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Novel Application of ReiThera's Gorilla-Derived Adenoviruses (GRAds) as Versatile Platforms for Oncolytic Virotherapy: Advancing Selectivity and Payload Delivery.

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Abbreviations

%	Percentage
°C	Centigrade degrees
Δ24	Delta24 mutation, deletion of 24 bp in E1A protein
μg	Microgram
μL	Microliter
μM	Micromolar
μm	Micrometer
ψ	Viral packaging signal
ADCs	Antibody-drug conjugates
ADP	Adenovirus Death Protein
AdVs	Adenoviruses
Amp	Ampicillin
AmpR	Ampicillin resistance
ATCC	American Type Cell Culture
BAC	Bacterial Artificial Chromosome
Bgh-polyA	Bovine growth hormone polyadenylation signal
Bp	Base pairs
BSA	Bovine Serum Albumin
CAR	Coxsackievirus B and Adenovirus Receptor
CD8	Cluster of differentiation 8
ChAd	Chimpanzee adenovirus
Chl	Chloramphenicol
CO ₂	Carbon dioxide
CPE	Cytopathic effect
CR	Conserved Region
CRA _d	Conditionally Replicative Adenovirus
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DAMP	Damage-associated Molecular Pattern
DBP	DNA Binding Protein
ddH ₂ O	Double-distilled water

ddPCR	Droplet Digital PCR
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DSG2	Desmoglein-2
<i>E. coli</i>	<i>Escherichia coli</i>
ECGS	Endothelial Cell Growth Supplement
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FIX	Coagulation factor IX
Fk	Fiber knob
FL	Full-length
FX	Coagulation factor X
g	Acceleration of gravity
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GRAd	Gorilla-derived Adenovirus owned by ReiThera
HAdVs	Human Adenoviruses
hCMV	Human Cytomegalovirus promoter
HSPG	Heparan sulfate proteoglycans
HSV	Herpes Simplex Virus
hTERT	Human Telomerase Reverse Transcriptase
HVRs	Hypervariable region
ICD	Immunogenic Cell Death
IFN	Interferon
IL	Interleukin
IRES	Internal Ribosome Entry Site
ITRs	Inverted Terminal Repeats

Kbp	Kilobase pair
ITR	Inverted Terminal Repeat
M	Molar
mg	Milligram
MHC	Major Histocompatibility Complex
mL	Milliliter
MLP	Major Late Promoter
MLPU	Major Late Promoter Unit
mM	Millimolar
MOI	Multiplicity of Infection
Mock	Uninfected cells
mRNA	Messenger Ribonucleic Acid
Mu	Polypeptide X
N	Normal
nAb	Neutralizing antibody
NaCl	Sodium chloride
ng	Nanogram
NHP	Non Human Primates
nhpAds	Non human primates Adenoviruses
nm	Nanometer
NPM1	Nucleophosmin-1
NRG-1 β	Neoregulin-1
NSCLC	Non-small Cell Lung Cancer
OAdVs	Oncolytic Adenoviruses
OD	Optical Density
ORF	Open Reading Frame
OVs	Oncolytic viruses
pAkt	Phosphorylated Akt
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PEG	Polyethylene Glycol
pGRAd	Vectorized version of GRAd genome in BAC plasmid
pHER3	Phosphorylated HER3
PS	Penicillin-Streptomycin
pRb	Retinoblastoma protein
pTp	Pre-Terminal protein
Rb	Retinoblastoma
RGD	Arginine-glycine-aspartic acid
RNA	Ribonucleic Acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SacB	Gene encoding <i>B. subtilis</i> levansucrose
SAd	Simian adenovirus
SC	Single-chain
SDS	Sodium dodecyl sulfate
ssOligo	Single-stranded Oligo
TAA	Tumor-associated antigens
TAP	Transporter Associated to Antigen Processing
TME	Tumor Microenvironment
TP	Terminal Protein
VA	Virus-Associated
vg	Viral genome
vp	Viral particle

Abstract

Oncolytic virotherapy has emerged as a promising strategy for cancer treatment, utilizing genetically engineered viruses to selectively infect and destroy tumor cells while sparing normal tissues. Among adenoviral platforms, human adenovirus type 5 (HAd5) has been widely studied; however, its clinical application requires genetic modifications to mitigate preexisting immunity and off-target effects. To address these challenges, this thesis explores the potential of ReiThera's proprietary gorilla-derived adenoviruses (GRAd), particularly GRAdB and GRAdC, as alternative oncolytic agents. These vectors, derived from nonhuman primates (NHP), exhibit low seroprevalence in humans, thereby reducing the impact of preexisting immunity and offering unique biological properties that could enhance therapeutic efficacy.

This work characterizes the replication, tropism, and selectivity of GRAdB and GRAdC, highlighting their distinct oncolytic profiles in tumor and normal cells. GRAdB demonstrated strong cytopathic effects in lung and glioblastoma tumor cells but also exhibited significant replication in normal cells, underscoring the need for further safety assessment. In contrast, GRAdC displayed a narrower tropism, selectively targeting tumor cells while sparing normal tissues, making it a promising candidate for further development. The study also investigates strategies to improve GRAdB's selectivity through genetic modifications, such as deletions in the E1A and E3 regions, and to broaden GRAdC's tropism by developing a chimeric fiber construct, GRAdC FkB. This construct integrates the GRAdC fiber shaft and tail with the GRAdB knob, combining the beneficial properties of both GRAd serotypes. The resulting design successfully broadens tumor tropism without compromising selectivity.

A key innovation of this research is the demonstration of GRAd vectors as biofactories for therapeutic payloads. The anti-HER3 antibody EV20 was successfully expressed in its functional form in both FL and SC formats, providing proof of concept for integrating therapeutic genes into GRAd vectors. This dual-action strategy combines the virus's lytic activity with the therapeutic effects of the payload, potentially enhancing immune responses and improving treatment outcomes.

The findings presented in this thesis establish GRAd vectors, particularly their chimeric derivatives, as versatile and innovative tools for oncolytic virotherapy. By addressing the limitations of existing platforms and demonstrating novel applications, this work paves the

way for the development of safer and more effective therapies for challenging cancers, which can be further validated through in vivo experiments.

Introduction

1. Oncolytic virotherapy

Oncolytic virotherapy is an innovative form of immunotherapy that uses natural or genetically modified viruses to selectively target and destroy cancer cells, while sparing normal cells. For this reason, oncolytic viruses (OVs) are emerging as powerful tools in cancer treatment. These viruses operate by infecting and replicating specifically within cancerous cells, ultimately causing the cells to rupture (lysis) and releasing viral progeny that can then spread to adjacent tumor cells (Figure 1). This self-amplifying property, where the virus replicates selectively within the target tissue, is rare in pharmacology, making OVs a form of “self-replicating” therapy within the patient’s body.

OVs show potential for both systemic and direct tumor site administration, allowing them to impact primary and metastatic tumors. They attack tumors via two primary mechanisms: (1) directly killing cancer cells through virus-induced cell death, and (2) stimulating an immune response that targets both infected and uninfected tumor cells (Figure 1). By inducing immunogenic cell death in tumors, OVs release tumor-associated antigens (TAAs) and immune-activating signals, including damage-associated molecular patterns (DAMPs), viral pathogen-associated molecular patterns (PAMPs), and pro-inflammatory cytokines, all of which help mobilize the immune system against the cancer[1].

The origins of virotherapy date back to the early 1900s, when Dr. George Dock reported a case of a 42-year-old woman with “myelogenous leukemia” who went into remission following an assumed influenza infection[2]. This report, made in 1896—37 years before it was known that influenza was caused by a virus—described the woman’s greatly enlarged liver and spleen returning to nearly normal size and her elevated leukocyte count decreasing by over 70-fold post-infection. Additionally, since the mid-1800s, there have been continuous reports of tumor regression occurring alongside natural viral infections[2]. However, limited medical conditions, technology, and understanding of viral and tumor mechanisms at the time prevented viruses from revolutionizing cancer treatment, leading to a gradual stagnation in OV development.

To date, many OVs have entered early-phase clinical trials, and currently, four OVs have gained regulatory approval for cancer treatment: the oncolytic adenovirus ONYX-015 (Oncorine) for head and neck cancer in China[3]; Rigvir, an oncolytic reovirus approved for

advanced melanoma in several Eastern European countries[4]; T-Vec (IMLYGIC), a granulocyte macrophage colony-stimulating factor (GM-CSF) -expressing oncolytic herpes simplex virus (HSV), approved in the USA, Europe, and Australia for treating advanced melanoma[5]; and in 2021, Deltalyact (Tesperpatricev/G47 Δ), a modified HSV, received regulatory approval and entered the market in Japan[6].

2. Oncolytic adenoviruses

Among all OV, adenoviruses (AdVs) are the most widely used in oncolytic virotherapy. An analysis of the 130 clinical trials involving OV listed on clinicaltrials.gov (accessed on 1 October 2022) shows that the largest proportion (31%) uses AdVs, followed by HSV (28%) and Vaccinia (21%). This prevalence likely results from extensive research over the years, particularly on human adenoviruses (HAdVs), and from the intrinsic characteristics that make these viruses well-suited for gene therapy—both as replication-competent and replication-defective adenoviral vectors.

HAdVs are highly suited for virotherapy due to several key advantages. Their genetics are well-understood, with genomes that are easy to manipulate without compromising viral replication, enabling the incorporation of diverse, large transgenes [7]. Clinically, they are both safe and reliable, with well-documented safety profiles [9] and stable genomes that support consistent performance in therapeutic applications. HAdVs exhibit broad cell tropism, infecting a wide range of cell types, including professional antigen-presenting cells. This enhances antigen presentation to T-cells, thereby amplifying the immune response against tumors[8], while also eliciting robust cell-mediated and humoral immune responses[9]. Finally, their lytic replication cycle directly lyses tumor cells (oncolysis), making them an effective tool for targeting cancer (Figure 1).

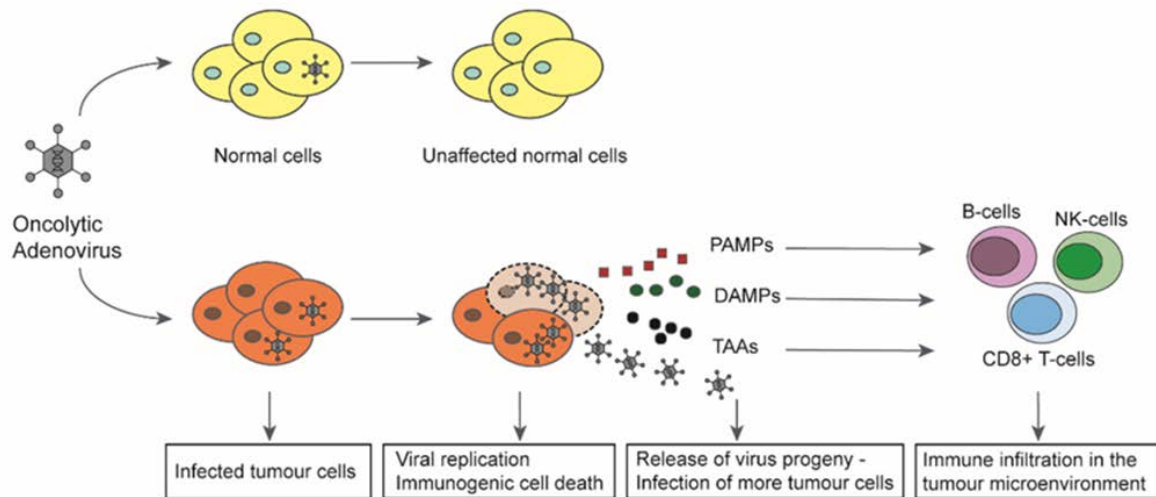


Figure 1 Oncolytic adenoviruses: mechanism of action.

The mechanism by which oncolytic adenoviruses act in cancer cells involves their selective replication within malignant cells. In healthy, non-cancerous cells, these viruses fail to replicate, leaving the cells unharmed. In contrast, within tumor cells, the viruses replicate effectively, generating new viral particles and triggering immunogenic cell death (ICD). This process results in the lysis of cancer cells, releasing viral progeny, PAMPs, DAMPs, and TAAs into the tumor microenvironment (TME). The newly produced viruses then infect nearby tumor cells, perpetuating the cycle of viral spread. Additionally, the release of immune-stimulatory molecules within the TME recruits immune cells to the tumor site, activating both innate and adaptive immune responses to target the tumor[10].

2.1 Classification

AdVs, members of the Adenoviridae family, are classified into five genera: Mastadenovirus, Avidadenovirus, Siadenovirus, Atadenovirus, and Ichtadenovirus. The Mastadenovirus genus includes adenoviruses that infect humans (HAdVs) and non-human primates (nhpAds)[11]. Currently, 116 HAdVs serotypes have been identified (hadvwg.gmu.edu) and grouped into seven species, A to G, previously referred to as subgroups. These species vary in morphology, DNA homology, and oncogenic potential [12].

HAdVs are globally distributed, with infections typically occurring within the first five years of life. Due to the high prevalence of these viruses, over half of the population develops immunity, particularly to human adenovirus type 5 (HAdV5). In healthy individuals, adenovirus infections generally result in mild symptoms; however, in immunosuppressed individuals, HAdVs can lead to severe illnesses[13].

HAdVs primarily target three main areas: the respiratory tract (species B, C, and E), the gastrointestinal tract (species F and G), and the ocular surface (species B, D, and E)[14]. A severe ocular infection known as epidemic keratoconjunctivitis (EKC) is caused by specific types within species D (types 8, 37, 53, 54, 56, and 64)[15]. EKC outbreaks are observed

worldwide but occur more frequently and on a larger scale in Southeast Asia, presenting significant health and socioeconomic challenges.

During infection, AdVs first interact with cell surface receptors, such as the coxsackievirus and adenovirus receptor (CAR), CD46, CD80/86, desmoglein-2 (DSG2), and heparan sulfate proteoglycans (HSPG)[12]. The viruses then enter and replicate within host cells.

A detailed overview of all identified HAdVs types, including receptor usage and tissue-specific tropism, is available in Table 1.

Table 1 Types and features of human adenoviruses

Species	Serotype	Identified Receptor(s)	Tropism
A	12,18,31,61	CAR	Cryptic (enteric, respiratory)
B	3,7,11,14,16,21,34,35,50,55,56,66,68,76-79	CD46,DSG2,CD80,CD86	Respiratory, renal, ocular
C	1,2,5,6,57,89	CAR,VCAM-1,HSPG,MHC1-a2,SR	Respiratory, ocular, lymphoid, hepatic
D	8-10,13,15,17,19,20,22-30,32,33,36,-39,42-49,51,53,54,56,58-60,62-65,67,69-75,80-88,90-103	SA,CD46,CAR	Ocular, enteric
E	4	CAR	Respiratory, ocular
F	40,41	CAR	Enteric
G	52	CAR,SA	Enteric

Note: CAR, coxsackievirus- and adenovirus receptor. DSG2, desmoglein-2. MHC1-a2, major histocompatibility complex-a2. SA, sialic acid. SR, scavenger receptor. VCAM-1, vascular cell adhesion molecule-1. HSPG, heparin sulfate proteoglycans.[12]

2.2 Structure

AdVs are non-enveloped icosahedral viruses with a double-stranded DNA genome that varies in size (26–45 Kbp) depending on the AdV serotype. The HAdV5 capsid consists of 13 proteins in total. Among these, the hexon (protein II, 109 kDa), penton base (protein III, 63.3 kDa), and fiber (protein IV, 61.9 kDa) are known as the major capsid proteins (Figure 2) [16].

The 720 hexon monomers, organized into 240 trimers, represent the most abundant component of the capsid and form the 12 faces of the icosahedron. Each monomer consists

of two eight-stranded β -barrels, or jellyroll domains, separated by a small β -sheet. These double jellyroll structures form the pseudo-hexagonal base of each trimeric hexon capsomer. Long loops between the β -strands make up the hexon towers and contain hypervariable regions (HVRs). This hexon architecture is highly conserved across the adenoviridae family, with variations mostly present in the HVRs, which show differing levels of flexibility in various virus types. Pentamers of the penton base fill gaps left by the five peripentonal hexons. The penton base protein itself consists of two domains: a single jellyroll structure and an upper domain that faces the solvent-exposed exterior. This upper domain features a hypervariable loop with an RGD sequence, an α v integrin-binding motif. Trimeric fibers attach to the penton base pentamer on its outer surface, forming a non-covalent complex. Each fiber comprises three regions: the head, shaft, and N-terminal tail. The C-terminal globular head, or "knob," enables fiber trimerization and receptor attachment at the host cell membrane. Additionally, the shaft's length and flexibility play a role in AdV cell entry by facilitating interactions between the virion, primary receptor (via the fiber), and integrins (via the penton base). The extended N-terminal tails, containing the conserved FNPVYPY sequence across all human adenoviruses, bind in the cleft between two adjacent penton base monomers to anchor the fiber to the capsid[17].

