, Orange= Added G's, Red= sgRNA sequence and Green= RNA scaffold, PAM= Prospacer Adjacent Motif			

Linearization of CAV2 genome vector using Cas9/sgRNA by double digestion

pICOCAV15, a modified oncolytic canine adenovirus derived from CAV2, a kind gift from Dr. Ramon Alemany (Translational Research Laboratory, IDIBELL-Institut Català d'Oncologia, Barcelona, Spain) [290]. Recombinant Cas9 Nuclease *(S. pyogenes*) was purchased from NEB (catalog no. M0386). For Cas9-mediated double digestion, two sgRNAs targeting two different locations were used and 3.5ug of template, 3.5ug of Cas9 and 7ug of each sgRNA (total 14ug of sgRNAs) with a ratio of 1:2:4 respectively were used to promote CRISPR/Cas9 mediated digestion. The reaction mixture with the appropriate ratio of template, Cas9, and sgRNA was then incubated in buffer 3.1 (NEB) at 37^0c overnight. The reaction was terminated by incubating the tubes at 65^0C for 10mins to inactivate Cas9 nuclease. To remove the sgRNA, the reaction was treated with 0.2mg/ml RNAse A (catalog no. EN0531, Thermo Scientific™) and incubated at 37^0C for 15 mins to 1 hour based the amount of sgRNA used. The reaction mixture was then treated with SDS (1%) and 1mg/ml proteinase K (Thermo Scientific™) and incubated at 55^0C for 30 min. Finally, the Cas9 digested vector was recovered by phenol/chloroform extraction and verified by gel electrophoresis.

Production of Insert and Homology directed repair (HDR)

The insert, consisting of FFIL2 or FF-anti-IL2Rα sdAb inserts flanking the homologous regions at both 5/- and 3/-end, were ordered from Gene Universal where the insert was synthesized and cloned into a standard pUC57 plasmid. The insert was PCR amplified using 0.5ng of pUC57 plasmid containing the insert sequence and the Phusion Hot start II high fidelity PCR master mix. Thermocycling condition was 1 cycle at 98^0 C for 30 seconds, 40 cycles of 98^0 C for 10 seconds, 62^0 C for 15 seconds (annealing), 72^0C for 1 min (extension) and 1 cycle at 72^0 C for 5 mins (final extension). The PCR product

was then gel purified by using GeneJET Gel Extraction Kit (Thermo Scientific™, catalog# K0691).

Homology-directed repair of Cas9 digested vector was conducted according to the protocol described in the NEBuilder® HiFi DNA Assembly Kit (catalog no. E5520). Briefly, Cas9 digested vector and HDR insert with a DNA molar ratio of 1:4 for vector and insert, respectively, for a total of 0.2 pmol, were incubated in the assembly buffer for 30 min at 50^0C in a thermocycler.

Transformation and positive colony selection

Following homologous recombination, 5 ul of the assembled product was transformed into NEB 5 alpha (catalog# C2987) competent cells according to the manufacturer's instruction. Positive clones were then selected by conducting colony PCR with forward primers located upstream and reverse primers located downstream of the insert (**Table 3.3**). Briefly, each colony was picked with a sterile toothpick and dipped into 40ul of nuclease free water (NFW), vortexed to mix and 5 ul from the mixture was added to the subsequent PCR reaction. The PCR assay was performed using GoTaq® Green Master Mix (Promega Corporation, catalog# M7122). Thermocycling conditions were: 1 cycle at 95^0 C for 2 min, 40 cycles of 95^0 C for 30 sec, 61^0C for 15 sec (annealing), 72^0C for 1 min 15 seconds (extension) and 1 cycle for 72^0C for 5 min (final extension). Positive colonies providing the desired amplicons in colony PCR were then used for further analysis. Preparation of plasmid DNA was carried out using a QIAprep spin Mini prep kit (Qiagen, catalog # 27106), and the insert was validated by sequencing (Eurofins MWG

Operon) followed by a larger scale preparation of positive plasmid DNA using a ZymoPURE™ II Plasmid Midiprep Kit (Zymo Research Corporation, catalog# D4200).

Table 3.3: FFIL2 PCR and sequencing primers

Purpose	Primers	Sequence (5' to 3')
PCR to produce FFIL2 Insert	Forward Primer	CTCACCCTCAACGAGGGCAAGTTAC
	Reverse Primer	CCGCTCGCGTGTATGAAAAATAAAGC
Colony PCR for FFIL2 Insert	Forward Primer	GCAAGGTCTCACTGAGTCAC
	Reverse Primer	TTGTTGAGATTACCGACCCTTG
Sequencing Primers	Forward Primer	GCAAGGTCTCACTGAGTCAC
	Reverse Primer	ATTGGCGTTACTATGGGAACATAC

Restriction digestion and Transfection

Linearized recombinant CAV2 plasmid vector was generated by double digestion with NotI-HF restriction endonuclease (NEB, catalog# R3189S). Briefly, 100ug of CAV2 vector was mixed with the required volume of cut-smart buffer and 200 units of NotI-HF, incubated 37^0C for 3hrs followed by gel purification with Zymoclean™ Large Fragment DNA Recovery Kit (Zymo Research Corporation, catalog# D4045). Linearized recombinant CAV2 vector was then used to transfect DKCre cells by using Lipofectamine P3000 transfection reagent (Thermo Scientific™, catalog# L3000015). The day before transfection, 2.5×10^5 cells adherent DKCre cells were seeded in a 12-well plate. On the day of transfection, cells were washed with 1 ml of 1X PBS and replaced with DMEM containing 5 % FBS without antibiotics or fungizone. Then, 1.5 ug of DNA was mixed with 6 ul of P3000 and the required volume of Opti-MEM media to bring the volume to 75 ul. This was mixed with 12 ul of Lipofectamine reagent diluted in 63 ul of Opti-MEM media (Thermo Scientific™) and incubated for 20 min at room temperature. The 150 ul of transfection mix was then added to the respective wells dropwise in serum containing

medium and incubated at 37^0C. After 24hrs, the media was changed with fresh DMEM containing 5% FBS without antibiotics or fungizone and incubated at 37^0 for 6 days.

3.3 Results

Canine lymphoma cell lines, OSW and 17-71, express IL2Rα mRNA

In order to be infected by IL2/anti-IL2Rα retargeted CAV2, the lymphoma cell lines should express cIL2Rα. Thus, cIL2Rα gene expression of PBMCs, CMT28, OSW and 17-71 was measured by RT-PCR followed by agarose gel electrophoresis. Result showed that the OSW and 17-71 provided a band size of 866bp (lanes 3 and 5 respectively) similar to that of positive control (PBMCs) (lane 7) with no band observed in the negative control cell line (CMT28) (lane 9) (**Fig. 3.2**). This data indicates that OSW and 17-71 cell lines successfully express the gene for cIL2Rα. As expected the canine mammary tumor line did not express IL2Rα.

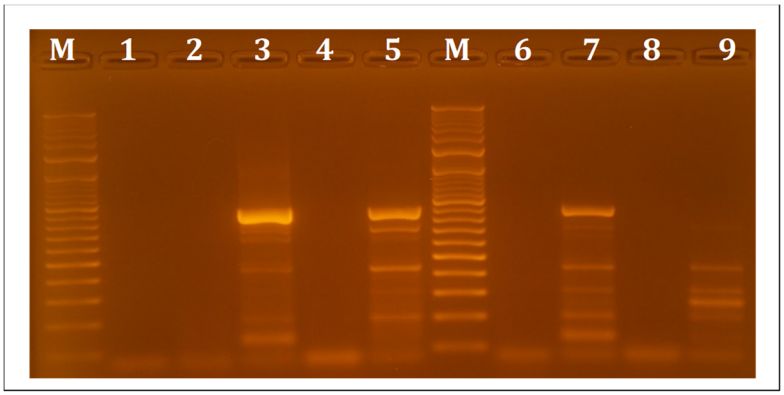

Figure 3.2: One step RT-PCR of OSW, 17-71, CMT28 and PBMC RNA with cIL2Rα primers

M=100bp + 500bp marker, 1= NTC, 2 = NRT for OSW, 3= OSW RNA, 4= NRT for 17-71, 5= 17-71 RNA, 6= NRT for PBMC, 7= PBMC RNA (positive control),

8= NRT for CMT28, 9= CMT28 RNA (negative control). NTC = No Template control, NRT = No Reverse transcriptase, cIL2Rα product size = 886bp.

sCAR-mIL2/ Ad5GL complex fails to infect canine lymphoma cell lines

Previous studies from our lab have shown that Ad5GFP virus fails to infect canine hematopoietic cell lines including canine lymphoma cell lines OSW and 17-71 [164, 353]. Our first goal was to infect canine lymphoma cell lines with sCAR-mIL2/Ad5GL complex to evaluate the potential of the bi-specific fusion protein adapter retargeted Ad5 to infect lymphoma cells lines (OSW, 17-71). In this regard, different MOIs (10, 100 and 1000) of Ad5GL were complexed with 5ng and 10ng of sCAR-mIL2 to generate sCAR-mIL2 fused Ad5GL complexes where sCAR will attach with the fiber knob of Ad5GL. Canine lymphoma cell lines 17–71 and OSW were then infected with sCAR-mIL2/Ad5GL complexes and positive control HEK293 cells were infected with Ad5GL, which has wild-type Ad5 tropism and expresses green fluorescent protein from the CMV immediate early promoter. Cells were examined by fluorescent microscopy to observe GFP reporter expression post-infection. HEK293 cell expressed green fluorescence 24hr post-Ad5GL infection at MOIs of 100 and 1000 virus particles/cell. In addition, HEK293 cells showed ~100% expression for GFP as well as cytopathic effect (CPE) 48hr post-infection at MOI 1000. Note that HEK cells transcomplemented the replication incompetent Ad5GL vector resulting in replication in these cells. Canine lymphoma cell lines, OSW and 17-71, had no GFP expression, even at the highest sCAR-mIL2/Ad5GL complex concentration (10ng of sCAR/mIL2 and 1000 MOI of Ad5GL) (**Fig. 3.3**). These results indicate that murine-IL2 based fusion protein adapter retargeted Ad5GL failed to infect canine lymphoma cell lines,

although this adapter has been previously shown to facilitate infection of murine lymphocytes [354].

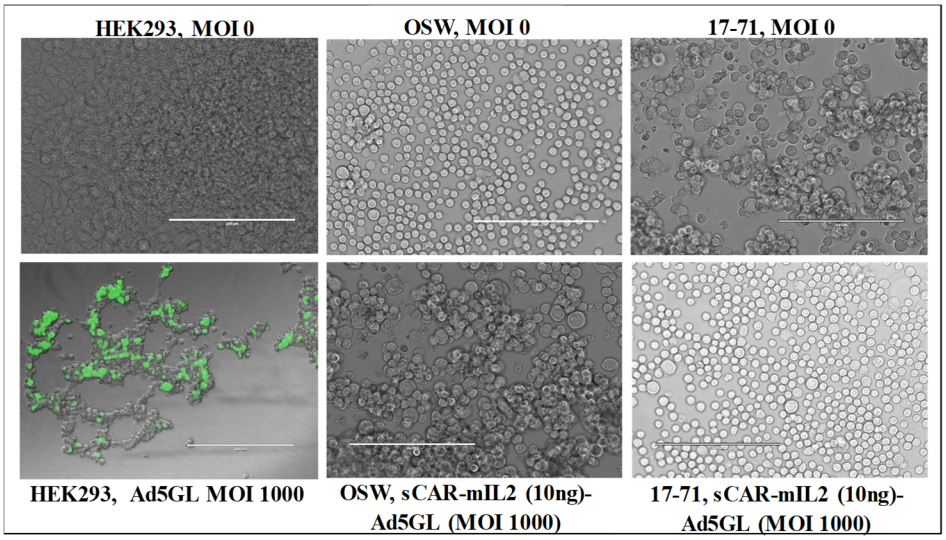

Figure 3.3: Infection of OSW and 17-71 cell lines with Ad5GL-sCAR-mIL2

Soluble coxsackie virus and adenovirus receptor murine interleukin 2 (sCAR-mIL2) complexed with Ad5GL failed to promote adenovirus transduction of canine lymphoma cell lines OSW and 17-71. 5ng and 10ng of sCAR-mIL2 were incubated with Ad5GL. Following incubation, lymphoma cells were infected with no virus (MOI = 0) and Ad5GL conjugated with the indicated amount of sCAR-mIL2 at 10, 100 and 1000 viral particles (VP) per cell. Forty eight hours post-infection, cells were observed for green fluorescence protein (GFP) expression using inverted fluorescent microscopy.

Murine IL2/IL2Rα sequences showed more diversity than that of human and canine sequences

To determine why sCAR-mIL2/Ad5GL failed to infect canine lymphoma cells, a similarity analysis was conducted between the murine, canine, and human IL2 and IL2Rα protein sequences using DNASTAR software.

According to the analysis, human and canine IL2 and IL2Rα showed evolutionary closeness compare to mouse IL2 and IL2Rα. The murine IL2 and IL2Rα protein showed significant divergence from canine of 78.1% and 67.5%, respectively. (**Fig 3.4 and 3.5**). When the IL2 protein sequences were aligned, the murine IL2 protein was shown to include a stretch of ten glutamine AA residues (**Fig. 3.4B, red arrow**) which are absent in human and canine IL2 protein (**Fig 3.4B**). The differences between murine and the canine sequences indicated that murine IL2 is unlikely to bind the canine IL2R. This explains the failure of the murine IL adapter to bind canine cells. As a consequence, canine IL2 and IL2Rα were chosen to be utilized as a targeting moiety to retarget CAV2 wild type fiber to canine cells. Specific differences potentially impacting the binding site of IL2 to its receptor could not be identified as the residues comprising the IL2R binding site have not yet been identified [355].

CAV2-AU-M1 showed variable levels of transduction capability in lymphoma cell lines

To further determine the background of adenoviral infection of canine lymphomas, canine lymphoma cell lines 17-71, OSW, CL-1, CLBL-1 and positive control DKCre were infected with a reporter canine adenovirus construct, CAV-AU-M1 which has wild type CAV2 tropism and expresses red fluorescent protein from the CMV immediate early promoter (developed in Chapter 2). Cells were examined by fluorescent microscopy and flow cytometry to determine the number of cells expressing the DsRed reporter gene post CAV-2-AU-M1 transduction (**Fig 3.6, 3.7, 3.8**). DKCre, OSW, 17-71 and CL-1 all expressed red fluorescence 48 hours post infection at MOIs of 10 and 100 virus particles/cell.