The minor capsid components, proteins IIIa (63.5 kDa), VIII (15.4 kDa), and IX (14.4 kDa), primarily function as cement proteins, linking the major structural units and the viral core (Figure 2) [18]. In addition to serving as a cement protein, protein VI plays key roles in virion maturation, entry, trafficking, and early gene expression. The viral core contains the double-stranded DNA genome (35,938 Kbp in HAdV5) associated with four proteins: V (41.6 kDa), VII (19.4 kDa), μ (4 kDa), and terminal protein (TP, 55 kDa), as well as the viral protease (23 kDa). The IVa2 protein, along with L4 33K, L4 22K, E2 72K, and L1 52/55K, is involved in capsid assembly and/or genome packaging[19].

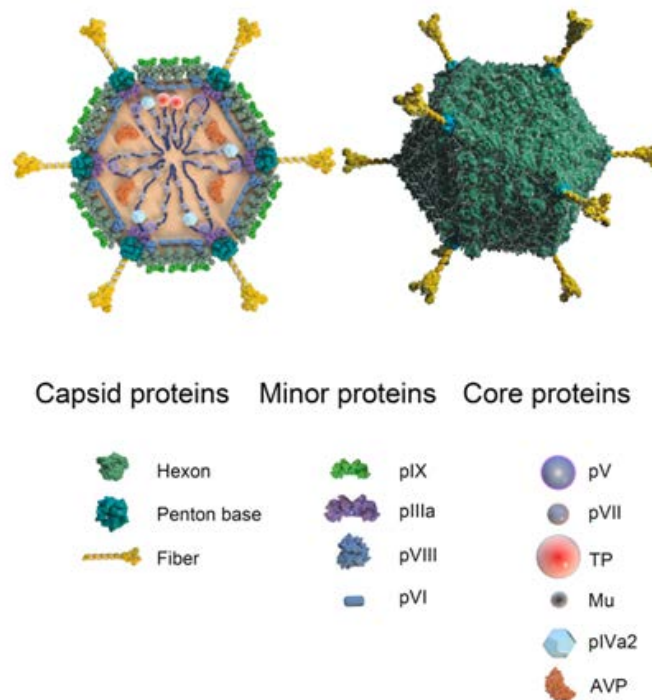


Figure 2 Virion structure of Human Adenovirus.

Virion structure of human adenovirus based on crystallography and cryo-electron microscopy. Fiber, Penton base and Hexon are capsid proteins. pIX, pIIIa, pVIII and pVI are minor proteins. Core proteins contains of pV, pVII, TP, Mu, pIVa2, AVP; TP (terminal protease). The lowercase "p" refers to protein[20].

2.3 Genome

The HAdV5 genome is a ~36 kb linear double-stranded DNA molecule flanked by two ~100 base pair (bp) inverted terminal repeats (ITRs) at both ends, which contain the viral DNA replication origins (Figure 3). These sequences are followed by the viral packaging signal (ψ) at the left end of the genome, which directs viral DNA encapsidation. The AdV pre-terminal protein (pTP) is covalently bound to the 5' ends of the genome and plays a critical role in initiating viral DNA replication. The HAdV genome encodes approximately 40 proteins, classified as early or late based on their expression timing, either before or after DNA replication, respectively. Generally, early proteins have regulatory functions, enabling the virus to take control of the host cell to replicate its own DNA, while late proteins are primarily structural. The genome specifically contains the immediate early E1A region, four early transcription units (E1B, E2, E3, and E4), and a set of delayed early units encoding proteins IX and IVa2 (Figure 3). The AdV major late promoter (MLP) drives the synthesis of a single late pre-mRNA that is alternatively polyadenylated and spliced to generate five families of late mRNA, the L1-L5 regions. Two additional small late transcripts, virus-associated (VA) RNAI and VA RNAII, are also produced. The transcription units of the AdV genome are transcribed from

both strands of the chromosome. AdVs exemplify viruses that efficiently utilize limited genetic space and information to maximize protein production for optimal viral propagation.

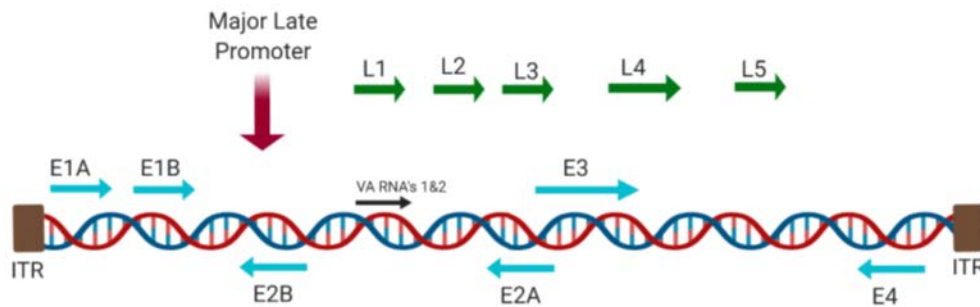


Figure 3 Schematic Representation of Adenoviral Genome Organization.

This diagram illustrates the adenoviral genome, highlighting key regions and their respective functions. The genome features inverted terminal repeats (ITRs) at both ends, essential for replication. Early genes (E1A, E1B, E2A, E2B, E3, E4) are indicated by blue arrows and are responsible for initiating viral replication, transcriptional regulation, and immune evasion. The major late promoter (MLP) activates the late genes (L1 to L5), shown in green arrows, which are primarily involved in viral particle assembly. Additionally, the VA RNAs 1 & 2 play a critical role in counteracting host antiviral defenses [21].

2.4 Infective cycle

2.4.1 Virus infection

The AdV infection cycle begins with the virus's contact with the host cell. The AdV fiber protein, through its knob domain, binds to the CAR, which serves as the primary receptor for both HAdV5 and coxsackie B viruses[18]. Attachment is further reinforced by the binding of the RGD peptide (Arg-Gly-Asp) on the penton base to cellular integrins $\alpha V\beta 3$ and $\alpha V\beta 5$, facilitating viral internalization (Figure 4.1). AdV can also exploit alternative receptors, such as HSPG and blood coagulation factors like factors IX (FIX) and X (FX). CD46 is used as a receptor by subgroup B human AdVs (HAdVB). This viral internalization is mediated via clathrin-coated vesicles for endocytosis, followed by partial capsid disassembly as the endosome acidifies (Figure 4.2-3) [22]. Endosomal escape is facilitated by the lytic action of protein VI, after which the nucleocapsid is translocated from the cytoplasm to the perinuclear envelope along the microtubule network, undergoing further disassembly (Figure 4.4). Upon reaching the nuclear pore complex, the protein VII-coated DNA enters the nucleus, where protein VII shields viral DNA from triggering the DNA damage response[23]. Viral DNA replication and assembly of progeny virions occur entirely within the nucleus of infected cells. The AdV life cycle spans 24-36 hours, with a single virus-infected cell producing approximately $1.0E+04$ progeny virions.

2.4.2 Early gene expression and Viral DNA replication

After the viral genome enters the nucleus, host RNA polymerase II begins transcribing the E1A gene, resulting in multiple mRNAs and proteins through alternative splicing. Early in infection, two key mRNAs, 12s and 13s, are generated, encoding the 243R and 289R proteins, respectively. These proteins play a central role in activating the early transcription units (E1B, E2A, E2B, E3, and E4) and driving the host cell into the S phase, a critical step for viral replication (Figure 4.5-9). This cell cycle progression is achieved by binding and sequestering the retinoblastoma protein (pRb) family, which are tumor suppressors that typically inhibit cell cycle progression by interacting with E2F transcription factors[24]. E2F factors promote S-phase gene expression, facilitating viral replication. Through E1A's role in activating S-phase entry and transcription, the remaining early transcription units are expressed, producing proteins essential for adenoviral replication[25].

The E1B gene products, primarily 19K and 55K, function to inhibit apoptosis. The 55K protein inactivates the pro-apoptotic tumor suppressor p53, which is triggered by E1A-induced cell cycle dysregulation, while the 19K protein blocks downstream mediators of p53-independent apoptotic pathways. E1A-driven cell cycle alterations cause p53 to accumulate, potentially triggering apoptosis as a defensive response[26]. The E2 region codes for key proteins required for viral DNA replication, including DNA polymerase (Ad pol), pTP, and single-stranded DNA binding protein (ssDBP) (Figure 4.10-13). The E3 region products are involved in immune evasion, helping the virus to evade the host antiviral response to complete its replication cycle. For instance, E3-19K sequesters major histocompatibility complex class I (MHC-I) molecules in the endoplasmic reticulum, preventing their display on the cell surface, while also binding to transporter associated with antigen processing (TAP) to block peptide loading on MHC-I molecules[27]. The E4 transcriptional unit supports viral replication, mRNA stability and transport, and plays a role in late gene expression.

2.4.3 Late gene expression and virion assembly

Once early gene products are sufficiently accumulated and viral DNA replication has initiated, the MLP is activated by a transcriptional complex of an IVa2 dimer and non-structural protein L4 33K (Figure 4.13-17) [18]. The MLP controls expression from the major late transcription unit (MLTU), which produces 15-20 mRNAs by differential splicing and polyadenylation of a single pre-mRNA. Most late proteins, originating from the L1-L5 regions, serve as structural components or assist in virion assembly and genome packaging.

When these late proteins are ready, they are transported to the nucleus, where DNA encapsidation and virion assembly occur (Figure 4.18-19). At this stage, the adenovirus protease cleaves certain structural proteins into their mature forms, making the virus particles fully infectious (Figure 4.20). Finally, cell lysis and release of progeny virions are triggered by the adenovirus death protein (ADP), expressed from the E3 transcription unit, which is the only early gene active in the late phase (Figure 4.21).

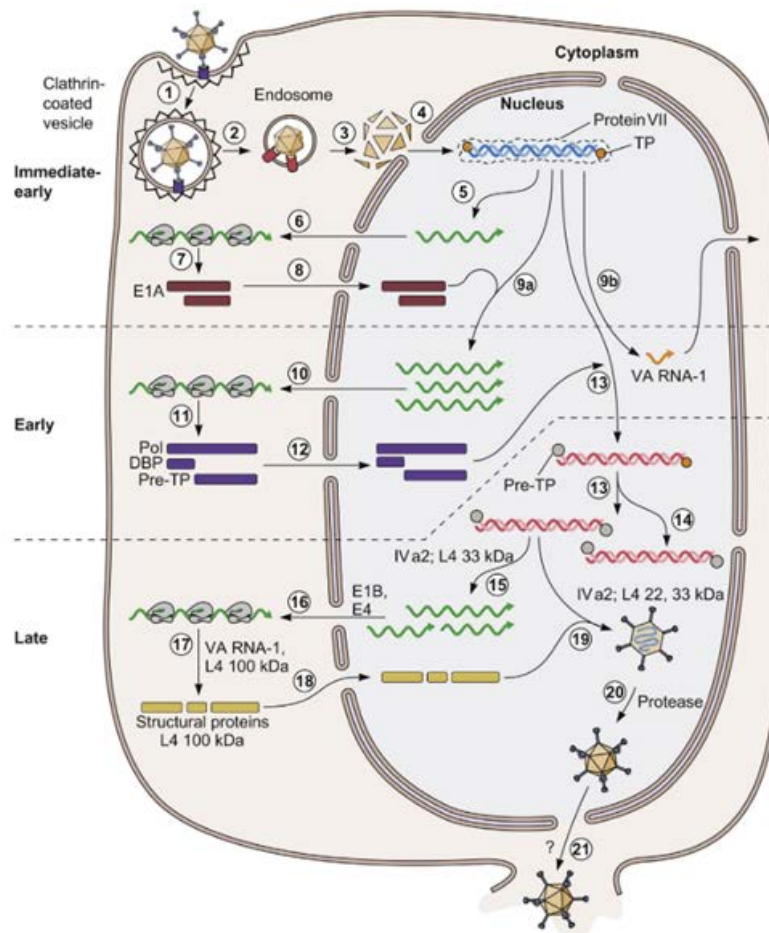


Figure 4 Adenoviral Life Cycle Within Host Cells.

This illustration depicts the adenoviral infection and replication cycle in a host cell. The virus enters the cell through clathrin-mediated endocytosis (1), followed by endosomal escape (2, 3) and transport of the viral genome to the nucleus (4). In the nucleus, transcription is initiated with immediate-early gene expression, including E1A (5), which activates the transcription of early genes such as E1B, E2, E3, and E4 (6-12). These early proteins support viral DNA replication and host immune evasion. Late genes are subsequently transcribed (13-19), producing structural proteins needed for capsid assembly. Viral assembly occurs in the nucleus, where new virions are packaged (20) and eventually released upon cell lysis (21)[28].

3. Limitations and Overcoming Strategies in Adenovirus-based Oncolytic Therapy

Adenovirus-based oncolytic virotherapy has shown great potential in cancer treatment due to the virus's innate ability to infect and lyse cells. However, employing adenoviruses, particularly HAdV5, presents several challenges. A major limitation is the high prevalence of pre-existing immunity to HAdV5 in populations where this virus is a common pathogen. This immunity can reduce therapeutic efficacy by promoting rapid clearance and destruction of the virus through the reticuloendothelial system, which diminishes its bioavailability and its capacity to effectively target tumors.

Another significant challenge stems from the adenovirus's natural cell tropism, which can lead to off-target effects and dose-limiting toxicity. The affinity of the HAdV5 vector for the CAR receptor, broadly expressed in various non-cancerous tissues, results in unintended interactions[21]. Additionally, the HAdV5 hexon protein binds with high affinity to FX, facilitating rapid liver sequestration[29]. This results in an elevated viral concentration in the liver, increasing the risk of hepatotoxicity. The penton protein is also implicated in off-target effects. The interaction between the RGD domain in the penton and integrins, which enables viral internalization into cells, appears to contribute to spleen uptake, triggering pro-inflammatory responses against the adenovirus (Figure 5) [30].

Collectively, these factors limit the selectivity and therapeutic index of HAdV5 for cancer-specific applications. To address these challenges, researchers have developed several strategies that combine viral detargeting and retargeting techniques to enhance tumor selectivity and reduce off-target effects[21].

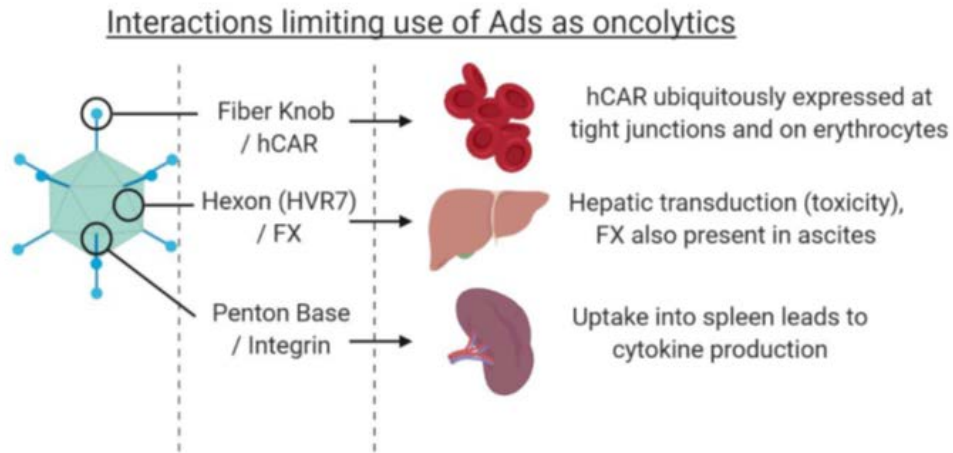


Figure 5 Challenges in Using Adenoviruses (AdVs) as Oncolytic Agents.

This diagram highlights key interactions that limit the effectiveness of AdVs in oncolytic therapy. The fiber knob interacts with the human coxsackievirus and adenovirus receptor (CAR), which is widely expressed at tight junctions and on erythrocytes, reducing tumor-specific targeting. The hexon protein, particularly its HVR7 region, binds to coagulation factor X (FX), leading to hepatic transduction and potential liver toxicity. Additionally, the penton base binds integrins, facilitating viral uptake into the spleen, which triggers cytokine production and systemic inflammation, further complicating therapeutic use[21].

3.1 Detargeting Strategies for Adenoviruses

In Ad-based therapies, the efficiency of treatment can be significantly impacted by the virus's native binding interactions. The CAR receptor, widely present in normal tissues, particularly at cell junctions, often has lower expression in aggressive cancers, which poses a challenge for HAdV5-based therapies that rely on this receptor for cell entry. As a result, modifying HAdV5 to bypass CAR dependency is essential for enhancing selectivity for cancer cells.

To address these challenges, researchers have developed a range of detargeting approaches, including genetic modifications (Figure 6). One such modification involves mutating specific amino acids in the HAdV5 fiber knob, such as the KO1 mutations (S408E and P409A) in the L5 gene, to disrupt CAR binding[31]. Another approach targets HAdV5's secondary receptor, the RGD domain, which enables virus internalization through integrin binding. Modifying this interaction, for example, by substituting RGD with RGE in the penton base, can reduce non-specific tissue interactions and improve cancer targeting[32].

A representative example that combines various detargeting techniques is the Ad5NULL: in this vector, all native tropisms of HAdV5 were eliminated by introducing mutations in key residues of the three main capsid proteins—hexon, fiber, and penton. These modifications included the KO1 mutation, the RGD-to-RGE substitution, and alterations to the hexon HVR7,

which prevent binding to FX and significantly reduce liver uptake [33]. Another effective modification involves replacing HVR7, HAdV5's primary FX-binding region, with that from a different adenovirus serotype, such as HAdV3[34]. This replacement reduces FX binding and limits liver sequestration, enhancing the vector's selectivity for cancer cells.

Alternatively, chemical approaches, such as coating AdV particles with polyethylene glycol (PEG), create a protective layer that helps the virus evade neutralization by pre-existing antibodies in patients previously exposed to HAdV5[35]. Such coatings shield the virus from antibody recognition while preserving its tumor-targeting abilities.

However, chemical modifications have limitations. Viral replication produces daughter virions that lack the protective polymer coat, meaning they may not retain tumor-targeting properties. This limitation has led many researchers to favor genetic modifications, which are heritable and persist in subsequent viral generations. Although detargeting strategies effectively limit non-specific interactions and toxicity, they underscore the complexity of engineering cancer-selective adenoviral vectors that balance specificity and therapeutic impact.

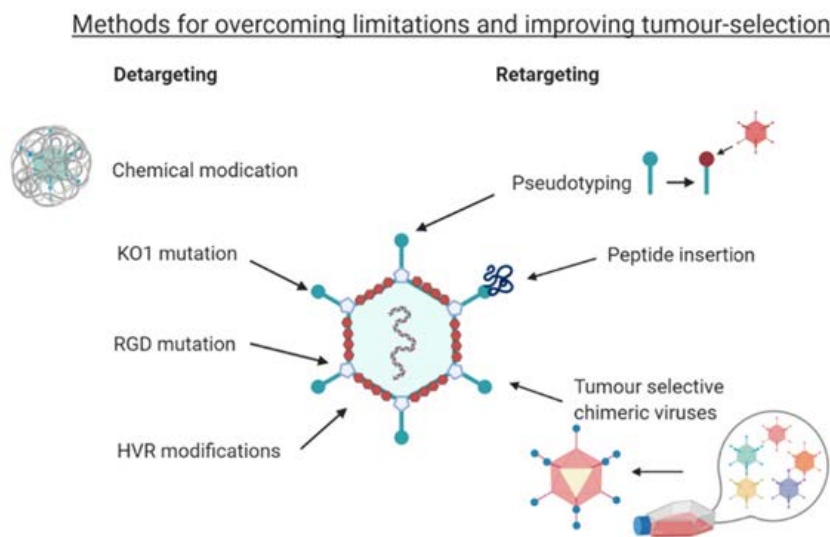


Figure 6 Strategies to Overcome Limitations and Enhance Tumor Selectivity of Adenoviruses.

This diagram illustrates approaches to improve the specificity and effectiveness of adenoviruses in targeting tumors. Detargeting methods include chemical modification, KO1 mutation, RGD mutation, and hypervariable region (HVR) modifications, all aimed at reducing off-target interactions and minimizing toxicity. Retargeting strategies focus on enhancing tumor specificity through pseudotyping, peptide insertion, and the development of tumor-selective chimeric viruses. These combined techniques enable the creation of adenoviral vectors with improved therapeutic potential and reduced systemic side effects[21].

3.2 Retargeting strategy for Adenovirus

Retargeting strategies for AdVs are essential for enhancing cancer cell specificity in oncolytic virotherapy (Figure 6). One popular method, pseudotyping, involves creating chimeric fiber knob or shaft proteins by combining structural elements from various adenovirus serotypes, thereby introducing alternative binding properties[36]. For instance, fiber knobs from serotypes that don't rely on CAR receptor are used to expand HAdV5's tropism without compromising the extensive knowledge available for HAdV5-based treatments. While promising, pseudotyping requires the chimeric fiber knob to correctly fold and form trimeric structures to ensure effective binding, and the range of known serotype-specific tropisms remains limited[37].

Peptide retargeting offers another viable approach to redirect AdV tropism toward tumor-specific receptors. This involves inserting targeting peptides into accessible regions of AdV capsid proteins, such as the HI loop of the fiber knob. For example, integrating the RGD-4C motif enhances the virus's ability to target $\alpha\beta3/5$ integrins commonly upregulated in cancers, such as glioma and ovarian cancer[38]. Other successful insertions, like the A20 peptide from the foot-and-mouth disease virus, enable high affinity for $\alpha\beta6$ integrins, a marker for certain epithelial cancers, yielding highly selective vectors like Ad5NULL-A20 for targeting ovarian and other integrin-positive cancers[33].

The hexon protein, which is abundant in the viral capsid, has also been explored as an insertion site. For instance, incorporating targeting peptides within the HVRs of the hexon protein, such as RGD-4C in HVR7, could yield capsids with hundreds of binding sites, enhancing cell specificity[39]. However, insert design demands careful consideration, as steric hindrance and charge effects can impact effectiveness, and peptides with defined secondary structures often yield better results in binding efficacy.

Another innovative retargeting strategy, "directed evolution", selects for adenoviral recombinants that naturally develop enhanced cancer-cell specificity (Figure 7) [40]. This approach bypasses traditional genetic modifications and instead uses a natural selection process. Enadenotucirev, developed by PsiOxus Therapeutics, serves as a prominent example, where recombinants from species B adenoviruses (derived from HAdV11 and HAdV3) were selected for their heightened cell-killing abilities in colon cancer cells. The resultant chimera demonstrated superior potency and selectivity in various solid cancers compared to earlier

therapies such as ONYX-015 and HAdV5[41], despite the exact mechanisms of its tumor specificity remaining under investigation. Early clinical trials indicate Enadenotucirev's promising efficacy and tolerability across multiple cancer types. This method highlights the potential for species B adenoviruses in cancer therapy, though challenges such as CD46 and DSG2 receptor interactions on normal cells remain and may require strategies to avoid non-target sequestration.

Future retargeting success will likely depend on advanced molecular techniques that allow high-throughput screening to identify novel peptides and capsid variants with enhanced tumor-specific binding.

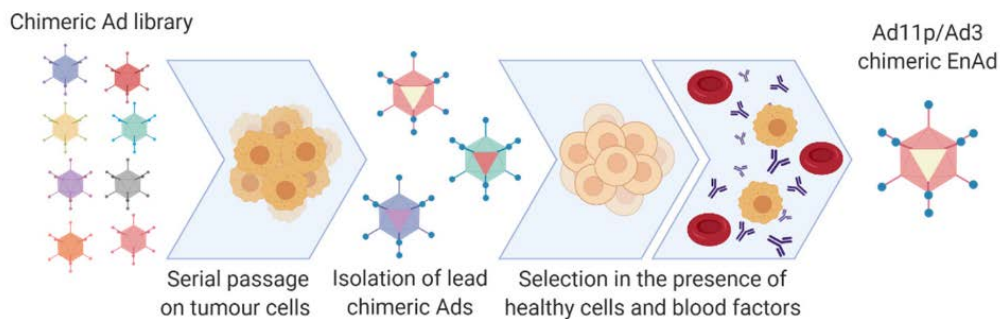


Figure 7 Directed Evolution mechanism.

A diverse chimeric AdV library is subjected to serial passage on tumor cells to promote viral recombination and tumor selectivity. Lead chimeric adenoviruses are then isolated and further refined through selection in the presence of healthy cells and blood factors, ensuring reduced off-target effects. The process results in the generation of optimized constructs such as the HAdV11p/HAdV3 chimeric Enadenotucirev (PsiOxus Therapeutics Ltd., Abingdon, UK)[21].

4. Advancements in Genetic Engineering of Oncolytic Adenoviruses for Cancer Therapy

The development of safe, cancer-selective, and efficient OV's for treating various tumors is strongly dependent on advancements in OV genetic engineering. Adenovirus, in particular, has shown significant promise as an oncolytic agent due to its natural ability to infect and lyse cells. However, despite its inherent potential, adenovirus is never used in its wild-type form for cancer therapy. Instead, it requires genetic modifications to enhance its selectivity, reduce toxicity, and improve its efficacy within the tumor microenvironment (TME). These modifications allow the virus to selectively replicate in cancer cells, activate immune responses against tumors, and limit off-target effects in healthy tissues.

Optimizing adenovirus as an oncolytic tool requires a thorough understanding of viral biology, host immunity interactions, and the immune evasion strategies employed by cancer cells. Genetic engineering strategies aim to overcome these barriers, making adenoviruses safer and more effective therapeutic agents. In the following sections, we will explore the three primary objectives in adenovirus engineering: restricting viral replication to cancer cells, increasing therapeutic efficacy by loading the virus with transgenes, and enhancing tumor cell targeting through fiber modifications. These targeted modifications are crucial to harnessing the full potential of adenovirus-based therapies in cancer treatment.

4.1 Influence on Adenovirus replication: Conditionally Replicating Adenovirus

The adenovirus E1 region, consisting of E1A and E1B genes, plays a crucial role in initiating viral replication by interacting with cellular proteins. In the design of oncolytic adenoviruses (OAdVs) for selective tumor targeting, researchers have focused on strategies that allow conditional activation of E1 genes, enabling the virus to replicate specifically within tumor cells and cause their destruction. This selective replication capability classifies these viruses as Conditionally Replicating Adenoviruses (CRADs)[42], which are widely used in oncolytic adenovirus therapies.

To achieve this conditional replication, several strategies are employed: tumor-specific promoter regulation of E1A, a 24 bp constant deletion in the E1A conserved region 2 (E1A Δ 24), a deletion in the conserved region 3 (CR3) of the E1A 13S transactivator protein and an E1B55K deletion or E1B-93R mutation (Figure 8).

These modifications enable the adenovirus to act selectively in cancerous tissues, as opposed to replication-deficient adenoviruses with E1 deletions, which are commonly used as vectors for gene therapy or vaccination due to their inability to replicate autonomously. The following sections will delve into these strategies, detailing how each modification contributes to tumor-selective replication in adenoviral vectors.

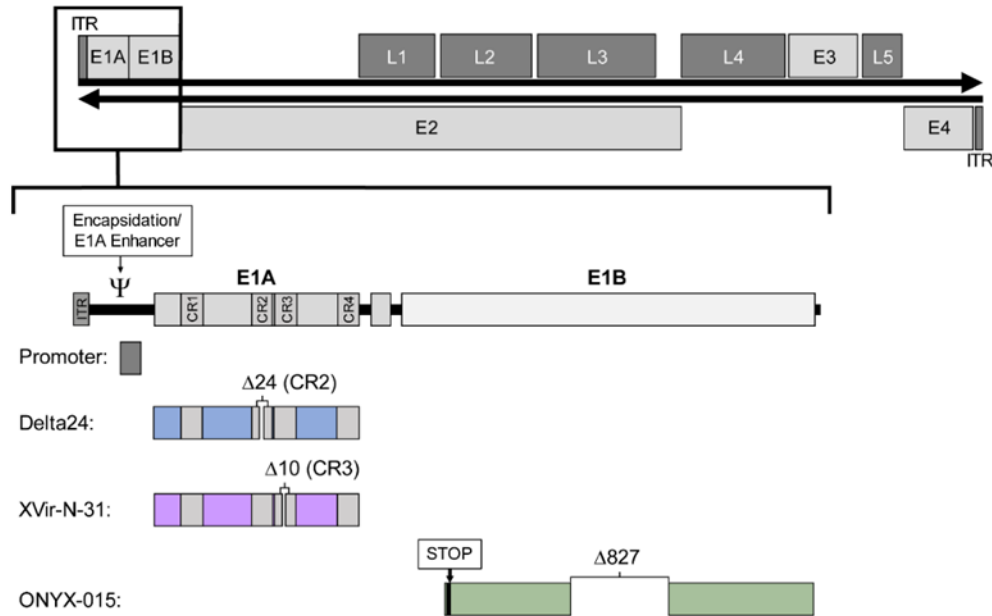


Figure 8 Schematic Representation of Adenoviral E1 Genome Modifications in Oncolytic Constructs.

The E1 region, critical for viral replication, is depicted with its regulatory sequences and enhancer elements. Specific modifications in promoter region or deletions, such as $\Delta 24$ (in CR2) and $\Delta 10$ (in CR3), are highlighted in constructs like Delta24 and XVir-N-31, which enhance tumor selectivity by restricting replication to cancer cells with defective Rb or p53 pathways. The ONYX-015 construct features a deletion ($\Delta 827$) in the E1B region, preventing replication in normal cells while maintaining its ability to target tumor cells. These modifications aim to improve tumor selectivity and safety profiles of oncolytic adenoviruses[10].

4.1.1 Tumor-specific promoters

To achieve selective replication within tumor cells, OAdVs often utilize tumor-specific promoters to control the expression of the E1A gene. These promoters are typically inactive in normal cells but become activated in tumor cells, enabling the virus to replicate selectively within malignant tissues (Figure 8). This strategy exploits the differential gene expression patterns between normal and cancerous cells, allowing the virus to proliferate in tumors while minimizing damage to healthy tissue.

Tumor-specific promoters, such as those regulating E2F-responsive elements, survivin, and human telomerase reverse transcriptase (hTERT), are commonly used in OAdVs. For instance, the adenovirus CG0070 (Ad5-E2F-E1A-GM-CSF) uses the E2F promoter to drive E1A expression selectively in retinoblastoma (Rb)-deficient cancer cells, leading to viral replication and cell lysis. CG0070 also encodes GM-CSF, enhancing the immune response by releasing tumor antigens and stimulating systemic antitumor immunity. This virus has shown efficacy in clinical trials and received Food and Drug Administration (FDA) approval for treating non-muscle invasive bladder cancer (NMIBC)[43].

Similarly, the Ad3-hTERT-CMV-CD40L vector leverages hTERT as a promoter for selective replication in cancer cells while incorporating CD40L to stimulate immune responses[44]. OBP-301 (Ad5-hTERT-E1A-IRES-E1B) also utilizes hTERT-driven replication, demonstrating cytotoxic effects on solid tumors with hypoxic regions where hTERT activity is upregulated[45].

Through these modifications, tumor-specific promoters play a critical role in enhancing the selectivity and safety of OAdVs, ensuring that replication and therapeutic effects are limited to cancer cells, thereby minimizing toxicity in normal tissues.

4.1.2 E1 Modifications: E1A and E1B Deletions for Tumor-Specific Replication

The early genes E1A and E1B are critical for adenoviral replication, orchestrating the viral life cycle, cell cycle modulation, and evasion of apoptosis. Genetic modifications targeting these genes have been key to engineering OAdVs with selective replication in tumor cells, sparing normal tissues (Figure 8).