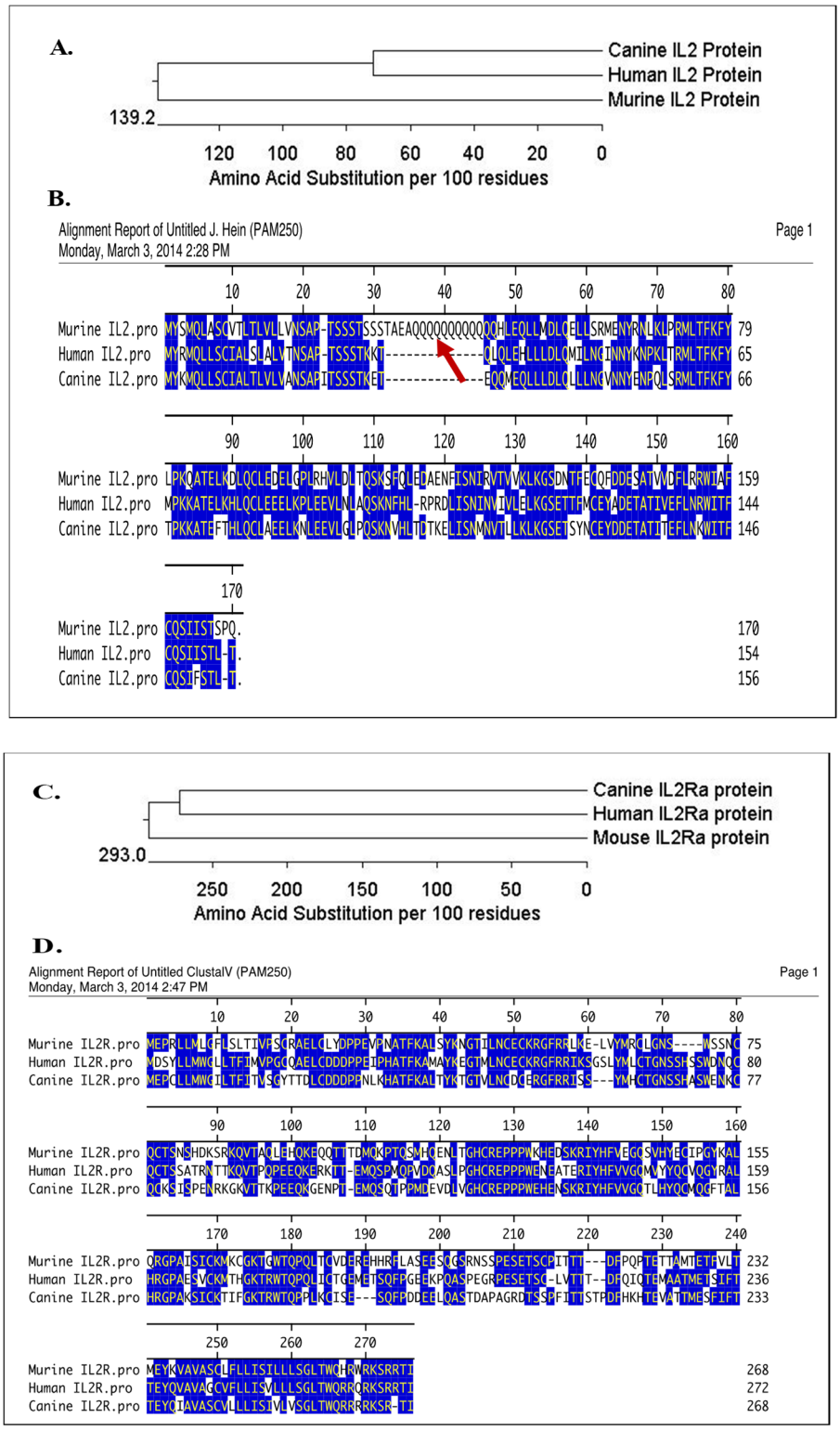

Figure 3.4: Similarity among canine, human and murine IL2 and IL2Ra proteins

In the diagram, i) IL2 protein (A, B) and ii) IL2Ra protein (C, D). A: The evolutionary relationship among canine, human and mouse IL2 protein via phylogenetic tree. B: Amino acid sequence variation present among canine, human and mouse IL2 amino acid sequences. Red arrow represents the presence of polyglumatine tracts. C: Evolutionary relationship among canine, human and mouse IL2Ra proteins via a phylogenetic tree. D: Amino acid sequence variation present among canine, human and mouse IL2Ra amino acid sequences. a= Alpha (α)

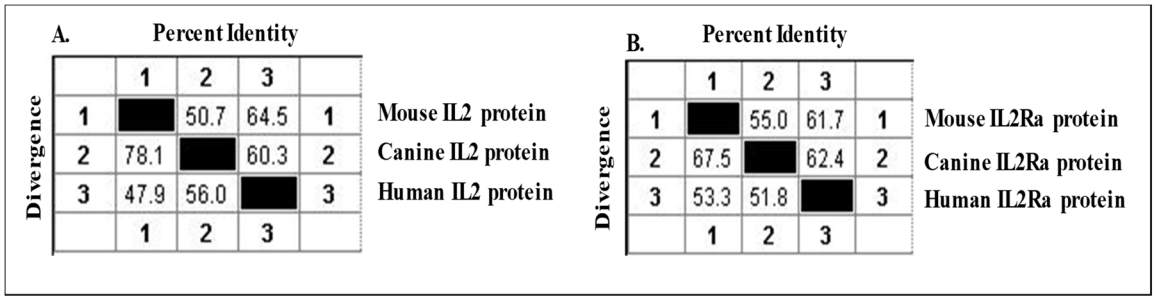

Figure 3.5: Sequence distance among mouse, canine and human A) IL2 and B) IL2Ra protein

This was evident by the increased percentage of CAV-AU-M1 transduced cells showing levels of fluorescence over the background of auto fluorescence in comparison to non-transduced cells. However, one of the canine B cell lymphoma cell line CLBL-1 showed no DsRed expression even at the highest virus concentration (100 MOI) (**Fig 3.6 and 3.7**).

The percentage of cells expressing DsRed in CAV2-AU-M1 infected cells is presented in **Fig. 3.8** and **Table 3.4**. When the percentage of cells expressing DsRed in comparison to non-infected cells was calculated the rate of infection was proportional to the virus particle MOI, except for CLBL-1. Virus infection rates in B cell lymphoma lines

varied, ranging from 0.2% in CLBL1 at MOI 100 to 97.45% in 17-71 at MOI 100 in these B cell lymphoma lines.

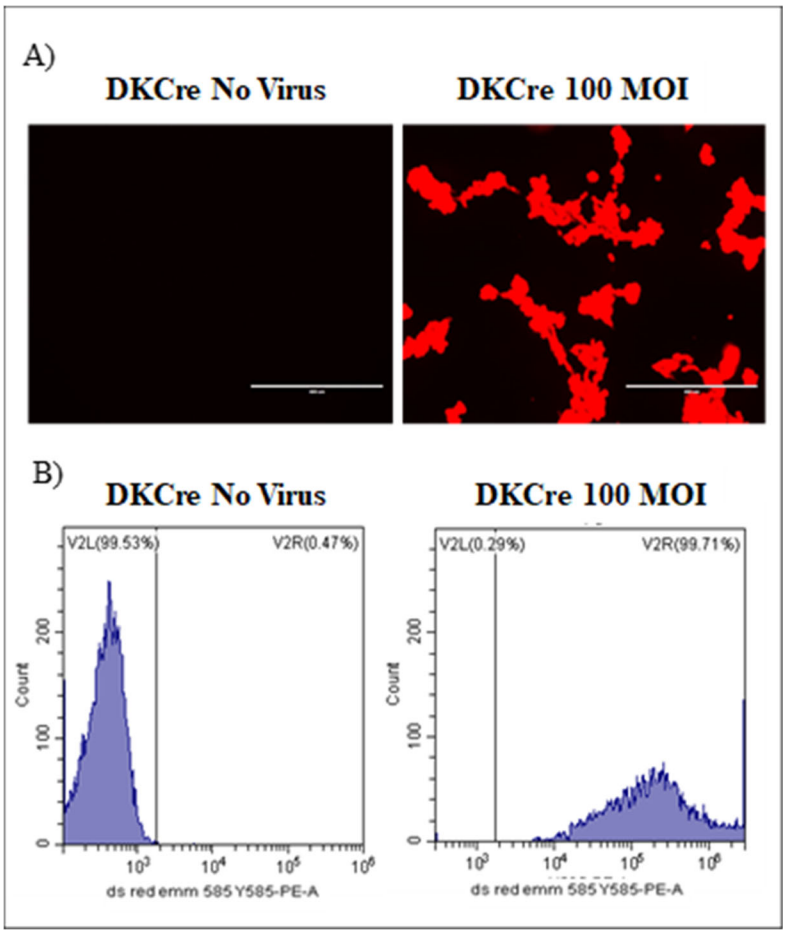

Figure 3.6: DsRed Expression by dog kidney cell line (DKCre)

DKCre cell line was transduced by CAV2-AU-M1 at multiplicity of infection of 100 (MOI 100 virus particles/cell). DsRed expression in cells were analyzed by A) Inverted fluorescent microscopy and B) flow cytometry 48 hours after CAV-AU-M1 infection.

However, infection rates at MOI 100 for two T lymphoma cell lines, OSW and CL1, were 44.99% and 68.39% respectively. These data indicate that the CAV2-AU-M1 virus transductional capability varies in lymphoma cell lines and it may infect cell lines by

using alternative receptors when compared to the human Ad5, given the lack of canonical adenoviral receptors in these cells.

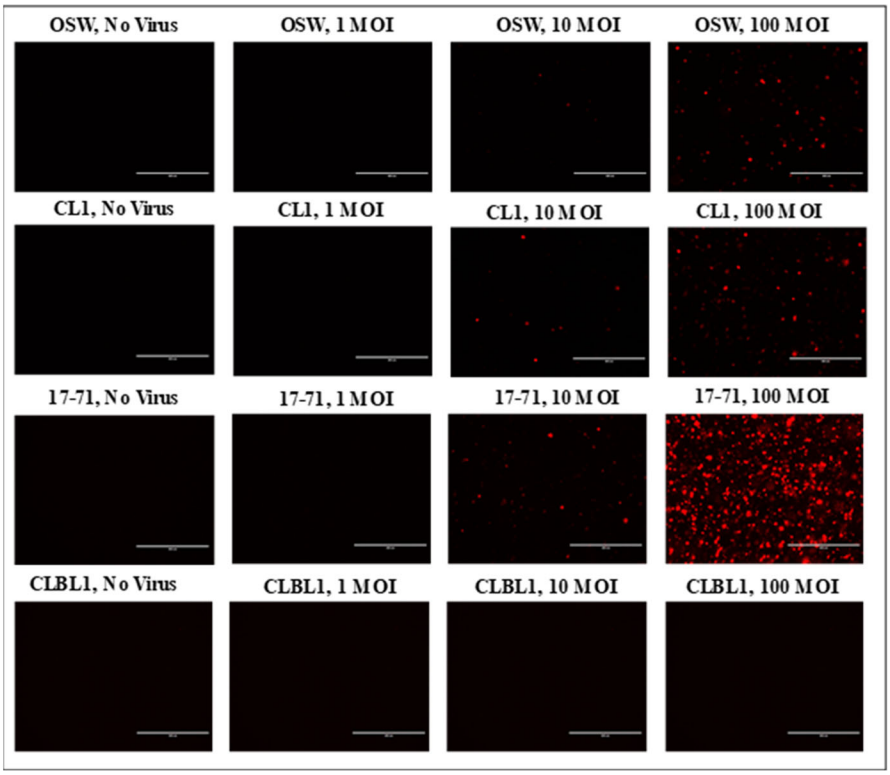

Figure 3.7: DsRed Expression by canine lymphoma lines

Canine T lymphoma cell lines (OSW and CL-1) and canine B lymphoma cell lines (17-71 and CLBL-1) were transduced by CAV2-AU-M1 at three different multiplicities of infection (MOI 1, 10 and 100 virus particles/cell). DsRed expression in cells were analyzed by inverted fluorescence microscopy 48hr after CAV2-AU-M1 infection. All fluorescent images were at 10X magnification.

Table 3.4: Percent cells infected by CAV2-AU-M1

Cell Lines	Tumor Types	0 MOI (% Population)	1 MOI (% Population)	10 MOI (% Population)	100 MOI (% Population)
OSW	T cell Lymphoma	0.24	0.52	6.20	44.99
CL1	T cell Lymphoma	0.01	0.57	24.58	68.39
17-71	B cell Lymphoma	0.07	0.29	18.57	97.45
CLBL1	B cell Lymphoma	0.09	0.44	0.11	0.20

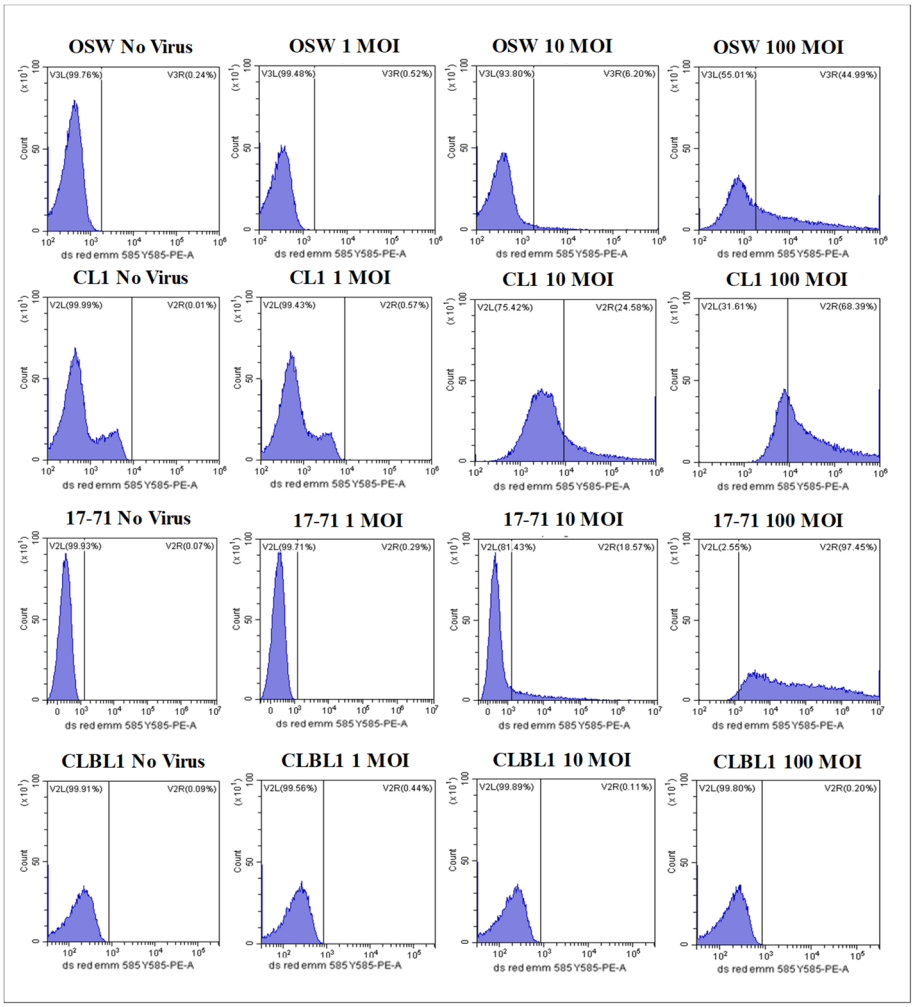

Figure 3.8: DsRed Expression by canine lymphoma lines

Canine T lymphoma cell lines (OSW and CL-1) and canine B lymphoma cell lines (17-71 and CLBL-1) were transduced by CAV2-AU-M1 at three different multiplicities of infection (MOI 1, 10 and 100 virus particles/cell). DsRed expression in cells were analyzed by flow cytometry 48 hours after CAV2-AU-M1 infection.