E1A Deletions

E1A proteins, expressed immediately after adenoviral infection, initiate viral replication by modulating the cell cycle and activating both viral and cellular genes. A well-established strategy involves a 24 bp deletion in the conserved region 2 (CR2) of E1A, referred to as E1A Δ 24[10]. This deletion disrupts E1A's interaction with the pRb, which regulates the release of the E2F transcription factor necessary for S-phase entry (Figure 8). In normal cells, the absence of this interaction prevents adenoviral replication. However, tumor cells, which often exhibit Rb pathway dysfunction and high levels of free E2F, allow E1A Δ 24 viruses to replicate selectively[46]. Clinical studies have demonstrated the safety and efficacy of E1A Δ 24-based OAdVs, such as Ad5- Δ 24, in targeting solid tumors via intratumoral and intravenous administration [47].

Further advancements have combined the E1A Δ 24 modification with tumor-specific promoters to enhance specificity. An example include AdCN103, which pairs E1A Δ 24 with an hTERT promoter[48].

Another approach involves the deletion of a splice acceptor site in CR3 region of E1A, leading to the loss of E1A13S expression. E1A13S is essential for the nuclear translocation of Y-box binding protein-1 (YB-1), which is overexpressed in many cancers[10]. Viruses such as DI520 and its derivative Ad-Delo3-RGD (XVir-N-31) leverage this mechanism for selective replication

in cancer cells while sparing normal tissues. XVir-N-31, featuring additional deletions in E1B19K and E3 and an RGD motif in the fiber protein, is undergoing clinical trials for glioblastoma (Figure 8) [49].

E1B Deletions

The E1B region encodes two proteins, 19K and 55K, that suppress apoptosis and stabilize viral replication in infected cells. The E1B55K protein binds to and inactivates the tumor suppressor p53, which is upregulated in response to E1A-induced cell cycle deregulation (Figure 8). Deletion of the E1B55K gene disrupts this interaction, rendering the virus unable to evade p53-mediated apoptosis in normal cells. However, tumor cells, often deficient in functional p53, allow replication of E1B-deleted viruses[47].

The prototypical E1B55K-deleted virus, DI1520 (ONYX-015), was one of the first clinically tested OAdVs (Figure 8). Despite challenges in achieving robust tumor selectivity, ONYX-015 laid the foundation for subsequent development[50]. Its derivative, H101 (Oncorine), which includes partial deletions in E3 for enhanced immune evasion, has been approved in China for the treatment of nasopharyngeal carcinoma[10]. Both viruses demonstrate the utility of E1B modifications in restricting replication to p53-deficient tumor cells.

In addition to E1B55K deletions, E1B93R mutations have emerged as a promising strategy. E1B93R plays a role in modulating p53-independent pathways. Its deletion creates an environment where tumor cells with alternative apoptosis resistance mechanisms still support viral replication, enhancing tumor selectivity while broadening the spectrum of targetable malignancies[47]. Building on this approach, Ixovex-1 introduces point mutations at the 3' splicing acceptor site of E1B-93R, enabling the production of the E1B-156R splice isoform while retaining a functional E3B region[51]. This precise modification improves replication competency compared to OAdVs with extensive deletions.

Preclinical studies in lung carcinoma models have shown that Ixovex-1 significantly enhances oncolytic efficacy, delays tumor progression, and improves survival outcomes, highlighting its potential as a refined and effective cancer therapy[51].

Modifications to the E1 region, encompassing both E1A and E1B deletions, are fundamental strategies in oncolytic virotherapy. These genetic alterations exploit the unique molecular landscapes of tumor cells, such as Rb and p53 pathway dysregulation, to achieve selective replication.

4.2 Comprehensive Insights into Arming Oncolytic Adenoviruses

Arming OAdVs with therapeutic payloads has significantly enhanced their versatility as tools in cancer therapy. By incorporating genetic payloads, these viruses can surpass their natural ability to selectively replicate and lyse tumor cells. Such modifications allow for precise tumor targeting, modulation of the TME, and the stimulation of robust anti-tumor immune responses. This dual approach enhances therapeutic efficacy while minimizing systemic toxicity, positioning OAdVs as promising candidates for advanced cancer treatments.

The success of an armed OAdV depends heavily on the careful selection of insertion sites within the adenoviral genome. Wild-type HAdV5 possesses a genome size of approximately 36 kbp and can accommodate up to 105% of its normal genome size without destabilization[52]. This limits the addition of foreign sequences to roughly 2 kbp without the need for genome deletions. To address this limitation, most recombinant AdVs incorporate deletions in non-essential regions of their genome, creating additional space for transgene integration without compromising the virus's structural integrity or replicative ability.

The E3 region is one of the most commonly deleted areas, as it encodes immune escape proteins that are not essential for viral replication in vitro. Partial deletions of this region can provide 2–3 kbp of additional capacity, while complete deletions can yield up to 4.5 kbp[52]. However, deletions larger than 3 kbp are less common as they tend to result in less robust viral particles. Expression cassettes inserted into the E3 region can utilize exogenous promoters such as the human cytomegalovirus (hCMV) or heat shock protein 70 promoters. Alternatively, the native E3 promoter can be utilized, in which case transcripts are derived not only from the E3 promoter but also from the major late promoter unit (MLPU) (Figure 9) [53]. Ribosome entry sites, splice acceptor sites, or 2A self-cleaving peptide linkers can further enhance the co-expression of transgenes on shared transcripts[10]. Additionally, the removal of the E3 region may offer a dual benefit by not only creating space for transgene insertion but also potentially enhancing anti-tumor immune responses through the elimination of viral immune evasion genes.

Another strategic site for transgene insertion is the E1B region. Both E1B-19K and E1B-55K can be deleted without significantly disrupting the viral lifecycle[54]. A full deletion of the E1B region creates approximately 1.8 kbp of additional capacity. Deleting E1B-55K transforms the adenovirus into a CRAd, restricting replication in non-cancerous cells while maintaining

replication in tumor cells. On the other hand, deleting E1B-19K enhances the virus's oncolytic properties and increases viral release, further amplifying its anti-tumor effects.

An additional approach involves inserting transgenes into the late gene regions of the adenoviral genome. This strategy offers potential advantages in terms of specificity, expression levels, and timing. Unlike other insertion sites, the expression of transgenes within the late gene regions is tightly linked to viral DNA replication, ensuring transgene expression occurs exclusively in actively replicating tumor cells[10].

By strategically selecting these genomic locations, researchers can maximize the expression of therapeutic transgenes while preserving the adenovirus's natural replicative and lytic potential. These advancements underscore the transformative potential of arming OAdVs in cancer therapy, providing a robust platform for targeted treatment and immune activation.

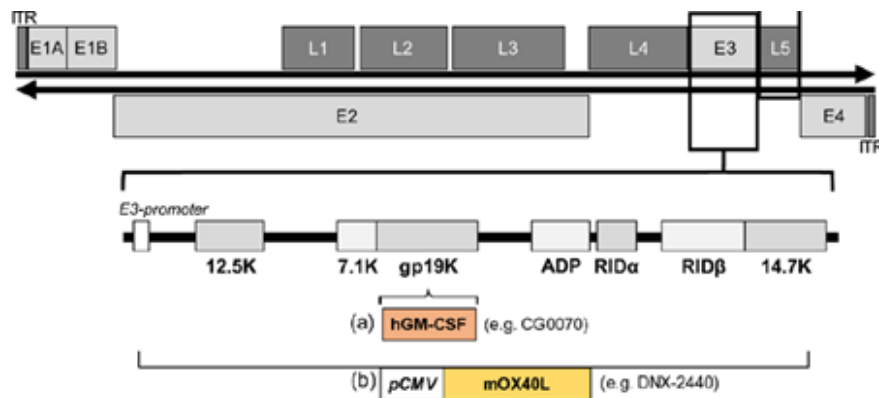


Figure 9 Engineering the E3 Region of Adenoviral Vectors for Therapeutic Gene Expression.

The E3 region is commonly modified in oncolytic adenoviruses to enhance therapeutic efficacy. The E3 promoter regulates the expression of genes such as 12.5K, 7.1K, gp19K, ADP, RIDα, RIDβ, and 14.7K, which contribute to immune evasion and apoptosis regulation. The E3 region is often partially or completely deleted to allow the expression of therapeutic transgenes, such as (a) hGM-CSF (e.g., in CG0070) to stimulate immune activation, and (b) mOX40L under the control of the CMV promoter (e.g., in DNX-2440) to enhance T-cell activation[10].

4.2.1 Diverse Payload Options for Armed Oncolytic Adenoviruses

The choice of payload plays a pivotal role in enhancing the therapeutic potential of OAdVs. These payloads can directly target tumors, stimulate immune responses, or modify the TME, creating a multi-faceted approach to cancer treatment. Key categories of payloads include:

Immune-Stimulating Agents

Immune-stimulating payloads amplify the body's natural defense mechanisms against tumors. Cytokines like interleukin-12 (IL-12), GM-CSF, and interferon-gamma (IFN-γ) enhance immune cell recruitment and activation within the TME, triggering robust inflammatory responses[55]. Additionally, encoding immune checkpoint inhibitors, such as PD-1 or CTLA-4

blockers, within the OAdV genome allows localized reactivation of T-cell-mediated immunity, avoiding the systemic toxicity associated with traditional therapies.

Enzymes and Matrix Modulators

Solid tumors often present dense extracellular matrices that act as barriers to therapeutic agents. Payloads like hyaluronidase and decorin degrade these structures, facilitating viral spread and improving penetration into rigid tumors[55]. By remodeling the extracellular matrices, these payloads enhance the virus's efficacy in targeting solid tumors.

Gene Silencing and Editing Tools

Arming OAdVs with gene-editing tools such as short hairpin RNAs (shRNAs) or CRISPR/Cas9 systems offers a genetic approach to suppress oncogenes or edit tumor-associated genes[55]. These payloads allow precise, targeted intervention at the molecular level, addressing malignancies at their genetic roots.

Monoclonal Antibodies and Bispecific T-Cell Engagers (BiTEs)

Among the payload options, antibodies hold significant promise due to their ability to target tumor cells while simultaneously modulating immune responses[56]. A compelling example is a study in which researchers armed an OAdV with the full-length anti-HER2 antibody Trastuzumab, achieving effective in situ antibody production alongside progressive oncolytic cancer cell killing. They engineered an infectivity-enhanced serotype 5 OAdV, Ad5/3-D24-tras, which encodes the heavy and light chains of human Trastuzumab, linked via an internal ribosome entry site (IRES). This approach successfully combined potent oncolytic activity with high localized production of Trastuzumab, leading to enhanced in vivo efficacy and robust antibody-dependent cell-mediated cytotoxicity (ADCC) in HER2-positive cancer cells[57].

Bispecific T-cell engagers (BiTEs), which simultaneously target T-cells (via CD3) and tumor antigens (like HER2 or Epithelial cell adhesion molecule - EpCAM), represent a highly innovative strategy[56]. These payloads facilitate potent immune-mediated clearance of cancer cells and have shown exceptional promise in preclinical models. Additionally, angiogenesis inhibitors targeting vascular endothelial growth factor (VEGF) disrupt tumor vasculature, starving tumors of nutrients and enhancing immune cell infiltration into the TME.

The arming of OAdV marks a significant advancement in cancer therapy, offering a dual mechanism of action—selective tumor replication and precise therapeutic delivery. By integrating therapeutic payloads into strategic genomic sites and employing diverse payload

options, armed OAdVs deliver unparalleled efficacy. These engineered viruses stand at the forefront of therapeutic innovation, holding the potential to reshape cancer treatment paradigms and provide new hope for patients with challenging malignancies.

4.3 Fiber Engineering for Enhanced Tropism and Tumor Selectivity

Fiber engineering has revolutionized the potential of OAdVs by enabling modifications that enhance their tropism and efficacy in cancer therapy. The fiber protein of AdVs, particularly its C-terminal globular knob domain, is crucial for mediating cell attachment and entry. HAdV5 primarily relies on the CAR receptor for cell binding. However, the heterogeneous and often low expression of CAR in many tumor types poses a significant limitation to the efficiency of HAdV5-based therapies. To address this, researchers have explored various strategies to modify the fiber protein and expand the range of receptors that adenoviruses can target, thereby improving their infectivity and specificity in cancer cells.

One of the most successful approaches has been serotype chimerism, which involves replacing the fiber knob domain of HAdV5 with that of another serotype that binds alternative receptors (Figure 10) [58]. For instance, HAdV5/3 chimeric vectors incorporate the knob domain of serotype 3, which targets DSG2, a receptor highly expressed in epithelial cancers. This modification significantly enhances infectivity in CAR-negative cells, such as melanoma and glioblastoma, as demonstrated in preclinical studies where HAdV5/3 exhibited superior transduction and cytotoxicity compared to unmodified HAdV5[59]. Similarly, HAdV5/35 vectors, which exploit the CD46 receptor, have shown remarkable efficacy in a wide range of cancer models, including glioblastoma and B-lymphocytic malignancies[60]. CD46 is ubiquitously expressed and upregulated in many cancers, making HAdV5/35 an effective tool for targeting tumors that lack CAR expression. These chimeric constructs improve tumor infectivity making them highly attractive for clinical applications.

Another promising strategy is the insertion of targeting peptides into the fiber knob domain, particularly in the HI loop or the C-terminus[58]. These regions are structurally flexible and do not interfere with CAR binding, allowing the incorporation of ligands that redirect the virus to alternative receptors. For example, among the first successful modifications was the incorporation of the RGD-4C peptide (CDCRGDCFC), which binds integrins $\alpha\beta3$ and $\alpha\beta5$, has enabled HAdV5 vectors to infect CAR-negative tumor cells efficiently (Figure 10). In glioblastoma models, RGD-modified adenoviruses demonstrated enhanced cell entry and

therapeutic efficacy, overcoming the limitations of native HAdV5 tropism[58]. The inclusion of the polylysine peptide (pK7), which interacts with heparan sulfate, further improved infectivity, particularly when combined with RGD-4C in the Ad5-RGD-pK7 vector. This double-modified vector outperformed unmodified HAdV5, achieving superior infectivity in CAR-negative cell lines such as glioma and lung carcinoma[61]. Similarly, the integration of peptides targeting HER3/ErbB3 into the HI loop has been shown to significantly enhance infectivity in HER3-positive breast cancer cells. In one study, an HAdV5 vector incorporating a 52-amino acid HER3-targeting peptide exhibited increased transduction and tumor-selective replication, highlighting the potential of peptide incorporation to improve both specificity and therapeutic outcomes[62].

Advanced modifications, such as fiber complex mosaicism, have further expanded the capabilities of fiber-engineered AdVs. This technique combines serotype chimerism with the integration of targeting peptides to create vectors capable of recognizing multiple receptors[58]. For instance, the HAdV5/3-RGD vector, which combines the DSG2-binding properties of HAdV3 with an RGD peptide for integrin targeting, demonstrated enhanced infectivity across a diverse range of cancer cell lines, including glioblastoma and lung carcinoma[63]. Another innovative approach is dual-fiber mosaicism, where a single adenovirus expresses two distinct fiber proteins with complementary receptor-binding specificities[64]. This strategy allows vectors to target heterogeneous tumors with mixed receptor expression profiles, improving the likelihood of effective infection throughout the TME.

These modifications not only improve the infectivity and therapeutic potential of adenoviral vectors but also address key challenges in their clinical application. By expanding the range of receptors targeted, fiber-engineered adenoviruses can achieve higher tumor-to-normal tissue selectivity, reducing off-target effects and systemic toxicity.

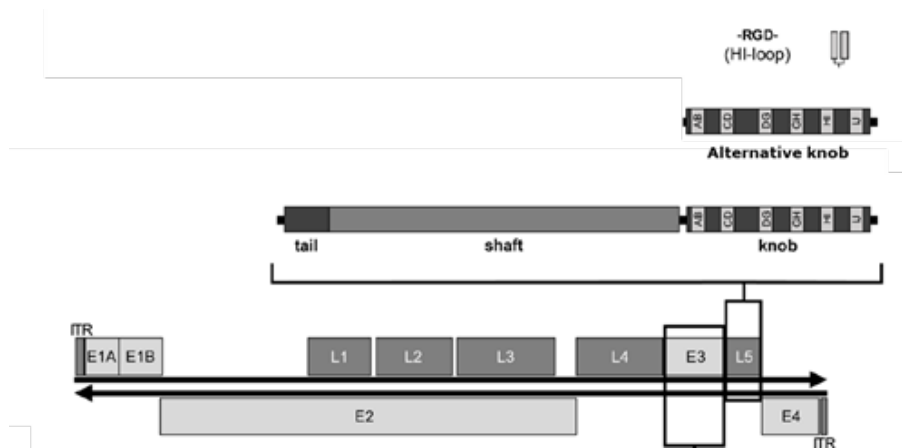


Figure 10 Modification of the Adenoviral Fiber Protein for Retargeting.