Generation of anti-cIL2Rα camelid single domain antibody (anti-sdAb-cIL2Rα)

In order to target canine IL2Rα, we sought to produce a single chain camelid antibody against a cloned canine IL2Rα. The canine IL2Rα protein is 268AA long and consists of extracellular (1 to 238 AA), short transmembrane (239 to 257 AA) and intracellular domains (258 to 268 AA).

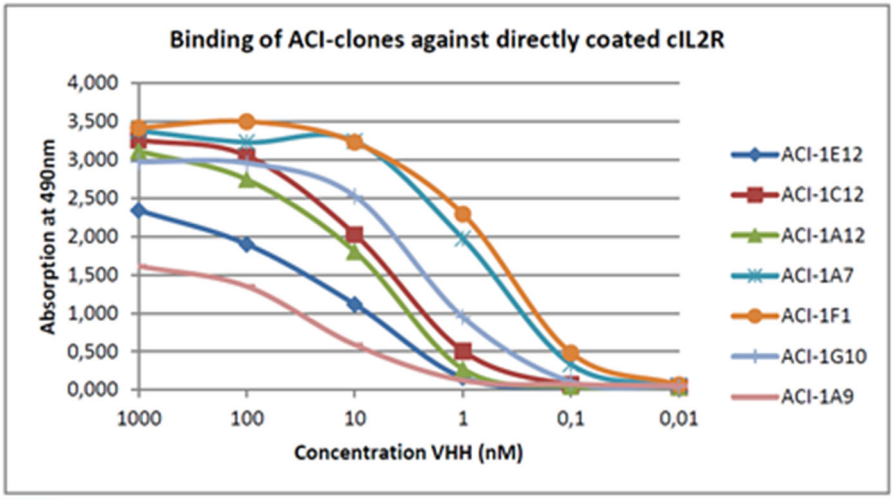

Figure 3.9: Dose response binding of selected VHH for anti-sdAb-cIL2Rα to cIL2Rα protein

This diagram is adapted from product data sheet of commercial sources (QVQ, Dutch company)

IL2 binding occurs in the extracellular region and, thus, antibody against the extracellular region would be sufficient to mimic receptor binding of ligand followed by internalization of the ligand. Commercial ecto-IL2Rα protein (1 to 238 AA) from R&D system (catalog # 6227-RC) was ordered and sent to a Dutch biotech company specializing in production of single chain camelid antibodies (QVQ, Dutch company). Two llamas were immunized with recombinant ecto-IL2Rα protein combined with standard adjuvant and following standard immunization schemes. The llamas were monitored for antibody

production, and when antibody was detected, lymphocytes were harvested. A phage display library of single domain antibodies was constructed using PCR products from cDNA produced from the llama lymphocytes. The phage were screened to select five unique clones for variable region heavy chain (V_HH) fragments specific for canine ecto-IL2Rα (cIL2Rα). (**Fig 3.8**). The commercial source (QVQ, Dutch company) provided protein, DNA clones, and DNA sequence data for each clone. These sdAbs were used to generate a panel of fiber retargeted CAV2.

Construction of IL2/IL2Rα-mediated fiber retargeted CAV2 by CRISPR/Cas9

For this study, two recombinant CAV2-based plasmid vectors were developed expressing the red fluorescent gene under transcriptional control of the human cytomegalovirus (CMV) major immediate-early enhancer/promoter element (CAV2-AU-M1). To construct the CAV2-FF-cIL2 (pCAV2-AU-M2) and CAV2-FF-anti-cIL2Rα-sdAb (pCAV2-AU-M3) viruses, DNA sequences were constructed *in silico* consisting of the N-terminal CAV2 fiber tail region followed by the entire fibritin protein, including the trimerizing foldon domain, of bacteriophage T4, a Gly-Gly-Gly-Gly-Ser peptide linker and either the entire canine IL2 sequence (Gene accession no. NM_001003305) or the sequence of an anti-IL2Rα sdAb (**Fig. 3.1**).

CRISPR-Cas9 technology was used to remove a section of the virus and replace it with alternative sequence. In this regard, two distinct cuts were made to remove a segment of the CAV2 genome containing most of the fiber gene and the alternative fiber-fibritin-IL2R binding domain construct was inserted by homology directed repair. The CAV2 fiber, like other adenovirus fibers, consists of tail, shaft, and knob domains. The fiber is 1657bp

long with a homotrimer of 542 amino acid (AA) peptides. The tail domain spans residues 1-43, the shaft domain contains 18 pseudo-repeats and spans residues 44 to 363, and the knob domain encompasses residues 364-542 [45, 227]. Based on the location of the 4th AA of the 3rd repeat of the CAV2 fiber shaft domain, it was determined that the Fibritin IL2 construct should be inserted at position 26967bp of CAV2-AU-M1. Two 20 bp sgRNAs, near the upstream and downstream ends of the DNA to be removed were identified that would create an appropriately double digested target vector **(Table 3.3 and Fig 3.9)**.

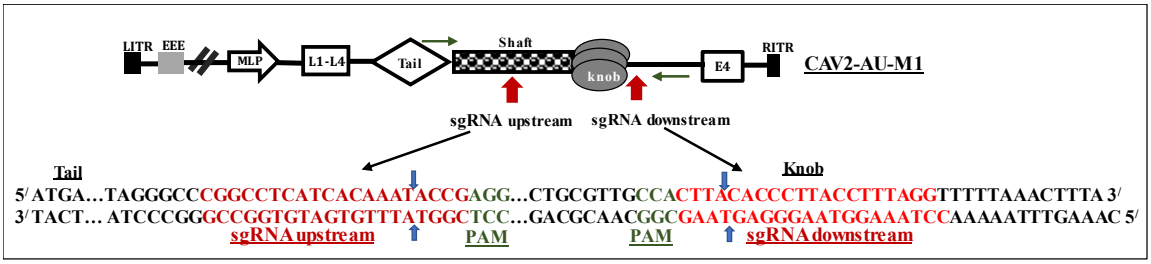

Figure 3.10: Schematic representation of the sequence and location of sgRNAs

The sgRNAs are located upstream and downstream region of CAV2 fiber followed by the PAM sequence. Red arrows represent sgRNAs location, blue arrows represent the position of Cas9 cut sites and green arrows represent the location of colony PCR and sequencing primers PAM= Protospacer adjacent (PAM) motif.

Two oligonucleotide were synthesized that contained the reverse complement of the target, additional RNA sequence for the common sgRNA scaffold and the T7 promoter. The sgRNA was synthesized by T7 polymerase **(Table 3.3)**. Once the sgRNAs were synthesized and validated, they was used to conduct the Cas9/sgRNA-mediated double digestion of the viral genome plasmid to remove part of the fiber shaft and the entire knob domain. Digestion of the plasmid resulted in a 36,667bp linear DNA and a 1330bp

fragment of truncated fiber (containing the rest of the shaft region and fiber knob) and was visualized by agarose gel electrophoresis (**Fig 3.10**).

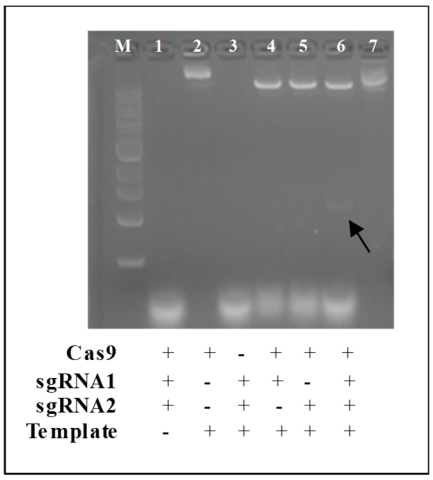

Figure 3.11: Diagram showing Cas9 mediated double digestion of fiber of CAV2-AU-M1

In the diagram, black arrow represents the 1330bp of truncated fiber. M= 1kb extended DNA ladder.

Homology directed repair (HDR) was used to insert the Fibritin-IL2 and Fibritin-anti-IL2Rα sdAb into the linearized pCAV2-AU-M1 vector in a process known as seamless cloning. The HDR insert was designed to contain 27 bp and 55 bp overlaps with CAV-2-AU-M1 in the left and right arms, respectively (**Fig 3.11A**).

The Cas9/sgRNA double digested, linearized CAV2-AU-M1 vector, along with either the Fibritin-IL2 or Fibritin-anti-sdAb-cIL2Rα insert, were incubated with Hifi DNA assembly master mix to generate recombinant vector via homology directed repair (**Fig 3.11B)**. The plasmid was then transformed into high efficiency competent cells. After 18hr of growth, 21 clones were randomly selected and colony PCR was performed. Results showed that 11 out of 21 clones were positive for the desired CAV2-AU-M2 vector (**Fig 3.11C**) and similar results were obtained for CAV2-AU-M3 vector.

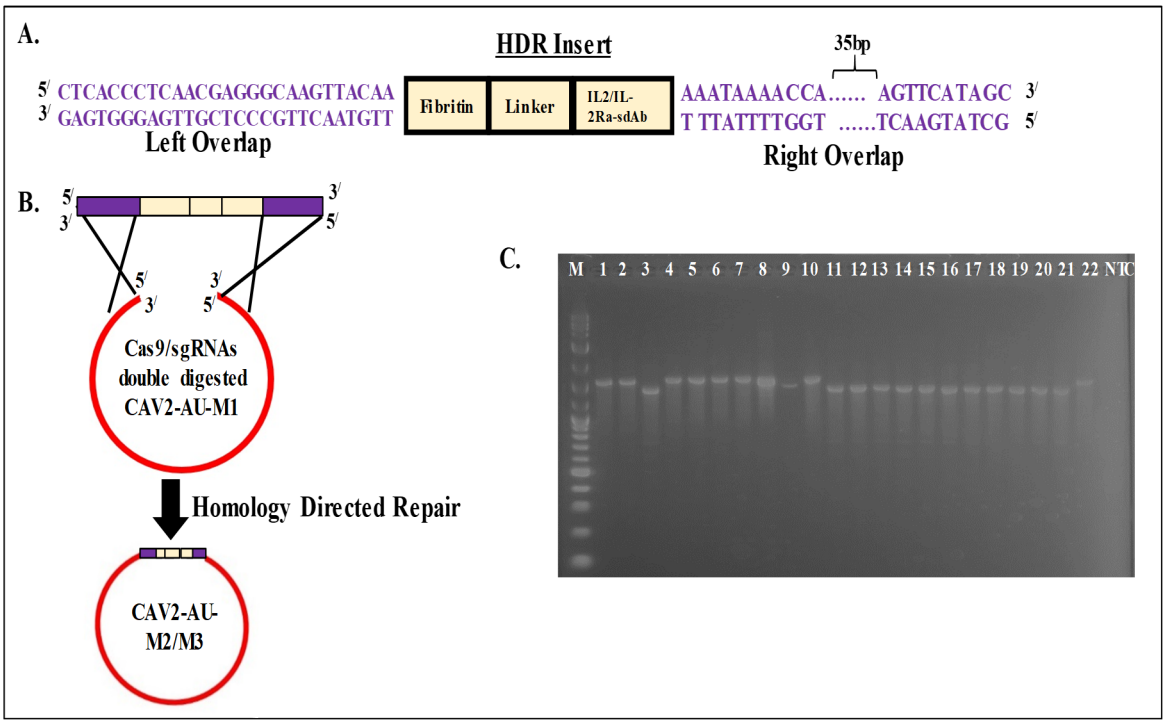

Figure 3.12: Diagram showing homology directed repair (HDR)-mediated generation of recombinant CAV2-AU-M2/M3 vector

A) Schematic representation of HDR insert sequence showing left and right overlaps encompassing the HDR insert. Left and right overlaps are 27 bp and 55 bp, respectively B) Schema for HDR strategy to insert HDR insert into the Cas9/sgRNA double digested vector C) Colony PCR amplification of the target region. M= Quick Load 2-log DNA ladder, 22= Amplification of wild type fiber (Product size= ~1656bp); FF-IL2 product size= 1450bp. NTC = No template Control.

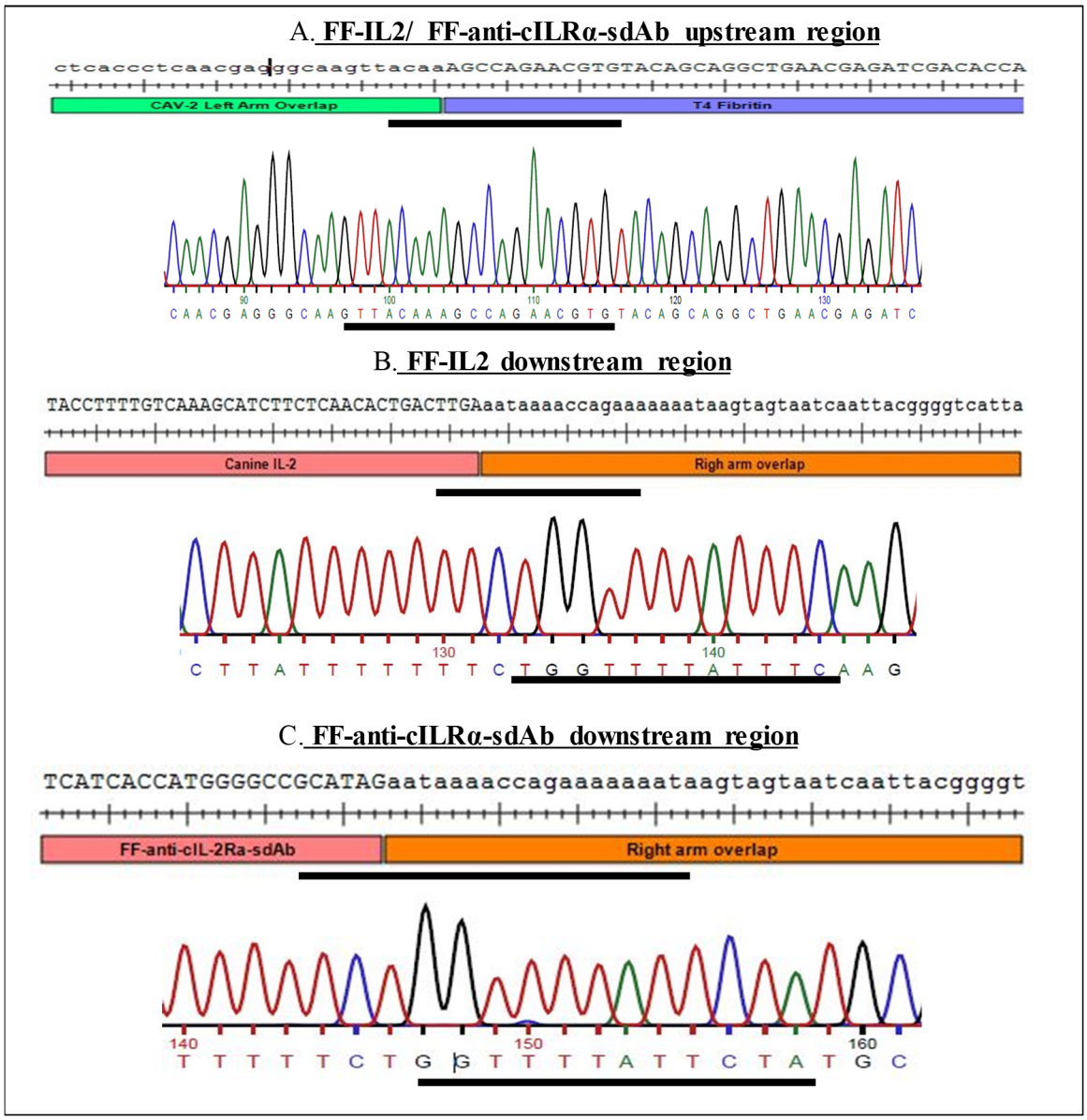

Figure 3.13: Sequencing analysis for insertional mutagenesis in recombinant vector

A) Top: Panel shows partial sequence for the junction point connecting fiber and fibritin. Bottom: Panel includes the chromatogram showing no insertional mutagenesis in the junction point. B) Top: Panel shows partial sequence for the junction point connecting the canine IL2 with right arm overlap. Bottom: Panel includes the chromatogram showing no insertional mutagenesis in the junction point. C) Top: Panel shows partial sequence for the junction point connecting the anti-

cIL2Rα-sdAb with right arm overlap. Bottom: Chromatogram shows no insertional mutagenesis in the junction point of. Black line represent the junction point- potential site for insertional mutagenesis.

DNA sequencing confirmed the presence of the desired insert in the desired location for both of the construct without any additional mutation at the junction point (**Fig 3.12 A, B and C**).

3.4 Discussion

Adenoviruses are outstanding vector systems to use for oncolytic virotherapy for cancer. However, lack of expression of CAR and integrin on the surface of cells of lymphocyte origin restrict the successful implementation of adenovirus-mediated gene therapy for hematologic malignancies. Despite promising new therapeutic approaches having been developed to generate transductionally targeted adenoviral vector, very few vectors have been successfully developed to target cells of lymphocyte origin. In this regard, we constructed ILR-targeted fiber modified CAV2 vector by genetically incorporating either anti-cIL2Rα sdAb VHH or cIL2 into a de-knobbed CAV2 fiber-fibritin protein (CAV2-AU-M2 and pCAV2-AU-M3).

Previously, extensive studies have been conducted to elucidate the infection mechanism of human adenoviruses for a broad range of cell types including the cells of lymphocyte origin. However, very few attempts have been made to study the status of CAV2 infection in lymphoma cells. Thus, in this study, CAV2-AU-M1 was used to infect four canine B- and T- cell lymphoma and result showed that CAV2-AU-M1 provided significant level of transduction in two T lymphoma (OSW and CL1) and one B lymphoma

(17-71) cell line but showed no transductional capability in one B lymphoma (CLBL1) cell line. CAV2-AU-M1 is a genetically modified version of the wild type strain of CAV2 and contains an RGD motif in its fiber peptide where wild type CAV2 lacks the RGD motif. Previous studies have shown that OSW and 17-71 cells express low levels of αvβ3/αvβ5 integrins but express significant levels of CAR receptor. So, it is possible that CAV2-AU-M1 infects the lymphoma cell lines by a CAR dependent pathway. Alternative to the CAR-dependent pathway, CAV2-AU-M1 may incorporate interaction with other alternative unknown cell type-specific receptors to promote enhanced gene transduction in those cell lines. However, this data indicates that CAV2-AU-M1 transduction capability varies based on cell types. Early investigations also reported variability in CAV2 transduction capability in cells of lymphocyte origin. Previously, no wild type CAV2 transduction was reported for human B cell lymphoma cell lines Daudi and Ramos expressing CAR but no αvβ3/αvβ5. Interestingly, CAV2 showed enhanced transduction for a human T lymphocyte cell line, Jurkat, expressing similar levels of CAR and αvβ3/αvβ5 to Daudi and Ramos cell lines and was also similar in transduction of human monocytic leukemia lines expressing little or no CAR, αvβ3 or αvβ5 integrins. Based on these findings, authors reported that CAV2 transduction varies based on the cell line and it may promote infection in both CAR- and αvβ3/αvβ5-dependent and independent manner or, in some cases, may utilize alternative cellular receptors [144]. However, further studies are needed to gather more in-depth knowledge regarding the cellular receptors utilized by CAV2.

Previously, in order to promote CAR- and integrin-independent transduction of murine T lymphocytes, Beatty *et al*. developed an adapter based strategy by generating a bi-specific fusion protein, soluble CAR murine interleukin 2 (sCAR-mIL2), to retarget

adenovirus to murine IL2-receptor (IL2R) and showed enhanced adenovirus transduction in a murine T cell line. However, the authors reported reduced transduction efficiency in activated primary T lymphocytes with sCAR-mIL2 retargeted adenovirus resulting from weak binding of the adapter to both adenovirus fiber knob and the cell surface IL2R receptor. In addition, the authors concluded that utilization of fiber retargeted virus containing a trimerization domain promoted strong binding followed by enhanced transduction. In this regard, this study aimed to replace the wild type adenovirus fiber with a fiber-fibritin chimera fused to either interleukin 2 or camelid single domain VHH antibody recognizing IL2Rα for targeting of CAV2-mediated gene delivery.

Several structural features have made T4 fibrtin an attractive candidate to be used as a fiber chimera. T4 Fibritin protein promotes assembly of the T4 long tail fiber to the tail basement. It also provides a stable trimeric structure that can absorb either extensive amino-terminal deletion or carboxy-terminal insertions without binding to any cellular receptor [238]. The assembly of adenoviral components occurs in the nucleus and cytoplasm to generate functional virus particles. The biological molecule that would be used as a fusion protein for fiber should also be folded and transported through the nucleus and cytoplasm. However, most of the biological ligands are thought to be folded and transported through the Golgi apparatus and, thus, these are not biologically compatible with adenovirus biology. In contrast, IL2is reported to be folded and translocated via the nucleus instead of the Golgi apparatus, and, thus, can assemble as part of the Ad fiber during adenovirus capsid synthesis [354]. In addition, upon receptor binding, IL2 is directly internalized without requiring any secondary interaction with any other receptor [355]. Conventional Abs are double chained and thus, complex to generate and biologically

incompatible with cytosolic Ad capsid synthesis and less stable [198, 356]. In contrast, camelid derived single domain antibody (sdAd) consisting of variable heavy chain (VHH) provides high cytosolic stability, ease of production by phage display and sdAd engineered fusion proteins showed effective targeting in model systems [357, 358].

In this study, IL2R-targeted fiber modified CAV2 were generated by employing CRISPR/Cas9 mediated replacement of the wild type fiber of CAV2. Two sgRNAs were designed and synthesized *in vitro* and utilized to successfully conduct CRISPR-Cas9-mediated double digestion of the target vector. One of the two sgRNA were designed in the bottom strand to maintain appropriate GC (40%) content of the sgRNA facilitating efficient cutting. Results showed that this method provided efficient double digestion following the linearization of the vector irrespective of the position of the sgRNA indicating the versatility and efficiency of CRISPR/Cas9 mediated editing of large viruses such as CAV2. This method provided efficient generation of positive clones where the cloning efficiency was ~52% for double digestion (Figure 3.3 11C). In addition, this study also showed that the *in vitro* CRISPR/Cas9 mediated technique to replace large fragments from the CAV2 genome with desired insert is very accurate as the junction points were perfectly conserved with no insertion or deletion occurring. No other changes were detected at any other location including inserts or recombination of the vector (Fig. 3.12). CRISPR/Cas9 has the potential to promote off-target effects resulting in cleavage of non-target sites that may limit its application in large complex genomes [319, 320]. However, in this study, no off-target cleavage has been observed during digestion at multiple sites. The presence of off-target cuts may result either in multiple bands after Cas9 digestion or the presence of the insert in an alternative location. However, these results have not been

observed in this study indicating the efficiency of this technique for the modification of a large vector.

3.5 Conclusions

Our goal was to utilize CRISPR/Cas9 *in vitro* to fiber modified recombinant CAV2 vector retargeted to the IL2R receptor to promote CAR- and integrin-independent CAV2 transduction in cells of lymphocyte origin. This study is the first attempt to utilize the feasibility of *in vitro* CRISPR/Cas9 to conduct genetic replacement of fiber in the large CAV2 vector. In addition, this study is also the first attempt to conduct genetic incorporation of either anti-cIL2Rα sdAb VHH or cIL2 into a de-knobbed CAV2 fiber-fibritin protein. Results showed the technique adopted provided a unique mechanism to generate functional, fiber-modified recombinant CAV2 vector in a rapid and less-complex manner. However, further experiments are needed to produce functional and purified virus particles. In addition, further validation is needed to characterize the fiber-modified vector as well as to study the functionality of IL2R targeted fiber modified CAV2 capable of enhanced gene transduction in cells of lymphocyte origin.

CHAPTER 4

Analysis of Endogenous and Exogenous Tumor Upregulated Promoter Expression in Canine Tumors

4.1 Introduction

Gene therapy is a promising approach to treat different types of cancer. Cancer gene therapy aims to modify or kill cancerous cells [359], however, if used indiscriminately, may lead to serious side effects such as peripheral neuropathy and immunosuppression. This issue can be resolved by cancer-specific conditional gene expression to enhance robust therapeutic outcomes with relatively minimal side-effects [328, 360]. One such strategy is to employ tumor-upregulated or tissue-specific promoters to express therapeutic transgenes [279].

Promoters that are broadly upregulated across a variety of cancers with low expression levels in normal cells can serve as excellent candidates for driving therapeutic genes in cancer gene therapy. Examples include prostate–specific antigen (PSA) [361, 362], tyrosinase-related protein 1 (TRP-1), melanoma inhibitory activity (MIA), [363], and hepatocyte specific alpha-fetoprotein (AFP) [364-366]. We have selected three such upregulated promoters to study; survivin, chemokine receptor 4 (CXCR4) and telomerase reverse transcriptase (TERT).

Survivin is a bi-functional protein that promotes cell growth by inhibiting apoptosis. It is overexpressed in many cancers including breast [367], esophagus [368], lung [369], lymphoma [370], and others [371-373]. CXCR4 is a chemokine receptor that is expressed on most hematopoietic cells [374]. CXCR4 binding to CXCL12 ligand

promotes gene transcription, chemotaxis, cell survival, proliferation, organ development, inflammation and immune surveillance of cells [375-377]. CXCR4 is also overexpressed in many cancers [378-380]. Telomerase reverse transcriptase (TERT) is an integral part of the telomerase enzyme complex. TERT restricts cell growth arrest and empowers the cells to undergo self-renewal [381-383]. TERT is highly upregulated in embryonic stem cells, progressively dividing cells, and cancer cells [384]. Likewise, TERT is overexpressed in many malignant diseases including lung cancer, gastric melanoma, prostate cancer, breast cancer, and various hematopoietic malignancies. [385-387].

Dogs are an outstanding translational animal cancer model for humans because they share the same environment, develop spontaneous cancers, and have similar genetic alterations and mechanisms to humans. Dogs are relatively outbred as compared to laboratory rodents (although purebred dogs present unique opportunities to study predisposition to certain cancer types) and represent an intermediate size that allows an approximation of the dose and scale that is required to successfully treat people [388-391]. While several studies reported successful utilization of TERT, survivin, and CXCR4 for transcriptional targeting in human cancers, none of these promoters have been investigated for their activity in canine tumors [392-397]. The goal of this study was to measure the activity of these promoters in a panel of canine tumors. In addition to these endogenous promoters, we also utilized a modified version of the canine adenoviral E1A promoter, containing four E2F binding sites and one Sp-I site (E2F enhanced E1A promoter, or EEE), which has previously been shown to be upregulated in activity in tumor cells [290].

4.2 Materials and methods

Cell culture

Canine melanoma (CML7, CML10), canine mammary tumor (CMT12, CMT28), canine histiocytoma (DH82), fetal dog kidney (FDK), Madin-Darby canine kidney cells (MDCK), and primary normal canine fibroblast (NCF) (**Table 4.1**) cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Corning) with penicillin (100 IU/ml, Corning), streptomycin (100 ug/ml, Corning), amphotericin B (0.5ug/ml, Corning), and 10% FBS (fetal bovine serum, Sigma). Canine B cell lymphoma line 17-71 [349] (gift from Dr. Steven Suter, North Carolina State University) and peripheral T cell lymphoma line OSW [352] (gift from Dr. William C. Kisseberth, The Ohio State University) (**Table 1**) were cultured in RPMI (Roswell Park Memorial Institute medium, Corning) with penicillin (100 IU/ml, Corning), streptomycin (100 ug/ml, Corning), amphotericin B (0.5ug/ml, Corning) and 10% FBS (Sigma). All cells were grown at 37°C and 5% CO_2 and were validated to be of canine origin by species-specific PCR [307]

Table 4.1: Cells and cell lines

Cell Name	Status	Description	Type
NCF	Adherent	Normal Canine Fibroblast	Primary
PBMC	Suspended	Peripheral Blood Mononuclear Cells	Primary
MDCK	Adherent	Madin-Darby Canine Kidney Cell lines	Cell line
FDK	Adherent	Fetal Dog Kidney	Cell line
CML7	Adherent	Canine Melanoma Cell line 7	Cell line
CML10	Adherent	Canine Melanoma Cell line 10	Cell line
CMT28	Adherent	Canine Mammary Tumor cell line 28	Cell line
DH82	Adherent	Canine Histiocytic Cell line 82	Cell line
OSW	Suspended	Canine Lymphoma Cell line	Cell line
17-71	Suspended	Canine Lymphoma Cell Line	Cell line

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated using SepMate™-50 tubes (Stem cell Technologies). 10 ml of blood was drawn from the cephalic vein of a normal dog into EDTA tubes (Tyco) and diluted with an equal amount of phosphate buffered saline (PBS, Corning) containing 2% FBS. The mixture was added to SepMate™-50 tubes containing Histopaque 1077 (Sigma). The mixture was centrifuged at 1200g for 10 min at room temperature. The supernatant was transferred to a 15ml conical tube, washed twice with PBS containing 2% FBS, and centrifuged at 300g for 8 min at room temperature. The supernatant was removed and the PBMC pellet was recovered for total RNA isolation. All animal work was performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health) and was approved by the Auburn University Institutional Animal Care and Use Committee.