The fiber is composed of three main regions: the tail, shaft, and knob. The knob domain, responsible for receptor binding, can be altered to include alternative targeting motifs, such as RGD sequences in the HI loop, to redirect viral tropism towards integrins highly expressed on tumor cells. Additionally, the fiber knob can be replaced with alternative knobs to enhance binding specificity for non-native receptors[10].

4.4 Clinical Progress and Challenges in Oncolytic Adenovirus Therapy

OAdVs have emerged as a promising tool in cancer therapy, with extensive clinical evaluation across a variety of tumors and treatment approaches. AdVs were among the earliest gene therapy vectors investigated in clinical trials, with initial studies dating back to 1993[65]. ONYX-015, developed by Onyx Pharmaceuticals, marked a pivotal moment in this field as one of the first genetically modified OAdVs to undergo clinical testing. This virus, featuring a deletion in the E1B-55K gene, was designed to selectively replicate in tumor cells by exploiting the loss of p53 function commonly found in cancer. Early phase I and II trials demonstrated its safety and moderate antitumor efficacy in head and neck squamous cell carcinoma (HNSCC) and pancreatic cancer[66]. Notably, when combined with cisplatin-based chemotherapy in HNSCC patients, ONYX-015 achieved a tumor response rate of 63%, including 27% complete responses[67]. However, limitations in long-term survival outcomes and efficacy in other aggressive cancers, such as glioblastoma, led to its discontinuation in Western markets. Its derivative, H101 (E1B-55kDa deletion and a partial E3 deletion), was later approved in China in 2005 as the first OV for human use, demonstrating efficacy in HNSCC but falling short as a monotherapy for refractory solid tumors.

The modest success of ONYX-015 prompted advancements in OAdV design to overcome its limitations. A key issue with E1B-55K deletion was its unintended attenuation of viral replication, which constrained therapeutic efficacy. Second-generation OAdVs, such as ONCOS-102, CG0070, and DNX-2401, have incorporated intact E1B-55K genes or alternative

cancer-targeting strategies to restore replication capacity[65]. These newer constructs utilize mechanisms like tumor-specific promoters or deletions in the Rb binding domain of the E1A gene to achieve selective replication in cancer cells while maintaining robust oncolytic activity. Additionally, many modern OAdVs feature fiber modifications, such as the incorporation of the RGD-4C motif, enabling integrin-mediated entry and bypassing the dependency on CAR, a receptor often downregulated in tumors. For instance, DNX-2401, which combines a 24-bp deletion in E1A with an RGD-4C motif, has shown remarkable efficacy in glioblastoma patients, inducing complete regressions and stimulating systemic antitumor immune responses[68]. This led to its fast-track and orphan drug designation by the FDA for treating malignant gliomas.

Other innovative OAdVs, such as CG0070 and ONCOS-102, are engineered to enhance immune activation. CG0070, designed to express GM-CSF under the control of an E2F promoter, has achieved high complete response rates in bladder cancer, particularly in patients unresponsive to bacillus Calmette-Guérin (BCG) therapy[69]. ONCOS-102, armed with GM-CSF and modifications to enhance tumor selectivity, has demonstrated the ability to stimulate cytotoxic CD8⁺ T cells and upregulate PD-L1 expression in tumors, paving the way for combination therapies with immune checkpoint inhibitors like Pembrolizumab (also known as Keytruda, anti PD-1 antibody)[65]. These findings suggest that arming OAdVs with immune-stimulatory transgenes can enhance systemic antitumor responses, addressing challenges associated with the treatment of metastatic and non-injectable lesions.

Despite significant progress, systemic administration of OAdVs remains a major hurdle. Intravenous delivery often results in suboptimal tumor accumulation and limited efficacy. For example, phase I trials of intravenously administered ICOVIR-5 and Enadenotucirev reported only modest outcomes, with progressive disease observed in a substantial proportion of patients[65]. To address these limitations, many ongoing clinical trials are investigating OAdVs engineered to express proinflammatory cytokines like GM-CSF, IL-12, or immune costimulatory molecules such as CD40L and 4-1BBL. These modifications aim to maximize systemic antitumor immune responses and improve therapeutic outcomes in metastatic settings. For instance, Ad5-yCD/mutTKSR39rep-hIL12, an OAdV expressing IL-12 and two suicide genes, has shown promising antitumor activity in preclinical models and is now under clinical evaluation for prostate and pancreatic cancers[70].

As clinical trials continue to expand (Table 2), the potential of OAdVs as a cornerstone of immuno-oncology becomes increasingly evident. Their ability to combine direct oncolysis with immune modulation offers unique advantages, particularly when integrated into combination regimens with chemotherapy, radiotherapy, or checkpoint inhibitors. However, achieving durable systemic responses remains a critical challenge, underscoring the need for continued innovation in vector design and delivery strategies. With ongoing advancements, OAdVs are poised to play an increasingly significant role in the fight against cancer, offering new hope for patients with advanced and refractory malignancies.

Table 2 Oncolytic adenoviruses tested in current clinical trials

Oncolytic adenovirus	Tumor Selectivity	Route of administration	Combination	Indication	Phases	NTC number	Sponsor	Status
CG0070	E2F promoter	Intravesical injection	None	Bladder Cancer	2	NCT02365818	Cold Genesys, Inc.	Completed
DNX2401 (Ad5-D24-RGD)	24-base pair deletion in the E1A gene	Intratumoral injection	Temozolomide, INF, Pembrolizumab	Brain Cancer	1,1,2	NCT01956734, NCT02197169, NCT02798406	DNatrix, Inc.	Completed, completed, Active, not recruiting
LOAd703 (oAd/CD40L and 4-BBL)	24-base pair deletion in the E1A gene	Intratumoral Injection	gemcitabine, paclitaxel, atezolizumab	Pancreatic Cancer	1/2	NCT02705196	Lokon Pharma AB	Recruiting
Enadenotucirev (ColoAd1; chimeric Ad11p/Ad3)	Unknown	intratumoral injection or Intravenous Infusion	none	Colon Cancer, Non-small Cell Lung Cancer, Bladder Cancer, Renal Cell Carcinoma	1	NCT02053220	PsiOxus Therapeutics Ltd	Completed
ONCOS-102 (oAd/GMCSF)	24-base pair deletion in the E1A gene serotype 3 knob	intratumoral injection or Intravenous Infusion	none	Malignant Solid Tumour	1	NCT01598129	Targovax	Completed
		Intratumoral injection	Cyclophosphamide, Pembrolizumab	Melanoma	1	NCT03003676		Recruiting
delta-24-RGD	24-base pair deletion in the E1A gene	Intracerebral infusion by convection enhanced delivery	none	Brain Tumor	1/2	NCT01582516		Completed
OBP-301	human telomerase reverse transcriptase gene (hTERT) promoter.	intratumoral injection	none	Melanoma		NCT03190824	Syneos Health, Oncolys BioPharma Inc	Active, not recruiting
ORCA-010	24-base pair deletion in the E1A gene	intratumoral injection	none	Prostate Cancer	1/2	NCT04097002	Orca Therapeutics B.V.	Not yet recruiting
ICOVIR (Ad-DM-E2F-KDelta24-RGD)	24-base pair deletion in the E1A gene and E2F promoter	endovenous injection	none	Melanoma	1	NCT01864759	Institut Català d'Oncologia	Completed
ICOVIR	24-base pair deletion in the E1A gene	intravenous injection	MSC as a delivery tool	Solid Tumors		NCT01844661	Hospital Universitario Niño Jesús	Completed
VCN-01 (oAd/HA)	E2F promoter regulating E1A gene	intravenous injection	Durvalumab	Head and Neck Squamous Cell Carcinoma,	1	NCT03799744	VCN Biosciences, S.L.	Recruiting
Ad5-yCD/mutTKSR39reph IL12	replication-competent adenovirus type 5 containing a yeast	Intraprostatic Injection	none	Prostate Cancer	1	NCT02555397	Henry Ford Health	Recruiting

	cytosine deaminase (yCD)/mutant sr39 herpes simplex virus thymidine kinase fusion (yCD/ mutTKsr39) gene(E1 and IL-12 gene (E3)							System Baylor College of Medicine	
--	--	--	--	--	--	--	--	--	--

Note: adapted from [65]

4.5 Non-Human Primates Adenoviruses

Adenoviruses derived from NHPs represent a promising avenue in the field of oncolytic virotherapy, particularly as an alternative to human HAdVs, which often face challenges such as pre-existing immunity in the human population. Historically, animal-origin viruses have been explored for their oncolytic potential due to their ability to exploit the lack of adaptive immunity in human hosts. Early research demonstrated that some non-human viruses could retain oncolytic activity in unusual hosts, a concept supported by Moore's work in 1951, which showed that the Russian Far East encephalitis virus—a human virus—exhibited oncolytic activity against murine tumors[71]. This insight spurred efforts to evaluate various non-human viruses, including Newcastle disease virus, vesicular stomatitis virus, and myxomavirus, for their efficacy against human cancers.

In recent years, attention has shifted to NHP-derived adenoviruses, which are particularly appealing due to their low seroprevalence in humans and high genomic similarity to HAdVs. Unlike HAdV5, which is commonly used in oncolytic virotherapy but suffers from moderate efficacy due to widespread pre-existing immunity, nhpAds circumvent this limitation[72]. These viruses do not circulate in the human population, significantly reducing the likelihood of neutralizing antibodies. Furthermore, many nhpAds are able to infect human cells, highlighting their potential as oncolytic agents.

nhpAds share many functional similarities with HAdVs, including receptor usage and immune modulation, making them viable candidates for virotherapy. Notably, evolutionary evidence indicates several instances of host-species switching between apes and humans, underscoring the genetic and functional compatibility of these viruses. The recent success of chimpanzee adenovirus vectors in COVID-19 vaccine development has further validated the utility of nhpAds in therapeutic contexts[73]. However, challenges remain, as the replication of adenoviruses is often species-specific, and the oncolytic potential of NHP-derived adenoviruses requires further exploration.

Groundbreaking work in this field includes the successful generation of a gorilla-derived oncolytic adenovirus, which exhibits low seroprevalence in humans and promising oncolytic activity[74]. Preliminary data suggest that this virus, named AdV-lumc007DRbD or "Goravir," a Group B adenovirus carrying the E1A Δ 24 deletion, demonstrates comparable or even superior efficacy to HAdV5 in various cellular models. These findings underscore the potential of NHP-derived adenoviruses to advance the development of oncolytic viral therapies while addressing the limitations associated with existing vectors.

4.5.1 ReiThera Gorilla Adenoviruses

Within this context, ReiThera has developed proprietary adenoviruses isolated from biological samples of gorillas, referred to as GRAd. Specifically, six distinct GRAd isolates have been classified as belonging to either Group B or Group C adenoviruses based on the amino acid sequence of their Hexon protein. Each Hexon sequence was analyzed using BLAST (<https://blast.ncbi.nlm.nih.gov/>), which confirmed their similarity to known Group B or C adenoviruses. The protein sequences were further aligned using Clustal Omega (<https://www.ebi.ac.uk/jdispatcher/msa/clustalo>), and phylogenetic trees were constructed using Jalview (<https://www.jalview.org/>) (Figure 12).

GRAdC1 and GRAdC2 were found to cluster closely with other known Group C adenoviruses, such as HAdV2, HAdV6, ChAd3, and HAdV5. On the other hand, GRAdB1 and GRAdB3 clustered with the chimpanzee-derived SAdV29, GRAdB2 with the gorilla-derived SAdV47, and GRAdB4 with the chimpanzee-derived SAdV32. Historically, ReiThera has leveraged simian adenoviruses as a vaccine platform, with one of the GRAdC isolates being utilized in the COVID-19 vaccination campaign.

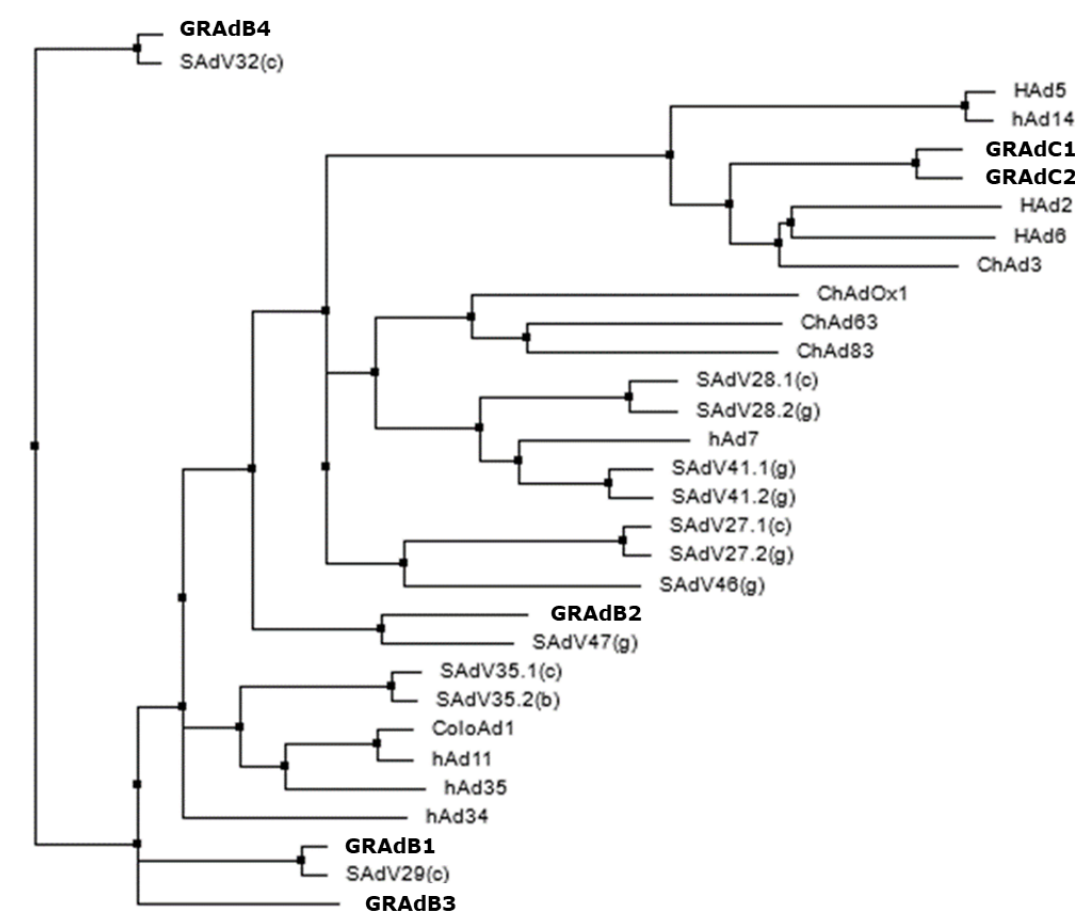


Figure 11 Classification of GRAdS to group B or group C adenoviruses.

This phylogenetic tree illustrates the evolutionary relationships among various adenoviral species, including human adenoviruses (hAd), chimpanzee adenoviruses (ChAd), and simian adenoviruses (SAd). The GRAd variants (GRAdB1, GRAdB2, GRAdB3, GRAdB4, GRAdC1, GRAdC2) are highlighted, showcasing their genetic divergence and clustering with related adenoviral species.

For the COVID-19 vaccine, ReiThera generated modified GRAdC vectors with specific genome deletions to optimize the backbone and inserted a transgene cassette encoding the full-length SARS-CoV-2 Spike protein in its stabilized form (S-2P)[75]. This design aimed to preserve neutralization-sensitive epitopes at the apex of the prefusion structure while enhancing expression levels in transduced cells. The safety and immunogenicity of this vaccine candidate, GRAd-COV2, were evaluated in a phase 2 trial (COVITAR study, ClinicalTrials.gov: NCT04791423), demonstrating that GRAd is a robust platform for genetic vaccine development, particularly when a strong CD8 T-cell response is required[76].