Table 4.2: Primary lymphoma tissues

Animal	Description
Zach	Diffuse lymphoblastic T cell lymphoma
Roxann	Diffuse large B cell lymphoma
Allie Bear	Diffuse large B cell lymphoma
Truffy	Diffuse large B cell lymphoma
Oliver	Diffuse large B cell lymphoma
Jack Kennedy	Diffuse large B cell lymphoma
Suzy	Diffuse large B cell lymphoma
Golda Spina	Diffuse large B cell lymphoma
Bitsi	Diffuse large B cell lymphoma
Nala	Diffuse large B cell lymphoma
Phumba	Diffuse large B cell lymphoma
Doggy	Diffuse large B cell lymphoma

Total RNA Isolation, Primer Design and Quantitative RT-PCR

Total RNA was isolated from cell lines, primary cells, normal canine tissues (lung, liver, heart, pancreas and kidney), and primary lymphoma tissue (**Table 4.2**) using Tri-reagent (Molecular Research Center, Inc.).

All cells and frozen tissue samples (40-80mg) were homogenized in 1 ml Tri-reagent. RNA was isolated from the aqueous phase by isopropanol precipitation. RNA concentration was determined by absorbance at 260 nm. The mRNA expression from canine survivin, CXCR4, TERT, and beta actin (cSurvivin, cCXCR4, cTERT) was measured by quantitative reverse transcriptase PCR (Q-RT-PCR).The cDNA from 1ug of RNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Catalog # 1708891). One tenth of the RT reaction volume was used for Q-RT-PCR assays using SSO-Advanced Universal syber green supermix (Bio-Rad) with 0.5uM forward and reverse primers in a volume of 20ul (**Table 4.3**). Thermocycling conditions were: 3 min at 95^0C, then 40 cycles of 95^0 C for 30 sec and 57^0C for 30 sec, on a Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System. The mRNA expression was analyzed by the ΔCt method using beta-actin as the comparator. Primer specificity was validated by sequencing the PCR product (Eurofins MWG Operon).

Table 4.3: Quantitative Reverse Transcription PCR (Q-RT-PCR) Primers

Gene	Primer (5/-3/)	Amplicon size (bp)	GeneBank Accession No.
β-actin Forward	ACGGGCAGGTCATCACTATT	220	NM_001195845.1
β-actin Reverse	ATCTCCTTCTGAATCCTGTCA		
cSurvivin Forward	AAGAACTGGCCGTTCCTGGAGG	149	AY741504.1
cSurvivin Reverse	TCATCATCTGGCTCCCAGCCTTC		
cCXCR4 Forward	GCGGGCGAGCGGTTACCA	167	NM_001048026.1
cCXCR4 Reverse	GAAGATGATGGAGTAGACTGTGGGCAG		
cTERT Forward	GTTCATCTCCCTGGGAAAGCACG	172	NM_001031630.1
cTERT Reverse	GCCCATCAACCAGCACAAGGAAC		
Primers were designed using NCBI Primer blast. Primers are based on canine sequences.			

Enhanced green fluorescent protein (eGFP) reporter plasmid constructs

Reporter plasmids were constructed using pDC311CMV-eGFP plasmid (gift from Dr. Yadvinder Ahi, NIH) (**Fig. 4.1A).** Canine Survivin, CXCR4 and TERT promoter

sequences were predicted from the canine genome using NCBI Blast (**Table 4.4**). The recombinant expression vectors pDC311-cSurvivin-eGFP, pDC311-cCXCR4-eGFP, and pDC311-cTERT-eGFP were commercially synthesized (Gene Script Inc.) (**Fig. 4.1B**).

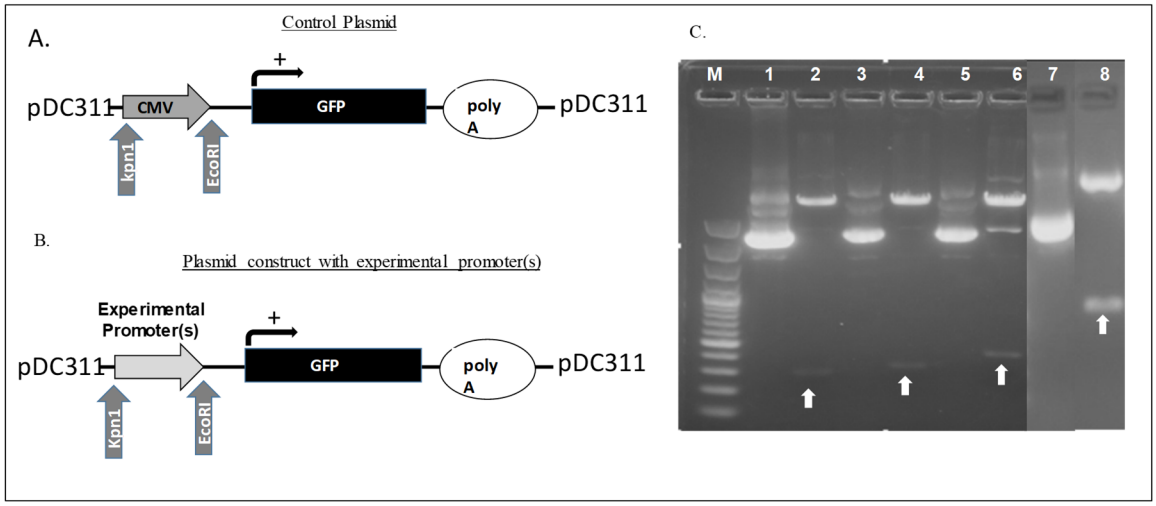

Figure 4.1: Construction of GFP reporter constructs and restriction digestion of experimental promoters

Construction of GFP reporter constructs driven by either (A) CMV promoter or (B) Experimental promoters. (C) Kpn1 and EcoRI-mediated restriction digestion of experimental promoters containing plasmid constructs. M= 100kb ladder; 1= pDC311-cSurvivin-GFP undigested plasmid (UDP); 2= Kpn1 and EcoR1 double digested (DD) clone for cSurvivin-GFP construct; 3= pDC311-cCXCR4-GFP UDP ; 4= Kpn1 and EcoR1 DD cCCXCR4-GFP construct; 5= pDC311-cTERT-GFP UDP; 6 = Kpn1 and EcoR1 DD cTERT-GFP construct; 7= pDC311-EEE-GFP UDP ; 8= EcoR1 and Kpn1 DD EEE-GFP construct. CMV = cytomegalovirus immediate early promoter, GFP = green fluorescent protein coding sequence, polyA = poly adenylation signal. White arrows indicate the location of the promoter fragment from the digested plasmid.

The EEE promoter sequence, with four imperfect palindromic E2F-binding sites and one Sp-I-binding site, was amplified using primers containing Kpn1 and EcoR1 restriction sites from pICOCAV15 (a gift from Dr. Ramon Alemany) [398]. The PCR product was digested with Kpn1 and EcoR1, and cloned into Kpn1/EcoR1 double digested pDC311-CMV-eGFP, replacing the MV promoter with EEE to create pDC311-EEE-eGFP (**Fig. 4.1B).** Recombinant plasmid constructs were confirmed by restriction digestion (**Fig. 4.1C**) and sequencing (Eurofins MWG Operon).

Table 4.4: Promoters

Promoter	Length (bp)	Chromosomal location	NCBI Gene ID
Canine Survivin	296bp	6	442936
Canine CXCR4	261bp	19	483900
Canine TERT	379bp	34	403312

Transfection and Flow cytometry

Adherent cells were transfected with jet prime transfection reagent (Polypus transfection, catalog# 114-07). Briefly, one day before transfection, 8 x 10^5 adherent cells were seeded in a 24 well plate. On the day of transfection, 0.5ug of DNA was mixed with 50ul of jet prime buffer, and added to 1ul of jet prime reagent and incubated for 10 min. The transfection mix was then added dropwise to the medium in each well and incubated at 37^0C for 48hr. Suspended cells were transfected via electroporation using the Neon Transfection System (Thermo Fisher Scientific, catalog # MPK1096). Briefly, the cells were washed with PBS and resuspended in resuspension buffer R at a final density of 2 × 10^7 cells/ml. Then, the cells were mixed with 1ug DNA/ 2× 10^5 cells and electroporated using 10ul Neon tips with the following conditions: pulse voltage: 1850, pulse width: 20,

and pulse no: 1. Following electroporation, the cells were immediately put on ice for 15 min. The cells were then placed in 500ul of prewarmed serum containing antibiotic-free media and incubated for 48hr.

After two days, transfected adherent cells were harvested by trypsinization and electroporated cells were harvested by centrifugation. Cells were washed twice with PBS. The cells were resuspended in PBS and 0.1% BSA, and analyzed for GFP expression by flow cytometry (Accuri C6).

Statistical Analysis

Promoter activity was compared using a two tailed student T test at the 95% confidence level by using Prism statistical software (GraphPad, San Diego, CA).

4.3 Results

The activity of cSurvivin, cCXCR4, cTERT, and EEE was evaluated in canine cells to determine whether their expression was upregulated in canine tumors, based on upregulated expression in human tumors [372, 373, 387, 399-402]. Both endogenous and exogenous promoter activity was analyzed, the former by Q-RT-PCR and the latter by reporter gene assays.

Analysis of endogenous promoter activity

Endogenous expression of cSurvivin, cCXCR4, and cTERT was analyzed by Q-RT-PCR in canine primary cells (NCF, PBMCs), cell lines (FDK, MDCK), cancer cell lines (CMT12, CMT28, CML7, CML10, DH82, 17-71, OSW), normal tissues (lung, liver,

heart, pancreas, kidney, intestine), and primary tumor tissues (1 T cell lymphoma, 11 B cell lymphomas) **(Table 4.1 & 4.2)**. The mRNA expression for each gene was normalized to B-actin expression (ΔCT) in each cell type (**Fig. 4.2**). Results showed that B actin levels varied depending on the source (cells/cell lines vs tissues) and relatively consistent B actin levels were observed within the cells/cell lines and within the tissue samples (**Table 4.5**). While it is possible that actual B actin expression levels may vary between cultured cells and tissue samples, given the consistency within each group, it is more likely that, these variations were the result of different efficiencies in RNA isolation between cultured cells and tissue samples. Such variation in RNA recovery should affect both B actin and the genes of interest proportionally, thus, B actin was used to normalize the mRNA expression for the tested promoters.

cSurvivin expression was not detectable in PBMCs, lung, pancreas, intestine, and one primary lymphoma (Suzzy). Low levels of expression were observed in NCF, kidney, heart, liver, CML7, 17-71, DH82, and four primary lymphomas (Zach, Oliver, Allie Bear, Truffy), ranging from 0.1% to 2.2% of beta-actin. Moderate levels of expression were observed in OSW and seven primary lymphoma samples (Phumba, Golda, Doggy, Bitsi, Jack, Roxann, Nala), ranging from 2.75% to 4.1%. The highest levels of expression were observed in CML10, CMT28, CMT12 and MDCK, varying from 4.7% to 10.4% (**Fig. 4.2A**).

cCXCR4 mRNA expression was undetectable in NCF, FDK, MDCK, DH82, normal lungs, liver, pancreas, and intestine. Low expression, ranging from barely detectable (0.05%) to 2.4% was seen in PBMCs, heart, kidney, CML7, CML10, CMT12, CMT28, and 17-71. Low level expression was also seen in lymphoma tissues (Doggy,

Golda, Roxann, Allie bear, Phumba, Jack, Bitsi, Truffy, Oliver), with the highest expression in this group reaching 3.1%. OSW (15%) and lymphoma tissue (Zach; 12.9%) showed clearly elevated expression levels (**Fig. 4.2B**).

Table 4.5: qRT-PCR CT (Cycle Threshold) values of B actin

Cell/Tissues	CT values of B-Actin				
	Exp1	Exp2	Exp3	Mean	Std. Error
NCF	21.36	21.41	21.42	21.40	0.019
PBMC	30.22	26.28	30.14	28.88	1.300
CML7	22.59	27.15	27.35	25.70	1.554
CML10	21.32	21.27	21.7	21.43	0.136
CMT28	27.07	26.06	25.9	26.34	0.366
CMT12	22.00	22.13	22.2	22.11	0.059
FDK	29.57	30.92	33.19	31.23	1.056
MDCK	25.72	26.09	25.67	25.83	0.132
DH82	29.58	29.7	31.22	30.17	0.528
OSW	24.96	29.25	30.2	28.14	1.612
17-71	23.59	28.18	30.59	27.45	2.053
Lung	36.43	31.23	33.71	33.79	1.502
Liver	32.58	31.31	34.79	32.89	1.017
Heart	29.09	27.63	29.3	28.67	0.525
Pancreas	34.14	31.7	35.68	33.84	1.159
Intestine	31.68	33.36	33.59	32.88	0.602
kidney	31.91	30.67	33.41	32.00	0.792
Allie Bear	28.19	27.14	28.01	27.78	0.324
Doggy	31.09	31.31	30.76	31.05	0.160
Nala	31.04	32.2	28.88	30.71	0.973
Zach	29.5	30.96	24.71	28.39	1.888
Jack	27.46	28.7	24.61	26.92	1.211
Roxann	25.7	33	29.4	29.37	2.107
Bitsi	27.46	31.58	28.22	29.09	1.266
Golda	29.23	33.77	27.17	30.06	1.950
Trufyy	20.74	34.18	26	26.97	3.910
Suzzy	34.69	34.95	34.42	34.69	0.153
Phumba	29.2	30.73	30.21	30.05	0.449
Oliver	25.53	28.28	29.12	27.64	1.084

ND= Not Detectable; Exp= Experiment; Std. Error = Standard error of mean

cTERT levels were 1 to 2 orders of magnitude lower than either cSurvivin or cCXCR4 in all tissues studied. cTERT was undetectable in most of the normal cells/tissues including NCF, PBMCs, lungs, liver, heart, pancreas, intestine and kidney. It was also undetectable in some cancer cell lines and primary lymphoma tissues including CML7, FDK, OSW, Nala, Zach, Truffy, Suzy, Bitsi, and Phumba. Low levels of expression were seen in CML10, CMT28, CMT12, and MDCK, and primary lymphoma tissue samples from Allie bear, Doggy, Jack, Roxann, and Golda. The highest expression of cTERT was seen in 17-71 (0.5%) (**Fig. 4.2C**).

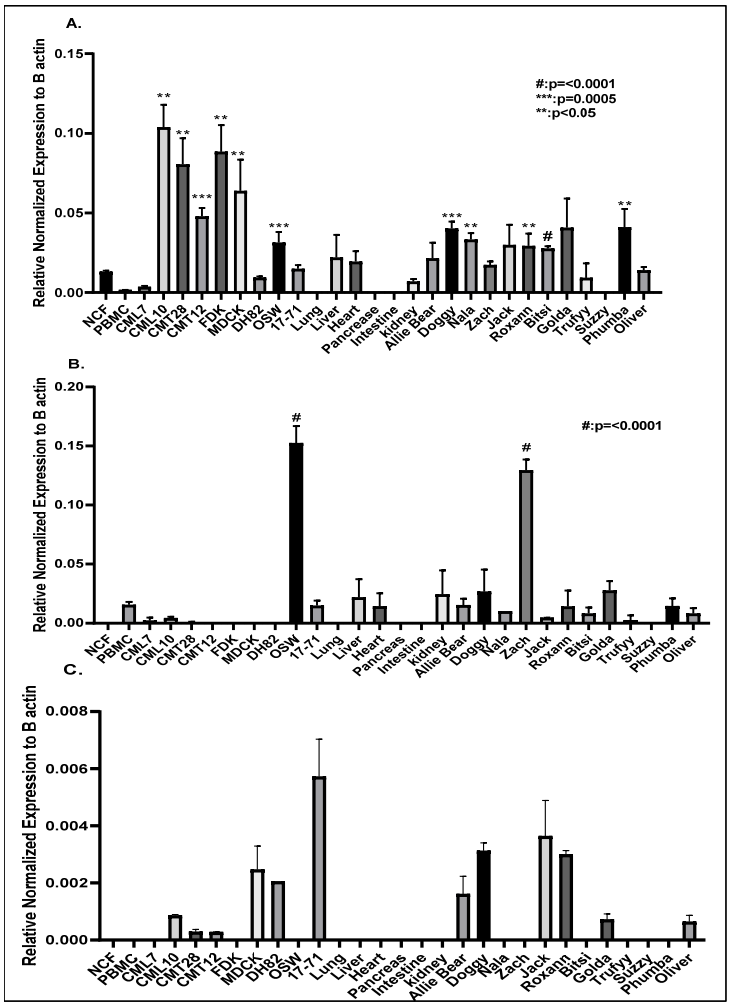

Figure 4.2: Quantification of endogenous mRNA expression

Quantification of endogenous mRNA expression of (A) cSurvivin, (B) cCXCR4 and (C) cTERT in normal canine cell lines /tissues and canine tumor cell lines/tissues using quantitative RT-PCR. Gene expression was normalized to β-actin (ΔC_t method). Bar graphs are representative of mean and SEM of three independent experiments. Two tailed Student's T test was conducted by using GraphPad prism statistical software. The p-values represent comparisons with (A) NCF and (B) PBMC. No normal cells expressed cTERT and therefore statistical significance could not be calculated for Fig. 4.2C.

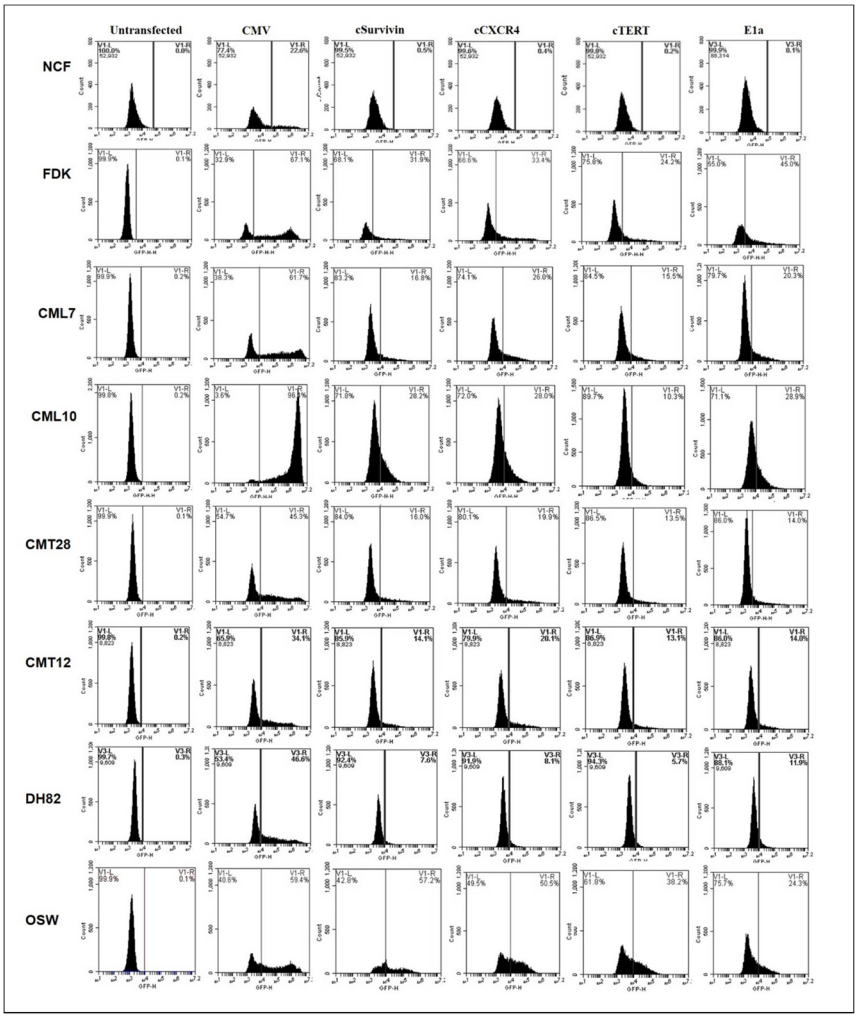

Figure 4.3: Flow cytometry analysis of exogenous promoter activity

Analysis of exogenous promoter activity in primary cancer cells and cell lines

The activity of exogenously provided cSurvivin, cCXCR4, cTERT, and EEE promoters was evaluated in normal and cancer cells. Reporter plasmids expressing GFP, driven by CMV (positive control), cSurvivin, cCXCR4, cTERT, or EEE promoters were transfected into NCF, FDK, CML7, CML10, CMT12, CMT28, DH82, and OSW cells. GFP expression was quantified by flow cytometry 48hrs after transfection. (**Fig. 4.3**). Flow cytometry provided results for both the number of cells expressing the experimental promoter and the intensity of the expression within a given cell. These parameters were analyzed independently.

Table 4.6: Percent cells expressing GFP

	cSurvivin	**cCXCR4**	**cTERT**	**EEE**
NCF	1.3	1.4	0.34	0.30
FDK	46.3	48.0	33.1	65.4
CML7	34.0	48.9	27.6	28.0
CML10	38.0	40.6	16.6	39.2
CMT12	42.2	54.6	43.8	38.0
CMT28	34.7	41.0	29.3	26.7
DH82	17.2	18.4	11.7	24.3
OSW	101.2	76.0	55.1	37.2

The percent of cells expressing GFP for each cell line are ranked by color as follows: Red > Yellow >Dark Green > Light Green. Color code changed if the difference is >0.3

In order to analyze differences in the number of cells expressing the GFP reporter from the experimental promoter, it was necessary to control for transfection efficiency.

Given the high level and ubiquitous nature of CMV immediate early promoter expression, the expression of GFP from this promoter was used to measure transfection efficiency [251, 403]. The number of cells expressing GFP from the experimental promoter was then normalized to the number of cells expressing CMV driven GFP in that cell line (**Fig. 4.4A, Table 4.6**). In NCF, all four promoters showed the fewest cells expressing GFP, relative to CMV. EEE activity was lowest at 0.30%, cTERT was slightly higher at 0.34%, and cSurvivin and cCXCR4 were the highest at 1.3% and 1.4% respectively. The number of cells showing promoter activity was notably increased in the remaining cells lines. The lowest expression was cTERT in DH82 cells at 11.7%. The highest percentage of cells expressing GFP occurred with the cSurvivin promoter in OSW cells (101.2%). The second highest percentage was cCXCR4 in OSW cells (76%). The third highest expression level was the EEE promoter in FDK cells (65.4%). The fourth highest level of expression was the cTERT promoter in OSW cells (55.1%) **(Fig. 4.4A, Table 4.6)**.

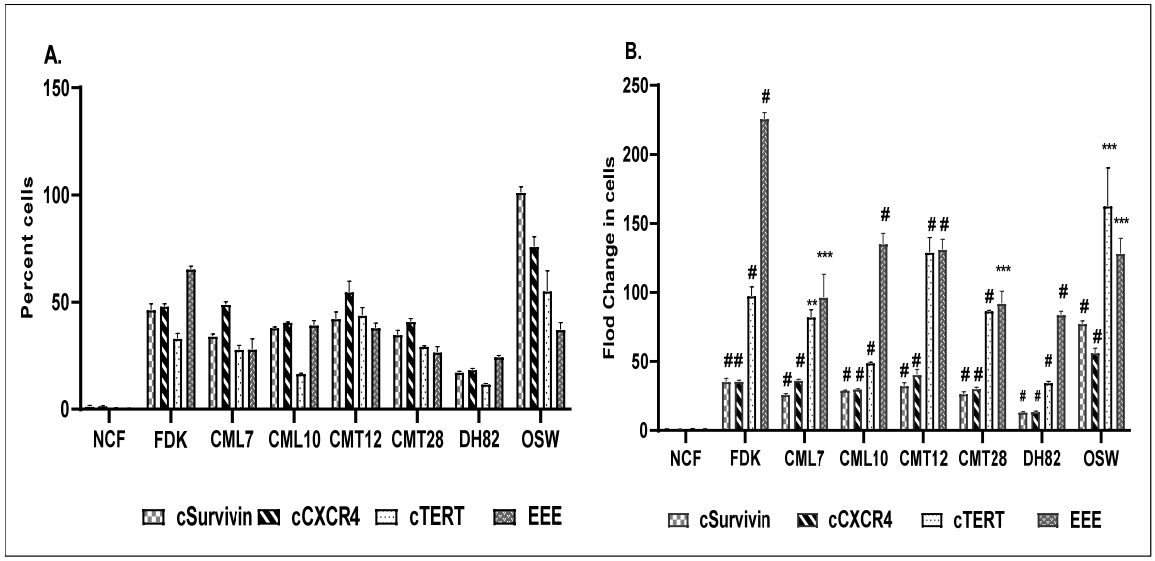

Figure 4.4: Quantification of the number of cell expressing GFP

Quantification of the number of cell expressing GFP normalized to either A) CMV-GFP expression or B) NCF to compare the significance of the observed variation in promoter activity. Bar graphs are representative of mean and SEM of three independent experiments. Two tailed Student's T test was conducted using GraphPad prism statistical software. P-values represent comparisons with NCF (B) and are represented as # ($p<0.001$), * ($p<0.005$), and ** ($p< 0.05$).**

In general, OSW cells had provided the highest or nearly the highest number of cells expressing GFP from the experimental promoters, while DH82 cells had provided the lowest number of cells expressing GFP from the experimental promoters when tumor lines were compared. Within each cell type, the promoters showed different relative activities. For example, promoter activity in OSW, as ranked from highest to lowest was: cSurvivin, cCXCR4, cTERT, and EEE; whereas CML10 activities were ordered: cCXCR4, EEE, cSurvivin, cTERT. **Table 4.6**, where the order of activity of each promoter is color-coded within each cell line, demonstrates that cCXCR4 has the highest activity in 5 cell lines and second highest in three others, while cSurvivin has the highest activity in two cell lines and the second highest activity in two other lines **(Figure 4.4A, Table 4.6)**.

To compare the activities (fold difference) of promoters between cancer cell lines and normal cells (NCF), an important measure of the specificity of the promoter for cancer cell gene expression, the CMV normalized expression values were normalized to values for NCF (**Fig. 4.4B, Table 4.7**). Among all of the tested promoters, EEE showed the highest relative activity, ranging from 83.8 to 225 times higher in cancer cell lines than in NCF. The EEE promoter generated the highest amount of relative expression in every cell line except OSW, where it was the second most active, following cTERT. cTERT was

second most active in all of the cell lines except OSW. cSurvivin and cCXCR4 expression levels were very similar, with cSurvivin slightly higher than cCXCR4 in 6 lines and lower than cCXC4 in one line (OSW). However, expression levels of both of these promoters were well below that of EEE and cTERT.

Analysis of promoter strength by measuring mean fluorescence intensity (MFI)

In order to gather quantitative information regarding promoter strength, we analyzed MFI from flow cytometry to estimate the amount of GFP produced in each transfected cell by each promoter.

Table 4.7: Fold GFP expression

	cSurvivin	p-Value	cCXCR4	p-Value	cTERT	p-Value	EEE	p-Value
NCF	1.0		1.0		1.0		1.0	
FDK	35.4	p=0.0001	35.5	p<0.0001	97.5	p=0.0001	225	p<0.0001
CML7	26.0	p<0.0001	36.2	p<0.0001	82.2	p=0.0001	96.5	p=0.0046
CML10	29.0	p<0.0001	30.0	p<0.0001	48.9	p<0.0001	135.1	p<0.0001
CMT12	32.5	p=0.0002	40.4	p=0.0004	129.0	p=0.0003	131.0	p<0.0001
CMT28	26.5	p=0.0001	30.3	p<0.0001	86.5	p<0.0001	92.0	p=0.0005
DH82	13.1	p<0.0001	13.6	p<0.0001	34.5	p<0.0001	83.8	p<0.0001
OSW	77.4	p<0.0001	56.1	p<0.0001	162.0	p=0.004	128	p=0.0003

The percent of cells expressing GFP for each cell line are ranked by color as follows: Red > Yellow >Dark Green > Light Green. Color code changed if the difference is >0.3

When the data from flow cytometry is examined (**Fig. 4.3)**, the maximum fluorescence intensity with the CMV promoter was fairly uniform in all cell types, however, the MFI varied between the cells/cell lines. Thus, while each cell type is capable of providing the same maximum intensity, the MFI varies based transfection efficiency. Low transfection efficiency results in less plasmid copy number within the target cells leading to variability in the MFI as observed for the tested cell lines. In order to compensate for the observed variability resulting from variation in transfection efficiency, the MFI

from each promoter was normalized to MFI for CMV-GFP expression in that cell line to directly compare the promoter strength within cell lines (**Fig. 4.5A**).

None of the promoters achieved levels of GFP expression seen with CMV. Significant variation in promoter strength was observed. Higher levels of expression for all 4 promoters were seen in NCF than in CML7, CML10, and DH82. CML10 showed the lowest fluorescent intensities, ranging from 1.0% to 1.9%. CMT12, CMT28 and OSW showed the highest fluorescent intensities with a range of 12.9% to 37.0%. cSurvivin showed a moderate level of strength in NCF, DH82, FDK, and CML7 ranging from 7.3% to 14.9% MFI (**Fig. 4.5A, Table 4.8**).