In this context, we also studied the prevalence of pre-existing immunity against GRAdC in a panel of 40 human sera of U.S. origin, which revealed significantly lower seroprevalence compared to HAdV5 (Figure 12) [75]. Additionally, we conducted a preliminary biodistribution experiment with GRAdC (unpublished data), providing initial insights into its in vivo behavior.

These studies indicated that GRAdC exhibits a natural tropism for the lung, likely due to its isolation from lung tumor cells. Interestingly, GRAdC appeared to be less susceptible to hepatic sequestration, a major limitation of HAdV5-based vectors (Figure 12).

These findings suggest that GRAdC has favorable characteristics not only for genetic vaccine applications but also potentially in the field of virotherapy. Regarding GRAdB, and given the company's focus on other areas, knowledge of these viruses has remained unexplored until now. However, this thesis aims to investigate and evaluate the applicability of both GRAdB and GRAdC in the context of oncolytic virotherapy.

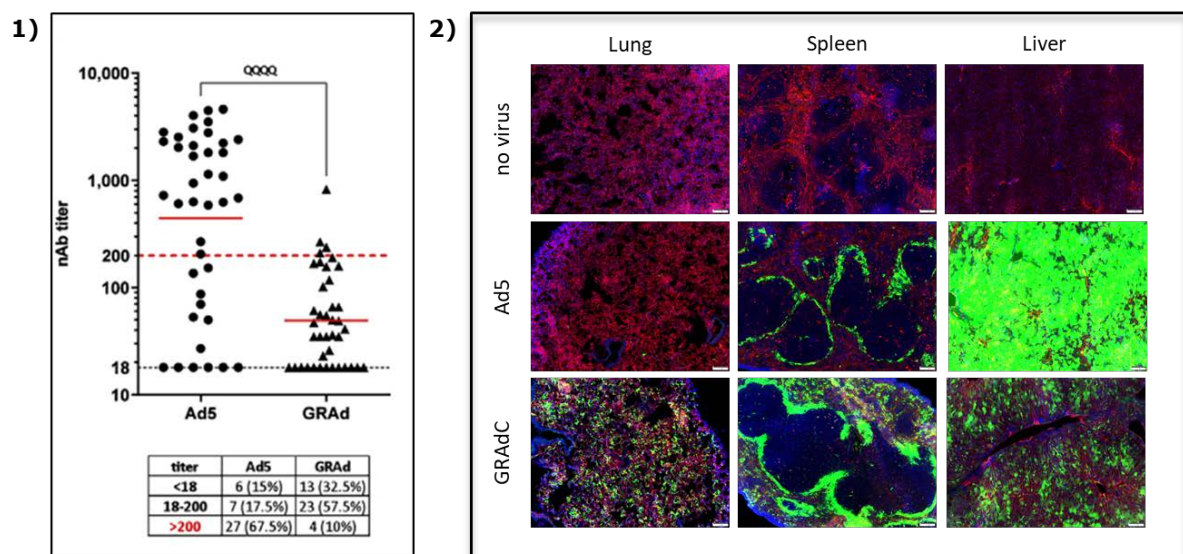


Figure 12 GRAdC Seroprevalence and Biodistribution.

1) The graph shows the neutralizing antibody (nAb) titers for adenovirus 5 (Ad5) and GRAd vectors in sera collected from a cohort of human healthy donors of US origin, demonstrating significantly lower nAb levels against GRAd, which reflects reduced pre-existing immunity compared to Ad5. The table below summarizes the distribution of nAb titers for the two vectors [75]. 2) The images illustrate viral particle distribution within several tissues, as visualized through immunofluorescence staining. GRAd vectors display a natural tropism for the lungs and reduced localization to the liver compared to Ad5.

5. Current Knowledge on EV20 and Its Therapeutic Potential

EV20, a humanized monoclonal antibody targeting the ErbB-3 receptor, represents a significant advancement in targeted cancer therapy. ErbB-3, a member of the epidermal growth factor receptor (EGFR) family, is frequently overexpressed in various malignancies and closely associated with therapeutic resistance, particularly through the activation of the PI3K/Akt pathway. The murine antibody MP-RM-1, which initially demonstrated promising efficacy against this receptor, was humanized into EV20 to improve its clinical applicability by reducing immunogenicity and enhancing its ability to internalize into tumor cells. The

humanization process involved grafting murine variable regions onto a human antibody framework, ensuring high affinity and specificity were retained [77].

EV20 exhibits multifaceted antitumor activity. As a naked antibody, it effectively blocks both ligand-dependent and independent ErbB-3 signaling, resulting in rapid receptor downregulation and suppression of downstream oncogenic pathways. Preclinical studies in xenograft models have demonstrated a significant reduction in tumor growth across various solid tumors, including melanoma, breast, ovarian, and pancreatic cancers[77]. However, the therapeutic innovation of EV20 is further extended through its conjugation with cytotoxic agents, such as saporin or monomethyl auristatin F (MMAF), to generate potent antibody-drug conjugates (ADCs)[78], [79]. These ADCs leverage EV20's specificity for HER3 to deliver cytotoxic payloads directly to tumor cells, demonstrating highly targeted activity with reduced systemic toxicity. For instance, EV20-MMAF has shown robust and selective cytotoxic activity against resistant melanoma and breast cancer cells, underscoring its capacity to overcome tumor escape mechanisms[79].

The therapeutic versatility of EV20 is further highlighted in combination with other targeted therapies, such as the third-generation EGFR tyrosine kinase inhibitor Osimertinib, particularly relevant in cases of resistant non-small-cell lung cancer (NSCLC). While Osimertinib is initially effective, it often induces compensatory HER3 upregulation, creating a vulnerability that EV20 can exploit. The combination enhances tumor cell elimination and activates immune-mediated mechanisms, including macrophage involvement and STING (Stimulator of Interferon Genes) pathway activation, amplifying antitumor effects. This innovative approach integrates targeted therapy with immunotherapeutic mechanisms to overcome advanced therapeutic resistance[80].

Ultimately, EV20 and its derivatives represent a groundbreaking model in cancer therapy, combining targeted specificity, therapeutic flexibility, and the ability to integrate into combination strategies. Its remarkable potential as a standalone therapy, coupled with its capacity to enhance the efficacy of other treatments, positions it as a cornerstone for developing advanced solutions against resistant malignancies. Inspired by these exceptional properties, we incorporated the coding sequence of EV20 into the genome of GRAd vectors to investigate the direct production of a fully functional antibody by the virus within tumor cells. The results obtained from this innovative approach are discussed in this thesis.

Aim of this thesis

Given the ongoing need for new therapies to treat advanced malignancies, recent years have seen a shift in focus toward developing more effective and selective treatments to address the limitations of current cancer therapies. Among these innovations, OAdVs—particularly HAdVs—have garnered significant interest due to their ability to selectively target and destroy tumor cells while potentially stimulating antitumor immunity.

In this context, the primary aim of this thesis is to explore and advance the application of proprietary gorilla-derived adenoviruses (GRAd) from ReiThera, specifically GRAdB and GRAdC, as innovative candidates for oncolytic virotherapy. This work seeks to address key challenges associated with existing adenoviral platforms, such as off-target effects, preexisting immunity, and limited tumor specificity. By leveraging the unique properties of GRAd vectors and employing advanced genetic engineering strategies, the objective is to enhance tumor selectivity, improve therapeutic efficacy, and broaden the potential clinical applications of these vectors, which have so far been utilized primarily as a vaccine platform.

A significant focus of this thesis is the evaluation of the replication dynamics and tropism of the GRAdB and GRAdC serotypes, investigating their distinct behaviors in both tumor and normal cells. Additionally, the research explores the potential of GRAd vectors to act as biofactories for therapeutic payloads, exemplified by the integration of the anti-HER3 antibody EV20. By incorporating such therapeutic payloads into the viral genome, this work aims to provide proof of concept for a dual-action therapeutic strategy that combines direct oncolysis with the localized production of immunomodulatory agents within the TME.

Ultimately, this thesis aspires to establish GRAd-based vectors as versatile and innovative tools in the field of oncolytic virotherapy, offering a safer and effective alternative for the treatment of refractory cancers while contributing to the development of next-generation cancer therapeutics.

Materials and Methods

1. Bacterial Manipulation

To obtain a sufficient quantity for manipulation, plasmid DNA was amplified in bacteria. For this purpose, we used the *Escherichia coli* (*E. coli*) strain DH5 α to amplify plasmids sourced from an external provider (Biofab), and the strain SW102 to engineer Bacterial Artificial Chromosome (BAC) plasmids containing the GRAd genome, incorporating the desired modifications through recombineering.

1.1 Bacterial Transformation

Plasmids containing the EV20 antibody sequence in both full-length (FL) and single-chain (SC) formats, flanked by homology sequences with the E3 region of GRAdB, were ordered from Biofab, our external plasmid synthesis and sequencing provider. To obtain a sufficient quantity for our experiments, 100 ng of plasmid DNA was used to transform commercial DH5 α bacterial cells via heat shock. The protocol was as follows: 50 μ l of competent cells were thawed on ice for 10 minutes, followed by the addition of the required amount of plasmid DNA. The mixture was incubated on ice for 30 minutes, then heat-shocked for 1 minute at 42°C, and placed back on ice for 5 minutes. Approximately 650 μ l of LB Broth was added, and the cells were grown for 1 hour at 37°C with shaking. 100 μ l of the transformation mixture was then plated on petri dishes containing LB agar and the appropriate antibiotic (Ampicillin-Amp) to allow growth of only the transformed cells carrying the plasmid of interest. The plates were incubated overnight at 37°C. The next day, a single colony from each transformation was inoculated in 300 ml of LB medium supplemented with the appropriate antibiotic. This bacterial culture was used for plasmid DNA extraction via Maxiprep, as described later.

1.2 Recombineering

In this work, we employed homologous recombination in bacteria to introduce all desired modifications into the GRAd genome. Recombineering involves using the *E. coli* SW102 strain, which carries a BAC containing the GRAd genome along with Chloramphenicol (Chl) resistance. The SW102 strain harbors a defective phage that expresses the lambda red genes, which are temperature-sensitive: they remain inactive when grown at 32°C but can be activated by heating the bacteria for 15 minutes at 42°C, promoting homologous

recombination between the BAC and an exogenous DNA sequence that has homology arms of 25 to 80 bp. The recombination process consists of two steps. In the first step, a selection cassette containing ampicillin resistance (Amp^R) and the SacB gene (conferring sucrose sensitivity - Amp^RSacB) is inserted into the target region of the virus for positive selection. After electroporation, plating the bacteria on LB agar supplemented with Chl and Amp allows for the selection of only those clones that have integrated the selection cassette. In the second step, the negative selection conferred by the SacB gene is used. The bacteria selected in the first recombineering step are electroporated with the DNA of interest, which is flanked by homology arms targeting the region where the Amp^RSacB cassette is located. At this point, only clones that have lost the SacB gene will be able to grow on LB agar plates containing Chl and sucrose, allowing selection of the bacteria that have acquired the DNA of interest.

The procedure was carried out according to the following protocol. Bacteria containing the BAC-GRAd were inoculated into 10 ml of LB medium supplemented with Chl and incubated overnight at 32°C with shaking. The next day, 1 ml of the overnight culture was inoculated into 50 ml of LB + Chl and incubated at 32°C with shaking until it reached an OD₆₀₀ of 0.5-0.6. At this point, 25 ml of the culture was transferred to a Falcon tube and subjected to heat shock at 42°C for 15 minutes in a water bath, followed by cooling on ice for another 15 minutes. From this point onward, all steps were carried out between 0°C and 4°C to ensure successful transformation. The culture was centrifuged for 5 minutes at 3750 RPM (revolutions per minute), the supernatant discarded, and the pellet washed with 25 ml of cold double-distilled water (ddH₂O). This process was repeated twice more, and after the final step, the pellet was resuspended in approximately 300 µl of ddH₂O. 50 µl of cells were transformed via electroporation with 200-400 ng of DNA corresponding to the Amp^RSacB cassette, which was obtained by Polymerase chain reaction (PCR) from an expression plasmid and carried homologous arms for the targeted region. The bacteria were then supplemented with 1 ml of LB and grown for 1 hour at 32°C with shaking. 100 µl of the culture were plated on LB agar supplemented with Chl and Amp, and incubated overnight at 32°C. The next day, the clones were screened by colony PCR to select positive clones for the next step.

The second step involved a similar procedure. Positive clones selected from the first step were inoculated into 10 ml of LB + Chl and grown overnight at 32°C. The next day, the bacteria were

made competent as described earlier. The DNA of interest (either a PCR amplicon, an insert obtained by plasmid digestion, or a single-stranded oligo - ssOligo) was added to the cells in amounts ranging from 200 to 400 ng, and the transformation was performed via electroporation. After approximately 3 hours of post-transformation growth, 100 and 200 µl of the culture were plated on LB agar containing sucrose and Chl, and incubated overnight at 32°C. The following day, the positive clones were screened by both colony PCR and streaking on Chl/Amp plates to exclude the presence of mixed clones or mutations in the SacB gene, which would impair counter-selection on sucrose. The positive clones were inoculated into 300 ml of LB + Chl to perform the Maxiprep. After confirming the mutation by sequencing, the plasmids were used for transfection.

1.3 Plasmid DNA Extraction from Bacteria

Plasmid DNA was obtained from 300 ml of bacterial culture grown in LB medium supplemented with the appropriate antibiotic. The plasmid DNA (Maxiprep) was prepared using the QIAGEN Plasmid Maxi Kit (QIAGEN), following the manufacturer's instructions.

2. Cell Culture

2.1 Cell Lines

The cell lines used in this study are listed in the Table 3 below:

Table 3 Cell lines used in this thesis

Cell line	Tumor type/Tissue	Origin	Medium	FBS	Supplement
A549	Lung cancer	Human	DMEM	10%	L-Glu, PS
NCI-H727	Lung cancer	Human	RPMI	10%	L-Glu, PS
NCI-H1975	Lung cancer	Human	RPMI	10%	L-Glu, PS
NCI-H1299	Lung cancer	Human	RPMI	10%	L-Glu, PS
GCh6	Glioblastoma (patient derived)	Human	DMEM	10%	L-Glu, PS
GCh14	Glioblastoma (patient derived)	Human	DMEM	10%	L-Glu, PS
U87MG	Glioblastoma	Human	DMEM	10%	L-Glu, PS
A375m	melanoma	Human		10%	
MRC5	Lung fibroblast	Human	DMEM	10%	L-Glu, PS

HUVEC	Umbilical cord	Human	DMEM F12K nut mix	10%	Heparin, ECGS
Mesenchymal	Human Amniotic Mesenchymal Stromal Cells	Human	CHANG medium	-	L- glutamina 2mM

The cancer cell lines A549, NCI-H727, NCI-H1975, and NCI-H1299, as well as the normal cell lines MRC5 and HUVEC, were purchased from the American Type Culture Collection (ATCC). The glioblastoma cell lines, both patient-derived (GCh6 and GCh14) and commercial (U87MG), along with the melanoma cell line (A375m), were provided by Professor Gianluca Sala (University "G. D'Annunzio" Chieti–Pescara, Italy). The Human Amniotic Mesenchymal Stromal Cells (Mesenchymal) were kindly provided by Dr. Ornella Parolini (Institute of Human Anatomy and Cell Biology, Catholic University of the Sacred Heart, Rome, Italy).

All cell lines were grown in their respective culture media: DMEM, RPMI, DMEM/F12K Nut Mix (all from Gibco - Thermo Fisher Scientific), or CHANG Medium (FUJIFILM IRVINE SCIENTIFIC). The media were supplemented with 10% Fetal Bovine Serum (FBS) (Gibco - Thermo Fisher Scientific), 2 mM L-Glutamine (Gibco - Thermo Fisher Scientific), 1% Penicillin-Streptomycin (PS, Gibco - Thermo Fisher Scientific), 0.1 mg/ml Heparin (Sigma), and 0.03 mg/ml Endothelial Cell Growth Supplement (ECGS, Sigma), as appropriate.