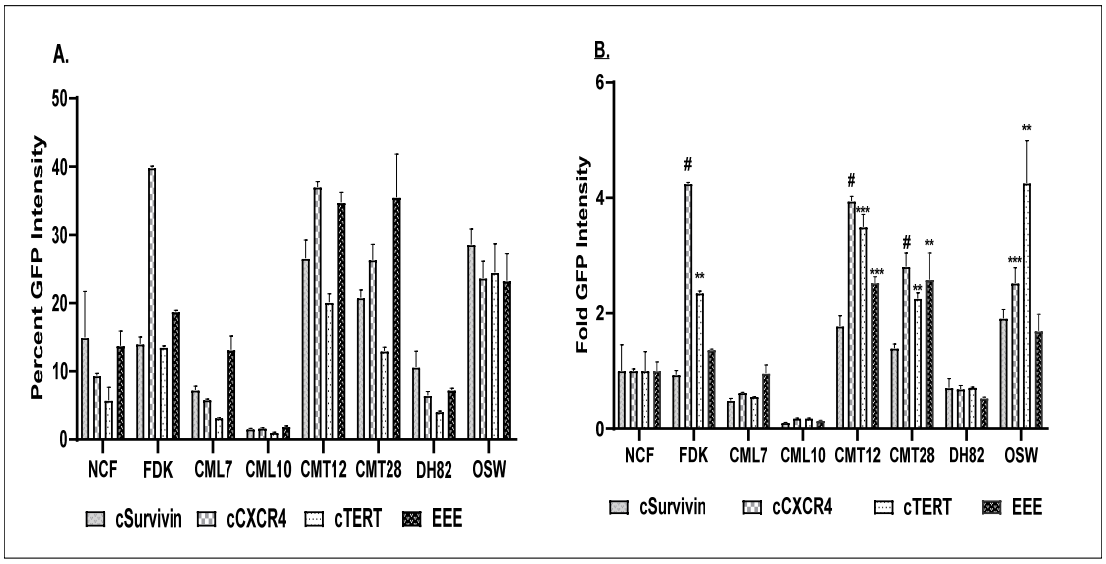

Figure 4.5: Quantification of mean fluorescent intensity (MFI)

Quantification of mean fluorescent intensity (MFI) normalized to either A) CMV-GFP expression or B) NCF to compare the significance of the observed variation in promoter the strength. Bar graphs are representative of mean and SEM of three independent experiments. Two tailed Student's T test was conducted by using

GraphPad prism statistical software. P-values represent comparisons with NCF (B) and are represented as # ($p \leq 0.0001$), * ($p < 0.005$), and ** ($p < 0.05$).**

cCXCR4 was the most active promoter, displaying 39.8% of the activity of CMV in FDK and slightly lower levels in CMT12, CMT28, and OSW (23.6% to 37%). cCXCR4 showed moderate strength in NCF at 3.4%. In contrast, cCXCR4 showed low strength in DH82, CML7, and CML10 (1.7% to 6.4% MFI). cTERT showed low strength in NCF, DH82, CML7, and CML10 (1.0% to 5.7%) and significantly higher levels of strength in FDK, CMT12, CMT28, and OSW (13.0% to 24.4%). EEE showed moderate strength in NCF, FDK, and CML7 (13.14% to 18.76%), and a high level of strength in CMT12, CMT28 and OSW (34.7%, 35.4%, 23.2%). The EEE promoter showed low levels of strength in CML10 and DH82 (1.92% and 7.2%) (**Table 4.8**).

When the order of activity of each promoter was color coded within each cell line (**Table 4.8**), several generalities about expression were seen. The cTERT promoter is almost uniformly the weakest, placing last in every cell line but one, OSW, where it was second strongest.

The remaining 3 promoters are each strongest in 2 or 3 cells lines, with a slight overall advantage in strength going to the EEE promoter, which was strongest in 3 cells lines (CML7, CML10, CMT28) and second strongest in an additional 4 cell lines (NCF, FDK, CMT12, DH82) .

Table 4.8: Promoter strength measured by MFI

	cSurvivin	cCXCR4	cTERT	EEE
NCF	14.9	9.4	5.7	13.7
FDK	14.0	39.8	13.5	18.8
CML7	7.3	5.2	3.2	13.1
CML10	1.6	1.7	1.0	1.9
CMT12	26.6	37.0	20.1	34.7
CMT28	20.6	26.4	12.8	35.5
DH82	10.6	6.5	4.1	7.3
OSW	28.5	23.6	24.4	23.2

The promoter strength for each cell line is ranked by color as follows: Red > Yellow >Dark Green > Light Green. Color code changed if the difference is >0.3

In order to compare the strength of the tested promoter in cancer cells to the strength of the same promoter in NCF, the normalized MFI for each promoter in each cell line was normalized to NCF. This comparison allows the relative expression in cancer versus normal to be determined, giving an indication of the potential specificity of expression. These values are relative and not absolute, thus it is understood that a promoter expressing at very high levels in a tumor cell, with moderate background in normal cells, may have a lower relative expression value than a promoter expressing at moderate levels in tumor and extremely low levels in normal cells. Three cell lines, CML7, CML10, and DH82, failed to achieve the same level of expression as NCF with any of the promoters. The remaining cell lines showed increased expression for most or all of the tested promoters, but the pattern was highly variable (**Fig. 4.5B, Table 4.9**).

Table 4.9: CMV-GFP expression-normalized MFI normalized to NCF

	cSurvivin	p-Value	cCXCR4	p-Value	cTERT	p-Value	EEE	p-Value
NCF	1.0		1.0		1.0		1.0	
FDK	0.936	p=0.895	4.24	p<0.0001	2.30	p=0.015	1.30	p=0.080
CML7	0.48	p=0.321	0.621	p=0.0004*	0.550	p=0.249	0.950	p=0.850
CML10	0.100	p=0.118	0.180	p<0.0001*	0.170	p=0.068	0.130	p=0.005*
CMT12	1.77	p=0.186	3.940	p<0.0001	3.49	p=0.003	2.52	p=0.001
CMT28	1.390	p=0.441	2.80	p=0.0017	2.250	p=0.022	2.580	p=0.031
DH82	0.700	p=0.577	0.680	p=0.011	0.710	p=0.437	0.520	p=0.040
OSW	1.910	p=0.118	2.52	p=0.004	4.250	p=0.015	1.690	p=0.103

The MFI normalized to NCF for each cell line is ranked by color as follows: Red > Yellow >Dark Green > Light Green
**: strength of promoter in this cell line is significantly less than that of NCF. Color code changed if the difference is >0.3*

cSurvivin failed to show a statistically significant increase in the intensity of GFP expression compared to NCF in any of the cell lines tested. cCXCR4 showed the highest increase of 4.24-fold in FDK and also showed significant increases over NCF in CMT12, CMT28, and OSW [3.9 ($p<0.0001$), 2.8 ($p<0.0001$), 2.5 ($p<0.0001$) respectively]. cTERT showed significantly enhanced GFP expression in OSW and CMT12 of 4.25 ($p<0.0001$,) and 3.4 ($p=0.0004$), respectively. EEE showed statistically significant increased GFP expression in two cell lines, CMT12 and CMT28, with 2.5 ($p=0.0020$) and 2.8 ($p=0.0014$) times higher MFI than NCF, respectively. The remaining cell lines, OSW, FDK, CML7, DH82 and CML10, failed to show any significant increase in expression with EEE over that seen in NCF.

4.4 Discussion

Utilization of transcriptional targeting could improve the safety and efficacy of cancer gene therapy approaches. The identification of suitable tumor-upregulated promoters is a prerequisite for this approach. Although several tumor-upregulated promoters have been utilized for human cancer gene therapy, none have been tested in

canine tumors [394, 395, 397, 404-406]. Given that many canine tumors are excellent models of their human counterparts, evaluation of these promoters in canine cells would provide valuable comparative information. In the present study, we selected the 5' upstream region of the cSurvivin, cCXCR4 and cTERT genes, based on their tumor-upregulated activity in human cancer. The endogenous activity of these promoters was assessed by Q-RT-PCR. The exogenous activity of these promoters, as well as a modified CAV2 E1A promoter (EEE) was evaluated using a GFP reporter gene.

Endogenous mRNA expression provides an estimate of activity of promoters in cells. In this regard, we measured mRNA expression levels of cSurvivin, cCXCR4 and cTERT genes (**Fig. 4.2**). All the promoters had very low levels of mRNA expression in normal cells and tissues. Among the tested genes, survivin showed the highest level of endogenous activity. Elevated survivin expression in human and murine melanoma, mammary cancer, and lymphoma have been previously reported [394, 395, 404, 407]. We have observed similar expression levels in canine cell lines. There was a moderate level of mRNA expression in one lymphoma line, and high levels of mRNA expression in one melanoma and two mammary tumor cell lines. Our normal tissue results are congruent with human and mouse data where low or no survivin transcript was observed in lung, liver, heart, pancreas and kidney etc. This is in contrast to a report of high levels of survivin mRNA in various normal dog tissues [408].

Elevated expression of CXCR4 has been reported in human mammary cancer, melanoma and B cell non-Hodgkin's lymphoma (NHL) [409-412]. However, we found little or no expression of cCXCR4 mRNA in most of the normal and cancer samples, including a B cell lymphoma cell line (17-71). CXCR4 mRNA overexpression has been

reported in T-cell NHLs [413, 414]. Likewise, high levels of CXCR4 mRNA expression were found in OSW and Zach, which are both T cell lymphomas, but no expression was seen in canine B cell lymphomas. cTERT showed the lowest level of endogenous mRNA expression in all of the cell lines and tissues, with maximal activity being one to two orders of magnitude lower than cSurvivin or CXCR4, indicating that TERT transcriptional activity is low in all canine tumors and tissues. Relative to NCF, cTERT expression increased in cell lines CML10, MDCK, DH82, and 17-71, as well as in primary lymphoma tissues from 6 of 12 dogs.

Based on these findings, cSurvivin gene expression was most consistently elevated in tumor samples, indicating that it might be a good choice for therapeutic applications. However, based on the endogenous expression data in normal cells and tissues, there could be significant off-target expression. The activity of endogenous cCXCR4 and cTERT appear to be limited to a few tumor types. The low level of endogenous expression of these genes relative to beta-actin also raises concern for their therapeutic utility. However, typical gene therapy applications rely on the use of exogenously provided promoter constructs. The alteration of chromosomal context combined with the selection of specific portions of the promoter might provide different expression profiles. For this reason, the three endogenous promoters, along with the modified CAV2 E1A promoter, EEE, were tested for their expression levels when provided exogenously.

The use of flow cytometry to assess exogenous expression allows parameters of expression to be evaluated, including the number of cells expressing the construct and the intensity of that expression in individual cells. The former indicates the breadth of

expression while the latter indicates its strength. The number of cells expressing the exogenous constructs was elevated for all of the promoters in all cancer cell lines tested when compared to NCF (**Fig. 4.4, Table 4.6**). The EEE promoter was active in a higher percentage of cells in the tumor for most lines, with the cTERT promoter performing second best. When the intensity of expression was evaluated, expression levels appear to correlate more with cell lines than with specific promoters (**Fig. 4.5, Table 4.7)**. For example, expression of all four promoters was elevated above that of NCF in both mammary carcinoma lines (CMT12, CMT28) and a T-cell lymphoma (OSW), while the intensity of expression was at or below the level of NCF in both melanoma lines (CML7, CML10) and the histiocytoma line, DH82. In cells with elevated fluorescent intensity, cCXCR4 had the greatest intensity in 3 lines and cTERT was highest in the 4th. It must be remembered that when comparing the MFI, very small numbers of NCF are being compared to much larger numbers of the other cells.

The immortalized fetal dog kidney (FDK) was ostensibly included as a second normal control, however, FDK showed increased endogenous expression with the survivin promoter. When exogenous expression was examined in FDK, the number of cells expressing GFP was elevated with all 4 promoters. When the intensity of expression was examined, FDK showed a range of increased expression over NCF, from no increase with cSurvivin to a moderate increase with cTERT and EEE, to a more than 4-fold increase with cCXCR4. Immortalization of FDK with SV40 Large-T antigen has likely resulted in FDK more closely resembling tumor cells than normal fibroblasts. Although, the fetal origin of FDK might also contribute to this phenotype

Comparison of the number of cells expressing the exogenous promoters to the level of expression with those promoters shows that these metrics are not well correlated across the panel of tumor cells tested. In some cases, such as CML7, CML10, and DH82, the tumor cells showed an increased number of cells expressing lower levels of GFP than are seen in normal fibroblasts. The low level of expression of the reporter in these tumor cells raises the concern that the use of these promoters might result in unacceptable side effects in normal cells. In contradistinction, in CMT12, CMT28, and OSW, both the number of cells and the intensity of fluorescence were elevated for all four promoters, indicating that more cells express these promoters and at a higher level than in normal canine fibroblasts, making these promoters potentially attractive for therapy in tumor cells of this type.

In this study, the mRNA level observed for endogenous expression differed from the high activity observed with exogenous expression from the same promoters. The data from the endogenous studies reflects the efficiency with which the chromosomal promoters mediate gene expression. Inside the cell, endogenous promoters maintain their own chromatin structure and are controlled by a complex network of regulatory elements such as transcription factors, activators and mediators. Regulation of mRNA levels in a cancer cell may be the outcome of multiple events such as mutation, chromatin remodeling, epigenetic modifications, and microRNA activity. In contrast, exogenously supplied promoters are putative promoter fragments cloned into a plasmid bearing reporter genes that lack the native chromatin structure and genomic context. As a consequence, mRNA and/or protein levels produced from the endogenous genes may not correlate with the exogenous activity derived from the same promoter in the same cell. In addition, positive

feed-back loops may also play an important role during exogenous activity promoting new protein to act as an activator resulting in high activity of the promoter [415-418].

Our primary objective was to find a tumor upregulated promoter that could be used as a transcriptional targeting tool for all types of cancer. Our data indicates that none of the promoters tested completely satisfy this requirement. Endogenous expression from cTERT, cSurvivin, and cCXCR4 was low, and increased expression was only seen in some cell types. Exogenously provided promoters were ubiquitous in their expression in more tumor cells than normal cells, but expression levels varied, with some being expressed at much lower intensities than in normal cells. Thus, it is clear that, among the promoters and cells studied, there is no “magic bullet” promoter that can be used in all, or even most cancers. The use of specific promoters will require individualized testing in a precision medicine-based approach. Even that approach has difficulties, as these results indicate that precision approaches such as transcriptome sequencing may seriously underestimate both the promoters that might be active, if provided exogenously, and the level of expression that could be expected from those constructs.