2.2 Maintenance of Cell Cultures

All cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO₂, using the appropriate culture medium. When cells reached approximately 90% confluence, they were washed with 1X Phosphate-Buffered Saline (PBS - Gibco) and detached using TrypLE Select (Gibco) after a 5-minute incubation at 37°C. Cells were then resuspended in a final volume of 15 ml of the appropriate culture medium and subcultured at ratios ranging from 1:2 to 1:10, depending on the cell type (lower splitting ratios for normal cells and higher ratios for cancer cells).

3. Engineering the GRAd Genome

3.1. Vector Construction

All modifications to the GRAd genome described in this thesis were introduced at the plasmid level using recombineering, as previously described. We already possessed vectorized

versions of both the GRAdC and GRAdB viruses (pGRAdC and pGRAdB), appropriately transformed into the *E. coli* strain SW102. The Amp^RSacB selection cassette was amplified by PCR from an expression plasmid using various primers, each containing homologous arms for the target insertion region. In the second recombineering step, either ssOligos were used to introduce deletions, or regions were PCR-amplified from the GRAdC or GRAdB genome depending on the intended substitution. Upon completion of homologous recombination, all plasmids underwent restriction analysis and sequencing for verification. Below are the details of all the modifications made, along with the primers used.

Table 4 Primers used for modifying the GRAd genome

Primer description	Primer sequence 5' → 3'	Recombineering step	Modification type
Amp in CR2 FW	Ggagagcggcagaggtgggaaaaaattgcctgaacttggtgctgctgaaatggacacccctattgtttatttttcta	1	GRAdB Δ24
Amp in CR2 RV	Ccgctgcggctcggatcgactgcacattttcctcctctcatcatcactcggaggtatttgtaactgttaattgtc		
CR2 Δ24	tgaacttggtgctgctgaaatggaccctccgagtgatgatgaagaggagg	2	
Amp in E3 CR1 βγδ FW	aaaagagaaaaatgccctgacattttttcctcacttttgttttacagacaatgaccctatttgtttatttttcta	1	GRAdB ΔE3
Amp in E3 CR1 βγδ RV	ccatactgtaaaagaaaagaatagatcttagatttcattaaagccatgtgaggtttatttgtaactgttaattgtc		
ssOligo ΔE3	aaaaactgagttcagaccctcctacatttaaaccacgcctctcatatgagctcggtc	2	
Swap GRAdC CR1 βγ FW	Aaaagagaaaaatgccctgacattttttcctcacttttgttttacagacaatgatgattggaatcaccctact	2	GRAdB with GRAdC E3 CR1βγ
Swap GRAdC CR1 βγ RV	ccatactgtaaaagaaaagaatagatcttagatttcattaaagccatgtgaggtttaccatgctgtaaaagaga		
Amp in pV GRAdB FW	acttagaagatactgagcagtcctccgatgtgtgtcccagcggcgaggatttattgttaactgttaattgtcct	1	GRAdB ΔpV
Amp in pV GRAdB RV	cgctgtggcgacgcacatccgcgccccaacatcctctacggcgaggtaccctattgtttattttctaaa		
GRAdB ΔpV ssOligo	Tctccgatgtgtgtcccagcggcgaggattgactgttgccgctgcctccttgagatatggccctcacttgccgcttcgctccccattactgaataaccgaggaataaactcgcgccgtagaaggatgttggggcgc	2	
Amp in pV GRAdB bis FW	Ccctgcacttagaagatactgagcagtcctccgatgtgtgtcccagcggcgaggaccctattgtttatttttctaa	1	GRAdB pVGRAdC
ALS in pV GRAdB bis RV	Gacgcgaaggcggcaagtgaaggccatatctgcaaggaggcagcggcaacagtcattattgttaactgttaattgtcc		
GRAdC pV in GRAdB FW	Ccctgcacttagaagatactgagcagtcctccgatgtgtgtcccagcggcgaggatgtccaagcgcaaaattaaag	2	

GRAdC pV in GRAdB RV	gacgcgaaggcggcaagtgagggccatatctgcaaggaggcagcgggaacagtcattaacaatgctgggggtgtag		
Amp in knob FW	aagctttgataattcaggagccattgtggtgggaacaaaaatgatgacaaacttaccctatttgtttatttttctaa	1	GRAdC Fk B
Amp in knob RV	aaataaataaaagcacacaattttgaaatcaaaaacaagcatctctgtgctttctttattgttaactgttaattgtcc		
GRAdB Knob in GRAdC fiber FW	Taagctttgataattcaggagccattgtggtgggaacaaaaatgatgacaaacttacactatggacgggcgcaa	2	
GRAdB Knob in GRAdC fiber RV	ataaataaaagcacacaattttgaaatcaaaaacaagcatctctgtgctttctcagtcattcttctaattgtaag		
Amp in GRAdC CR2 FW	gccggagcccagtggtataccttatatgcctgaactgcttcccgaagtggtagaccccctatttgtttatttttctaa	1	GRAd C Δ24 Fk B
Amp in GRAdC CR2 RV	caggtatctcagcatagtctaaagcaaaaggctcacccatcgtcgtgggcggttattgttaactgttaattgtcc		
GRAdC Δ24 ssOligo	agcccagtggtataccttatatgcctgaactgcttcccgaagtggtagacccgccagcgacgagtgggtgagccttttgccttagactatgctgagat	2	

3.1.1 GRAdB Variants

GRAdB Δ24

The region corresponding to the Rb-binding domain within the E1A gene was identified based on amino acid sequence homology with HAdV5. Consequently, the 24 nucleotides corresponding to the LHCYEEGF amino acid sequence in the CR2 region of the pGRAdB plasmid were first replaced with the Amp^RSacB cassette and subsequently deleted using a ssOligo, resulting in pGRAdB Δ24.

GRAdB ΔE3

In this case, the deletion region was selected based on the E3 deletion in GRAdC, which had been successfully utilized in our experience engineering this virus for vaccine applications. To achieve this, oligos were designed with homologous arms corresponding to the E3 12.5K and E3 14.7K regions, replacing the intervening sequence with the Amp^RSacB cassette. As in the previous modification, the use of a ssOligo in the second recombination step enabled the desired deletion (pGRAdB ΔE3).

GRAdB Δ24 ΔE3

The combination of these two deletions was achieved by subjecting the pGRAdB Δ24 vector to an additional recombineering step using the same inserts as those for pGRAdB ΔE3, resulting in the pGRAdB Δ24 ΔE3 vector.

GRAdB with GRAdC E3 CR1 δ

Through sequence alignment of the E3 regions of GRAdC and GRAdB, we identified an additional open reading frame (ORF), CR1 δ , in GRAdB. We decided to replace the native GRAdB ORFs with those of GRAdC in hopes of improving its selectivity. The intermediate recombineering product containing the Amp^RSacB cassette in E3 was used, which was then replaced by the E3 CR1 $\beta\gamma$ ORFs of GRAdC obtained by PCR, resulting in the plasmid pGRAdB with GRAdC E3 CR1 $\beta\gamma$.

GRAdB Δ pV

We aimed to replicate a deletion of the pV protein, known in the literature for enhancing the replication of HAdV5 in tumor cells. This modification involved the complete deletion of pV along with the introduction of suppressor mutations in the precursor region of polypeptide X (Mu). The Amp^RSacB cassette was inserted between nucleotides 16331 and 17460, and the second recombination step achieved both the deletion and the insertion of mutations 17447-17448 (G to A and C to A; G13E) and 17459 (G to T; R17I), resulting in pGRAdB Δ pV.

GRAdB pV^{GRAdC}

As an alternative approach, we aimed to replace the pV protein of GRAdB with that of GRAdC. In this case, the L2 V region in the plasmid was replaced by the Amp^RSacB cassette in the first recombination step. The L2 V region from GRAdC, amplified by PCR, was subsequently inserted in place of the selection cassette, resulting in the plasmid pGRAdB pV^{GRAdC}.

3.1.2 GRAdC Variants

GRAdC Fk B

For the construction of GRAdC with a chimeric fiber, the region corresponding to the knob was replaced by the Amp^RSacB cassette. The GRAdB knob was amplified by PCR and used in the second recombineering step. These two homologous recombination steps led to the construction of the chimeric pGRAdC Fk B.

GRAdC Δ 24 Fk B

The pGRAdC Fk B vector was subjected to an additional two recombineering steps to delete the region corresponding to the Rb-binding domain within the E1A gene. The Amp^RSacB cassette replaced the 24 nucleotides corresponding to the LTCHEPGF amino acid sequence in the CR2 region. A ssOligo was used in the second recombination to achieve the desired deletion, generating the plasmid pGRAdC Δ 24 Fk B.

4. GRAd Expressing the EV20 Antibody

4.1 Antibody Design

The sequence of the humanized anti-HER3 antibody, EV20, was provided by Prof. Gianluca Sala. To test the antibody's expression by the GRAd vectors, we chose to insert it into the virus's E3 region without adding an exogenous promoter. We designed two constructs: one encoding the FL antibody and the other encoding only the variable regions of the light and heavy chains complexed with the Fc domain, referred to as the SC version.

In the FL construct, the two chains were joined using an IRES to allow the expression of both chains from a single transcript. The bovine growth hormone polyadenylation (bgh-PolyA) signal was added downstream of the antibody sequence as a termination signal. For the SC construct, the variable regions of the light and heavy chains were connected with a linker consisting of the amino acid sequence "ADAAPTVSGSGGGSGGGSGGGS." The bgh-PolyA signal was also added after the Fc domain in this construct. For both versions, 50-nucleotide homology arms were added to the sequence upstream and downstream of the portion remaining after the E3 deletion in GRAdB: the left homology arm in the E3 CR1 α region and the right homology arm in the E3 14.7k region. Unique restriction sites (PacI and EcoRV) were introduced at the ends of the cassette to facilitate the extraction of the insert for recombineering through digestion. These sequences were synthesized within a shuttle plasmid by our provider, Biofab, and subsequently amplified as previously described.

4.2 Vector Construction

The GRAd vectors expressing the EV20 antibody were obtained through recombineering. In the case of GRAdB, we could proceed directly with the second step using the intermediate vector containing the Amp^RSacB cassette in E3 (created to generate pGRAdB Δ E3). Since GRAdC FkB was only available in its "full genome" form, we performed the standard two-step recombineering process. The detailed procedures and the sequences of the primers used are outlined below:

Table 5 Primers used for inserting the EV20 antibody into the GRAd genome

Primer description	Primer sequence 5' $\rightarrow$ 3'	Recombineering step	Modification type
Amp in E3 Fk B Fw	ctgtcatttgtgtgctgagtataataaaggctgagatcagaatctactcgacccctatttgttta tttttctaa	1	GRAdC Fk B EV20 SC
Amp in E3 Fk B Rw	agtgattttttattgattacagtatgatcaattgaaaggataaggtcTTatttgttaactgtt aattgtcc		
EV20 in GRAdC E3 FW	Tgtcatttgtgtgctgagtataataaaggctgagatcagaatctactcgccaccatggaatc tcagacacaggt	2	

EV20 in GRAdC E3 RV	caagtaagtgattttttattgattacagttatgatcaattgaaagggataaggtcccatagagc ccaccgcat		
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GRAdB EV20 FL and SC

To generate GRAdB expressing the FL and SC versions of the EV20 antibody, we used the previously constructed recombineering intermediate (pGRAdB Δ E3) containing the Amp^RSacB cassette in E3. The plasmids containing the two antibody versions were digested with PacI and EcoRV to prepare the insert, which was then used in the second step of recombineering to produce pGRAdB EV20 FL and pGRAdB EV20 SC. The plasmids were verified by restriction analysis before being used for transfection.

GRAdC FkB EV20 SC

The pGRAdC FkB construct underwent additional homologous recombination steps to insert the SC version of the EV20 antibody into the E3 region. In the first step, the Amp^RSacB cassette replaced the E3 region of the virus. The insert for the subsequent recombination step was prepared by PCR, allowing for the incorporation of homology arms for GRAdC at the ends of the cassette. The recombined plasmid was identified through colony PCR and verified by restriction analysis and sequencing. The pGRAdC FkB EV20 SC plasmid was then amplified and used for transfection.

5. Growth, Amplification, and Purification of GRAds

5.1 Rescue of GRAds by Transfection

Once the viral DNA has been engineered with the appropriate modifications through bacterial recombination, the next step is to grow the viruses in cells. Since these are replication-competent viruses, we decided to grow them in the same cell line from which they were originally isolated, A549 cells. To do this, the amplified and extracted plasmid DNA was digested with the PmeI enzyme, which removes the plasmid portion and exposes the viral ITRs that serve as the origin of replication for viral DNA synthesis. The linearized DNA was then introduced into A549 cells via transfection using Lipofectamine 2000 (Invitrogen-Thermo Fisher Scientific). Lipofectamine is a lipid-based transfection reagent that forms liposomes in aqueous environments, trapping the DNA. The DNA-containing liposomes (positively charged on their surface) can fuse with the negatively charged plasma membrane of living cells, allowing the nucleic acid molecules to enter the cytoplasm for replication.

The transfection protocol used in this study followed these steps: 8 µg of digested DNA and 16 µl of Lipofectamine were each diluted separately in 500 µl of non-supplemented DMEM. The two mixtures were combined and incubated for 20 minutes at room temperature (RT). The mixture was then added to A549 cells, which had been cultured in a T25 flask at 60-80% confluency after a media change with non-supplemented DMEM. The following day, the supernatant in the flask was removed and replaced with complete DMEM (containing 10% FBS). The day after the media change, the cells were split at a 1:3 ratio into T75 flasks. The transfection was left to proceed until the cells reached a complete cytopathic effect (CPE), which typically occurred 7-10 days post-transfection.

At this point, both the cells and the supernatant were collected and subjected to three cycles of freezing and thawing to ensure the complete release of viral particles from the cells. The cell lysate was then centrifuged for 10 minutes at 2000 RPM, generating the clarified lysate P0 (initial rescue passage), which was used for the subsequent amplification cycle of the virus.

5.2 Amplification of GRAds

The P0 obtained from the transfection serves as the starting point for virus amplification, both in terms of quantity and quality. As amplification progresses through the different stages, the number of viral particles increases, and the proportion of non-encapsidated virus decreases compared to mature virus (detected by droplet digital PCR – ddPCR - with or without nuclease treatment before processing, which releases the viral DNA, as described later). In this thesis, for the first amplification step, we infected A549 cells in a T75 flask with 5 to 7 ml of P0 lysate at an uncontrolled multiplicity of infection (MOI). When the cells showed a complete CPE (between 5 and 7 days post-infection), they were harvested and subjected to three cycles of freezing and thawing, followed by clarification, resulting in the production of clarified P1 lysate (first amplification passage). The lysate was then quantified by ddPCR, and the next amplification cycle was initiated by infecting a larger number of cells. A T150 flask of A549 cells was infected with P1 lysate at an MOI of 100, and once again, the infection was allowed to proceed until a complete CPE was observed, which typically occurred after 2-3 days for GRAdB and 4-5 days for GRAdC. The cells were harvested and subjected to freeze-thaw cycles and clarification, generating the clarified P2 lysate (second amplification passage). At this point, the virus was quantified and could either be further amplified (P3), purified, or used in experiments as clarified lysate

5.3 Purification of GRAdS

The GRAdS were purified using the Vivapure® Adenopack 20 RT kit (Sartorius). This kit allows for column chromatography purification of up to 1.0×10^{12} group C adenoviral particles from a 20 ml culture. We efficiently used it to purify both GRAdC and GRAdB. The purification protocol followed in this thesis included the following steps: 18 ml of clarified lysate (if the volume was smaller, it was brought up to 18 ml by adding lysis buffer (Table 6); for larger volumes, we used more than one column to purify the same virus) was filtered through a $0.45 \mu\text{m}$ filter. The column was equilibrated with 5 ml of 1x wash buffer (prepared in water from a 10x stock solution) and centrifuged for 5 minutes at 500g force (g). To the 18 ml of filtered lysate, 2 ml of loading buffer was added drop by drop before being applied to the column and centrifuged for 5 minutes at 500g. The flow-through was discarded, and the column was washed twice with 18 ml of 1x wash buffer, followed by centrifugation for 5 minutes at 500g. Elution occurred in two consecutive steps by adding 1 ml of elution buffer to the column, incubating for 10 minutes at RT, and centrifuging for 5 minutes at 500g (final elution volume: 2 ml). The eluate was subjected to buffer exchange and concentration using Vivaspin® 20 devices provided in the kit. The 2 ml of eluate was brought up to 10 ml with the formulation buffer (A195 for GRAdC and A438 for GRAdB - Table 6), then centrifuged for 30-40 minutes at 500g until the volume was reduced to 1 ml. This step was repeated twice. Finally, 1 ml of purified virus was filtered through a $0.22 \mu\text{m}$ filter and quantified by ddPCR. The virus was then aliquoted into smaller volumes and stored at -80°C .