CHAPTER 5

Conclusions and Future Directions

Gene therapy is a promising modality, encompassing a diverse set of therapeutic approaches that deliver therapeutic genes to modify or kill abnormal cells [328]. A subset of gene therapy, oncolytic virotherapy, which is the use of conditionally replicative adenoviruses to selectively eradicate cancer cells while sparing normal cells, is a promising technique. Among many advantages, the self-perpetuating nature of replicative adenoviruses make them an ideal vector to be used in a variety of monogenic diseases and cancers [298, 419]. Adenoviruses are an attractive vector systems to use for oncolytic virotherapies due to their high transduction efficiencies in both dividing and quiescent cells, low risk for insertional mutagenesis, and robust gene expression [329, 420]. However, several genetic modifications at the transductional and transcriptional level are necessary to generate a successful tumor-targeted conditionally replicating adenoviral vector (CRAd). Transductional targeting will facilitate the utilization of cancer-cell specific cell surface receptors to allow viral entry in the target cell by bypassing the primary cellular receptors utilized by virus which may either be overexpressed in normal cells or may be absent in the target cell. Transcriptional targeting will control viral replication at the level of transcription by regulating essential viral genes under the control of transcriptional regulatory sequences that are upregulated in tumor cells but not in normal cells. Thus, both transductional and transcriptional targeting approaches can work collaboratively to enhance the tumor specificity of oncolytic virotherapy by complementing each other and can reduce normal cell toxicities.

Despite promising new approaches that have been developed to generate successful adenoviral vectors by employing transductional and transcriptional targeting, successful application to adenoviral vectors are still limited. Thus, new transductional and transcriptional modification strategies need to be developed to enhance the potency of adenovirus mediated oncolytic virotherapy. This is particularly true for disseminated tumors, including malignant cells of lymphocyte origin. Until recently, most studies have been conducted utilizing human adenoviruses. These have shown enhanced liver tropism as well as rapid viral clearance by preexisting immunities, resulting in low therapeutic efficacy after administration. One way to circumvent the limitations of human adenovirus is to use non-human adenoviruses such as CAV2. These viruses show promise as they have many of the same properties as Human Ad, without pre-existing immunity. They may also be used to treat dogs with cancer, both in the capacity of models of human disease, as well as for the dog's benefit. However, few attempts have been made to improve the transductional and transcriptional targeting abilities of CAV2. In addition, the conventional approach to the genetic modification of CAV2 is time consuming, inefficient and, thus, also needs improvement. In this regard, this dissertation attempts to develop new and improved strategies to facilitate transductional and transcriptional targeting of CAV2, leading to enhancement of CAV2 mediated oncolytic virotherapy in disseminated tumor such as hematologic malignancies, while utilizing a selective, less-time consuming and effective approach to make these vectors.

In Chapter 2, *In vitro* CRISPR/Cas9-mediated insertional mutagenesis was used to develop a recombinant CAV2 vector containing CMV-DsRed inserted between the fiber and E4 region (Cav2-AU-M1). This work successfully demonstrated that functional

recombinant CAV2 vector could be generated without the need for homologous recombination, shuttle plasmids or the presence of unique restriction enzyme sites. This technique allows the generation of functional virus in a less-time consuming and less complex manner than previously, and free of contamination from any parental virus. In addition, the CRISPR/Cas9 modified CAV-AU-M1 is now available to be used as a parental vector for other modifications to promote transductional and transcriptional targeting. Moreover, CRISPR/Cas9 based modification can also be utilized, in the future, to engineer a diverse range of modifications to the CAV2 vector, more efficiently and rapidly. Subsequent to the application of CRISPR/Cas9 to insert a transgene construct into CAV2, two guide RNA's were utilized to "replace" a piece of CAV2 for a new insert. This removed most of CAV2 fiber and inserted novel targeting fiber-fibritin constructs as described in Chapter 3 (CAV2-AU-M2, CAV2-AU-M3). This work also implies that CRISPR/ Cas9 can be successfully used to modify other non-human adenoviruses such as bovine or avian adenoviruses.

Some challenges were encountered in the construction of new vectors by CRISPR/Cas9. While the ability to identify sites for guide RNAs vastly exceeds the availability of useful restriction sites, there are still locations where identification of suitable guide RNA sites is difficult. When trying to identify two sites, to allow for CAV2 genome segment replacement, this was amplified by the need to find two sites. This can be partially addressed by adjusting the overlap sequences upstream or downstream of the CRISPR/Cas9 cut sites. Additional issues include the failure of some insertion sites to produce viable virus or expression of the inserted cassette. This may be due to the interference of the insertion with some, as yet undescribed, component of packaging. It

was immediately clear that CAV is relative sensitivity to the size of its genome and care must be taken to limit added bases.

Successful adenovirus infection incorporates the initial binding of virus fiber knob to the cell surface coxsackie-adenovirus receptor (CAR) followed by secondary binding of an RGD motif in the penton protein with $\alpha_v\beta_{3/5}$ integrins on the cell surface. Both steps are prerequisites for successful viral internalization into the target cell. However, cells of lymphocyte origin lack CAR and integrin on their surface and, thus, adenoviruses fail to infect these cells, restricting adenoviruses utility as a gene delivery tool for these cells. Chapter 3 of this dissertation effectively addresses this need. A highly novel targeting approach that will overcome the natural barriers to adenovirus infection in lymphocytes has been developed. In an attempt to identify appropriate ligands, we have shown that IL2R is expressed on some lymphocytes and lymphomas. We also demonstrated that murine IL2 and IL2R sequences are evolutionarily more distant than human and canine IL-2/IL-2R whereas human and canine IL2 and IL2R are evolutionary close to each other. This context may explain why murine IL2 was unable to facilitate adenoviral infection of canine lymphoma cells. Since earlier work demonstrated the inability of human Ad5 virus to infect canine lymphoma cells, we tested CAV2 for its ability to infect these cells and found that infection status may vary based on the cell line tested. CAV2 infection was shown to be both CAR and integrin dependent and independent, the latter potentially utilizing one or more alternative cellular receptors. While IL2 targeted virus is likely to provide an excellent ligand receptor system for lymphoma, further studies are needed to completely explore the CAV2 infection riddle in cells of lymphocyte origin.

Having shown the potential of IL2/IL2R targeting, we designed an approach to use and generate fiber modified CAV2 vectors by replacing wild type fiber with interleukin 2 and/or single domain camelid antibodies (sdAb) to IL2R. These targeting moieties, cloned as fusion proteins with phage T4 fibritin and the basal portion of CAV2 fiber generated Fiber-Fibritin-IL2/sdAbIL2R (FF-IL2, FF-sdAbIL2R) chimeras where fibritin will retain the trimerization potential of the fiber shaft region. As noted above, CRISPR/Cas9 technology was utilized to conduct double digestion of CAV2 genome to replace the wild type fiber. In congruence with the data from first chapter, the utilization of CRISPR/Cas9 allowed quick and efficient replacement of CAV2 wild type fiber to generate the pCAV-AU-M2 and pCAV2-AU-M3 vectors. This process provided seamless insertion of the desired insert without providing a single base pair insertion or deletion at the junction point. However, future studies are needed to evaluate the scope, feasibility, and success of this approach.

Although we have seen successful virus spread in the level of transfection, these vectors need to be tested to determine their ability to form infectious particles. As fiber modified virus lacks native fiber, making of this virus requires its assembly be conducted in a wild type fiber expressing cell. This approach will generate chimeric viruses containing both native and modified fiber. Generation of purified fiber modified virus, lacking native fiber, requires several rounds of amplification in wild type fiber expressing cells followed by infection into wild type DKCre cells. This final infection would lead to the production of pure fiber modified virus. In addition, in this study, we only utilized one sdAb to retarget CAV2 vector. Thus, in future, up to 5 different CAV2-anti-cIL2Rα-sdAb-DsRed viruses will be constructed using CRISPR/Cas9 technology.

Once the virus is amplified, additional experiments should be conducted to analyze the functionality of the fiber modified virus. In order to analyze the target binding and internalization capability of the fiber modified virus future studies will employ the murine T-cell line, CTLL-2. CTTL-2 cells depend on IL2R signaling for survival and proliferation and also expresses high levels of IL2R [354]. Canine IL2 can also activate the IL2R signaling cascade in CTLL-2 [421]. Thus, this cell line is an ideal tool for analyzing the transductional targeting capacity of IL2/IL2R retargeted CAV2. CTLL-2 cells can be transduced with fiber retargeted CAV2-AU-M1/M2 and with wild type fiber (CAV2-AU-M1) (negative control) with increasing multiplicities of infectious (MOI) of viral particles (1, 10, 100, 1000). DsRed expression will be measured by flow cytometry 24 hours post-infection. Additionally, canine lymphoma cell lines OSW, 17-71, CLBL-1 and CL-1, which we have shown to be IL2Rα positive by RT-PCR, would also be infected with retargeted virus and DsRed expression would be measured by flow cytometry and quantitative PCR (qPCR). Further validation of IL2/IL2R mediated retargeting of CAV-2 would be conducted by blocking experiments with anti-canine IL2 antibody and anti-canine IL2Rα antibody (R&D Systems), as well as sdAbs expressed from the same cloned sdAbs used to make the vectors. CAV2-AU-M1, CAV2-AU-M2/M3 and up to 5 different CAV2-anti-cIL2Rα-sdAb-DsRed viruses will be compared.

In addition, the transduction potential of retargeted CAV2 in canine peripheral blood mononuclear cells (PBMCs) would also be tested. In order to achieve this goal, we will isolate fresh canine PBMCs from dogs, which are enriched in lymphocytes such as T-cells and B-cells. Isolated PBMCs will then be infected with untargeted and retargeted CAV2 with increasing MOIs. Flow cytometry will be used to measure GFP expression in

lymphocyte subsets such as helper, cytotoxic and regulatory T-cells using canine anti-CD4 (R&D system), anti-CD8 (Bio-Rad) and anti-Foxp3 antibodies (ThermoFisher) as well as in B cells with anti-CD20 antibody (InvivoGen).

Systemic administration of IL2 for therapeutic purposes has the potential to induce inflammatory cytokine releases, or "storms" that may generate adverse side effects or vascular leak syndrome (VLS) [422, 423]. Thus, experiments would be conducted to investigate the potential of generating cytokine storms following IL2R signaling cascade activation. In this aspect, the cytokine profile of lymphoma cells and PBMCs infected with retargeted and wild type CAV2 will be measured by multiplex ELISAs in a *Luminex MAGPIX*® system (R&D).

Apart from *in vitro* studies, the transductional potential of retargeted CAV2 will be assessed *in vivo* in murine xenografts. In order to analyze whether retargeted virus will infect IL2R expressing lymphocytes *in vivo*, we will assay viral genome magnitudes in non-tumor sites in *in vivo*. 1 x 10^6 17-71 or OSW cells will be implanted subcutaneously into 6-8 week-old SCID mice. After the xenograft tumor becomes palpable, 1 x 10^9, 5 x 10^9 or 1 x 10^{10} VP of virus containing IL2, anti-IL2R sdAb, or wild type fiber will be intravenously injected. Virion localization to tumor and non-tumor sites will be assessed by using in vivo fluorescent imaging (IVIS Lumina XRM). Virion localization to tumor and non-tumor sites will be also assessed by qPCR of harvested organs. Anti-tumor efficacy of retargeted CAV2 will also be analyzed by measuring tumor size after virus infection. Mice reaching their humane endpoint will be euthanized by CO2 asphyxiation

according to institutional regulations. The average survival time (days ± SD) will be calculated for each group.

Although, CAV2 fiber modification by utilizing the IL2/IL-2R signaling cascade potentiates a highly novel targeting approach to promote adenovirus transduction in lymphocytes, it also incorporates several contingencies that may need to be addressed to enhance success. Firstly, while CTLL2 cells have been described as binding canine IL2, CAV2-AU-M2 or CAV2-AU-M3 virus may not bind. As described, canine IL2R expressing cells will also be employed to measure virus infection. In addition, early investigations showed that hAd5 showed post-internalization defects. In the HSB-2 T cell line, Ad5 was internalized via an alternative clathrin-independent pathway leading to failure of Ad5 in escaping the endosomal compartment [331]. Interestingly, IL2 ligand upon binding to its cognate IL2R receptor has been reported to be internalized via a clathrin-independent pathway and this may generate post-internalization defect for IL2/IL2R retargeted CAV2 virus, including failure in endosomal escape [424]. In addition, IL2 targeted virus may generate potential adverse cytokine storms and in turn, unwanted side effects. Our sdAb retargeted viruses should not produce this side effect, although that would need to be tested to be sure. In the case of lymphoma therapy, Treg cells also express high levels of IL2Rα subunit and may be activated or infected with retargeted CAV2. Effector T-lymphocytes also express significant levels of IL2Rα and also have a chance to be activated after infection with retargeted viruses. These cells should not be killed by the virus as it should only replicate in tumors, further emphasizing the value of using multiple layers if targeting. If, however, the transcriptional targeting of these viruses is functional in activated lymphocytes, these cells may be vulnerable. The outcome may be determined

by the balance between activation and expansion of tumor-reactive effector T cells relative to Treg cells upon infection with retargeted virus. However, if these contingencies could be overcome then these fiber-modified CAV2 would have far greater significance, in that this approach can be translated to human patients and will allow adenoviral vectors to be successfully utilized for genetic therapy vector for a wide range of autoimmune and other diseases beyond hematopoietic malignancies.

Regarding transcriptional targeting, several tumor upregulated promoters (TUPs) have been tested for human cancers but very few have been tested for canine tumors. Chapter 4 of this dissertation involves a series of studies to measure the activity of three endogenous canine promoters, Survivin, CXCR4 and CTERT, in a panel of canine tumors. In addition to these endogenous promoters, a modified version of the canine adenoviral E1A promoter, containing four E2F binding sites and one Sp-I site (E2F enhanced E1A promoter, or EEE), which has previously shown upregulated activity in tumor cells, was also investigated for its tumor upregulated activity in canine tumors. Endogenous expression levels were measured for cTERT, cSurvivin, and cCXCR4 and were low for all three, with some non-malignant and some tumor cell lines and tissues expressing the gene. Expression levels from exogenously supplied promoters were measured by both the number of cells expressing the construct and the intensity of expression in individual cells. Exogenously supplied promoters were active in more cells in all tumor lines than in normal cells, with the EEE promoter being most active, followed by cTERT. The intensity of expression varied more with cell type than with specific promoters. Ultimately, no single promoter was identified that would result in reliable expression, regardless of the tumor type. Thus, these findings imply that identification of a pan-cancer promoter may be

difficult. In addition, this data raises the concern that endogenous expression analysis may not accurately predict exogenous promoter activity. In the future, these promoters will be utilized to generate CRAd where the replication of the virus is controlled in a tumor upregulated manner. In addition, these promoters would also be used to drive transgene expression in a tumor selective manner to enhance the potency of CRAd- mediated oncolytic virotherapy.

To sum up the research described in this dissertation, an alternative method to produce recombinant and functional CAV2 vector has been developed by employing the gene editing tool, CRISPR/Cas9 and successfully produced functional recombinant CAV2 vector. This is a significant step forward in the utilization of CAV2 viruses for novel applications. A novel, transductionally retargeted CAV2 vector employing a highly novel targeting approach to overcome the natural barriers to adenovirus infection in lymphocytes has also been developed. Finally, the current research has also identified potential canine TUPs that may promote tumor selective functionality of oncolytic viruses for canine tumors.

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