Table 6 Composition of buffers used for purification

Buffer	Composition
Lysis Buffer	10mM Tris pH 8 135mM NaCl 1mM MgCl_2
A195 Buffer pH 7.4	10mM Tromethamine (TRIS) 10 mM Histidine 5% sucrose 75 mM NaCl 1 mM MgCl_2 0.02% PS-80 0.1 mM EDTA 0.5% (v/v) Ethanol
A438 Buffer pH 6.6	10 mM Histidine 7.5% sucrose 35 mM NaCl 1 mM MgCl_2 0.1% PS-80 0.1 mM EDTA 0.5% (v/v) Ethanol

5.4 Viral Titration by Digital PCR (ddPCR)

The viral samples, either in the form of lysates or purified viruses, were titrated using ddPCR. This method employed primers and probes (labeled with FAM and HEX) targeting a conserved region of the hexon gene present in both GRAdC and GRAdB, allowing quantification of viral genomes (vg) or viral particles (vp) in the given preparation. ddPCR enables absolute quantification of nucleic acid target sequences by counting nucleic acid molecules encapsulated in water-in-oil droplets where PCR amplification occurs. The droplet reader then counts the fluorescent positive and negative droplets to calculate the concentration of the target DNA.

Samples were processed differently based on whether they were viral lysates or purified viruses. Lysates were further processed differently depending on the type of nuclease used, with each sample prepared in duplicates for both +/- nuclease data. For DNaseI (Roche), a mix was prepared containing 5 µl of virus, 10 µl of buffer, 5 µl of DNaseI or 5 µl of ddH₂O, and 75 µl of ddH₂O. The mix was incubated for 30 minutes at 37°C, followed by the addition of 5 µl of 0.5M EDTA (pH 8) and incubation at 80°C for 20 minutes to inactivate the enzyme. For SAN nuclease, the mix was prepared by adding 0.05 units/µl of enzyme to 100 µl of virus, and the mixture was incubated for 1 hour at 37°C. From this point onwards, both types of samples underwent the same processing: diluted 1:2 with 0.2% SDS and incubated for 10 minutes at 56°C to break the viral capsid and release the viral DNA. The sample was then diluted in ddH₂O to a final dilution factor of 1:4000 or 1:20000, depending on the amplification passage being quantified.

For purified virus samples, 10 µl of virus was diluted in 90 µl of 0.1% SDS and incubated at 56°C for 10 minutes. In this case, a series of 10-fold serial dilutions in ddH₂O were performed to reach dilution factors of 1:10000, 1:100000, and 1:1000000 before loading the samples onto the plate. For each sample, 5 µl was added to 15 µl of PCR reaction mix. The PCR mix was prepared with specific primers and probes for the GRAd hexon gene (listed below) and aliquoted into a 96-well plate:

PCR Mix Components (1X):

- ddPCR Bio-Rad Mix 2x: 10 µl
- Hexon Forward Primer (100 µM): 0.08 µl
- Hexon Reverse Primer (100 µM): 0.08 µl

- Hexon Probe (100 μ M): 0.04 μ l
- ddH₂O: 4.8 μ l

Primers and Probe Sequences (5'→3'):

- Hexon Forward Primer: GCTTCGGAGTACCTGAG
- Hexon Reverse Primer: GTGGTCACATCGTGGGTGGG
- Hexon Probe: CTGGTGCAGTTCGCCCCG

The plate was then transferred to a droplet generator, which partitioned each sample into 20,000 nanoliter-sized droplets. The plate was sealed and placed in a thermocycler to perform the following thermal PCR protocol:

- Stage 1: 2 min at 50°C
- Stage 2: 10 min at 95°C
- Stage 3: 15 sec at 95°C; 1 min at 60°C (40 cycles)

After the PCR reaction, the plate was transferred to the droplet reader, which produced a file containing the number of positive droplets (containing at least one copy of the target DNA) for each well. The file reported the concentration corresponding to vg (or vp)/ μ l and the total vg in each well (20 μ l). Considering the final dilution factor of the sample and the fact that 5 μ l of the sample was loaded into each well, the concentration of the original sample was calculated using the following formula:

$$vp/\mu l = \text{Total vp} \times \frac{\text{Dilution Factor}}{5 (\mu l)} \Rightarrow vp/ml = vp/\mu l \times 1000$$

6. Oncolytic Adenovirus In Vitro Assays

All our wild-type and engineered viruses were characterized in vitro through a series of experiments assessing their effects on cellular metabolism, replication, and production of newly formed viruses. Additionally, in some instances, optical microscopy images were included to visually demonstrate the CPE induced by the viruses. The tests performed are described below in detail.

6.1 MTS Assay

One of the primary parameters to evaluate the oncolytic properties of a virus is its effect on the cellular metabolism of both normal and tumor cells. To assess this, a cell proliferation assay was performed using the MTS assay (CellTiter 96® AQueous Non-Radioactive Cell

Proliferation Assay – Promega). This colorimetric test measures the number of living, proliferating cells through the bioreduction of the MTS tetrazolium salt into formazan by dehydrogenase enzymes in metabolically active cells. The amount of formazan produced, measured by absorbance at 490 nm, is directly proportional to the number of live cells in culture.

Before starting the main experiment, cells were seeded in 96-well plates at varying confluences to determine the optimal cell density for each cell line to allow the assay to proceed for 3 and 8 days, which were the selected time points. The chosen cellular confluences are listed in Table 7. On the day of the experiment, the 96-well plates were coated with 20 µl of Applied Cell Extracellular Matrix (ECM matrix – abm), and cells were seeded at 100 µl per well. That evening, cells were infected with the appropriate amount of virus to achieve the desired MOI (1000, 100, 10, 1, 0.1), with each infection performed in triplicate. The assay was developed on days 3 and 8 post-infection by adding 20 µl of MTS solution to each well and incubating for 1 hour at 37°C in the dark. The absorbance at 490 nm was measured using an EnSight plate reader. Results were expressed as a percentage of metabolic activity, setting the uninfected cells (mock) as 100% and calculating the ratio of the absorbance (OD490) from infected cells to that of the mock controls. The lower the resulting percentage, the higher the impact of the virus on cellular metabolism.

Table 7 Confluence of cell lines for the MTS assay

Cell line	Confluence (cells/well) → Day 3 post infection	Confluence (cells/well) → Day 8 post infection
A549	1.1E+04	3.7E+03
NCI-H727	3.3E+04	1.1E+04
NCI-H1975	1.0E+04	3.3E+03
NCI-H1299	3.7E+03	1.2E+03
GCh6	1.0E+04	1.1E+03
GCh14	1.0E+04	3.3E+03
U87MG	1.0E+04	2.2E+03
MRC5	1.1E+04	7.3E+03
HUVEC	6.7E+03	4.4E+03
Mesenchymal	6.6E+03	3.3E+03

6.2 Infectivity Assay

This technique is based on detecting hexon-positive cells through immunostaining in monolayers of both tumor and normal cells, which are infected with different viruses (either purified or as lysates) at various MOIs (100, 10, 1, 0.1). This assay allows to determine the extent to which the virus replicates its viral genome within different cell lines. Specifically, a

higher number of counted spots in a well indicates greater replication capacity for that particular virus in the specific cell line.

On the day of the assay, 96-well plates were coated with 20 μ l of ECM matrix, and all cell lines were seeded at 2.1×10^4 cells/well in a final volume of 100 μ l. That evening, the viruses were diluted in 100 μ l of the appropriate culture medium (depending on the cell type being infected) to achieve the desired MOI, with each infection performed in triplicate. Plates were then incubated at 37°C in a humidified atmosphere with 5% CO₂ for 48 hours. After incubation, the medium was aspirated, and cells were fixed and permeabilized with 50 μ l of cold methanol, incubated at -20°C for 20 minutes. The methanol was then removed, and plates were left to dry and stored at -20°C until assay development. For development, plates were washed three times with PBS 1X, blocked with 100 μ l/well of 1% Bovine Serum Albumin (BSA) in PBS 1X for 1 hour at RT, and then incubated for another hour with the primary anti-Hexon antibody (AbCAM cat N° BO25 AD 05) diluted 1:400 in PBS 1X with 1% BSA (100 μ l/well). After washing three times with PBS 1X, cells were incubated for 1 hour with an anti-mouse AF647 secondary antibody (AlexaFluor647 goat anti-mouse, Invitrogen A21235) diluted 1:500 in PBS 1X with 1% BSA. Finally, cells were washed three times, PBS 1X was removed, and the plates were read with a CTL Immunospot reader, which provided the number of spots counted per well.

6.3 Productivity Assay

This assay was designed to measure the quantity of newly produced virus (wild-type and/or engineered) after controlled MOI infection in both tumor and normal cell lines. A preliminary experiment was conducted to determine the optimal cell seeding density for each cell line tested (see Table 8). Cells were seeded in 24-well plates coated with 70 μ l of ECM matrix in a final volume of 1 ml per well. In the evening, cells were infected with different viruses at the desired MOI (1000 and 100) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 3 and 8 days, with each infection performed in duplicate. After incubation, cells and supernatants were collected, lysed, and clarified through three cycles of freeze-thawing followed by centrifugation. The clarified lysate was quantified by ddPCR, and the results were expressed as productivity (vp/cell), taking into account the physical titer obtained from the experiment and the number of cells per well on the day of infection, calculated with the formula:

$$\text{vp/cell} = \frac{\text{Total vp}}{\text{Total cells}}$$

Table 8 Confluence of cell lines for the productivity assay

Cell line	Confluence (cells/well) → Day 3-8 post infection
A549	2.6E+05
NCI-H727	4.6E+05
NCI-H1975	2.2E+05
NCI-H1299	3.2E+05
GCh6	2.1E+05
GCh14	1.8E+05
U87MG	2.7E+05
MRC5	6.0E+04
HUVEC	6.7E+04
Mesenchymal	1.3E+05

6.4 Microscopy Analysis

6.4.1 Evaluation of Cytopathic Effect

The CPE refers to the morphological and structural changes that cells undergo following viral infection. These changes are clearly visible under a microscope. In several cases, we captured microscopic images to provide a qualitative assessment of the effect of our GRAd viruses on normal cells. MRC5 and/or HUVEC cells were plated at a known confluence in 6-well or 24-well plates and infected with GRAd C and B, either wild-type or engineered, at MOIs of 100 and/or 1000. Images were taken at 3 and/or 8 days post-infection. The CPE of each GRAd variant was then compared to its wild-type counterpart and to the mock.

6.4.2 GFP Expression

In this experiment, we used GRAdC ΔE1 GFP, a replication-defective virus where Green Fluorescent Protein (GFP) expression is under the control of a constitutive exogenous promoter, to evaluate its ability to enter cells where its wild-type version is unable to replicate, such as MRC5 cells. We plated 1.0E+06 MRC5 cells and A549 cells (used as a positive control) in 6-well plates and infected them with the virus at MOIs of 100 and 1000. Images were taken 48 hours later in both bright field and GFP signal.

7. GRAd-derived EV20 Antibody Expression and Characterization

7.1 Expression by Western Blot Analysis

These experiments were conducted to evaluate the expression of the EV20 antibody, in both FL and SC forms, by GRAdB EV20 FL, GRAdB EV20 SC, and GRAdC EV20 SC in lung tumor cells (A549, NCI-H727, and NCI-H1975) and in MRC5 cells. In the morning, we plated 1.0E+06 lung tumor cells and 5.0E+05 MRC5 cells in 6-well plates. In the evening, the cells were infected

with various viruses at different MOIs (1, 10, 50, and 100). 48 hours post-infection, the supernatants were collected and centrifuged at 4000 RPM to remove cell debris. The samples were then used for Western blot analysis. A total of 26 μ l of supernatant was mixed with 10 μ l of NuPAGE™ LDS Sample Buffer (4X) and 4 μ l of NuPAGE™ Sample Reducing Agent (10X) in a final volume of 40 μ l, denatured at 99°C for 10 minutes, and loaded onto NuPAGE® 4-12% Bis-Tris gradient gels for SDS-PAGE. The gel was transferred onto a nitrocellulose membrane and incubated for one hour with a blocking solution (5% dried milk in PBS 1% + Tween20 0.1%). After three washes with PBS 1% + Tween20 0.1%, the membrane was incubated for one hour with an anti-Human secondary antibody diluted 1:20000 in the washing solution. After another three washes, the signal was detected using Pierce™ ECL Western Blotting Substrate and analyzed using the ChemiDoc image system (Bio-Rad).

7.2 EV20 Antibody binding capacity

7.2.1 ELISA

For the evaluation of in vitro binding affinity of EV20 antibodies contained in supernatant from adenoviral transduced cells, Maxisorp 96-well plates (Nunc, Thermo-Fisher) were coated with ECD (HER3 C-terminal domain) [1 μ g/mL, 1:100 in PBS] overnight at 4°C. After blocking with 1% BSA in PBS for 1 hour at RT, 100 μ l of 1:2, 1:5, 1:10 diluted (in PBS) adenoviral supernatants were added and incubated for 1 h at RT.

For the detection, after three washes with PBS-0.05% Tween20, anti-human IgG-HRP (#A01070; Sigma-Aldrich) was added (1:5000) and incubated for 1 hour at RT. After three washes, stabilized chromogens were added for at least 10 minutes in the dark before stopping the reaction with the addition of 1N H₂SO₄. The resulting color was finally read at 450nm with an ELISA plate reader (Tecan, Switzerland).

7.2.2 FACS Assay

For the evaluation of HER3 binding by EV20 antibody contained in supernatant from adenoviral transduced cells, 3.0E+05 A375M cells were collected and incubated for 30 minutes at 4°C with supernatants 1:2 diluted. Supernatants were heated at 56°C for 30 minutes for adenovirus inactivation, then as control EV20 was heated also and used in all the experiments at the same concentration (10 μ g/mL). As positive control, 10 μ g/mL EV20 was used. Cells were then washed with PBS and incubated for 30 minutes at 4°C in the dark with

anti-human IgG AlexaFluor-488 conjugated secondary antibody (1:300, #A11013 Thermo-Fisher). Cells were analyzed using a FACS Canto II cytometer (BD Biosciences).

7.3 Impact of EV20-GRAd Derived Antibody on Cellular Signaling

2.0E+05 A375M cells were seeded in 6-well plates in complete medium. After 24 hours, the cells were starved overnight in serum-free medium. The day after, the cells were washed with PBS and then incubated for 2 hours with supernatant from adenoviral transduced cells (1:20 dilution in serum-free medium), with EV20 (10µg/ml) as positive control and with heated EV20 (10µg/ml). After the incubation, cells were washed with PBS and treated for 10 minutes with 10ng/ml Human Neuregulin-1 (NRG-1β - #5218, Cell Signaling) in serum-free medium.

Cells were lysed in RIPA buffer (50mM Tris/HCl pH 7.6, 150mM NaCl, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, 1mM EDTA pH 8) containing protease inhibitor cocktail (Sigma-Aldrich), phosphatase inhibitor cocktail (Roche, Thermo-Fisher), and Na₃VO₄ (Sigma-Aldrich). Lysates were clarified by centrifugation at 13000 RPM for 30 minutes at 4°C. Equal amounts of protein lysates (20 µg per sample), previously heated at 95°C for 5 minutes, were subjected to SDS-PAGE electrophoresis and then electro transferred to nitrocellulose membranes for Western blot analysis. Membranes were blocked for 1 hour at RT with 5% non-fat dry milk in PBS-0.1% Tween20. Membranes were then incubated overnight at 4°C with the following primary antibodies: Akt Antibody (#9272, Cell Signaling, Danvers, Massachusetts, USA), anti HER3/ErbB3 (D22C5, Rabbit mAb, #12708s, Cell Signaling), anti Phospho-HER3/ErbB3 (p-HER3 - Tyr1289, 21D3, Rabbit mAb, #4791s, Cell Signaling), anti Phospho-Akt (p-Akt - Ser473, D9E, Rabbit mAb, #4060, Cell Signaling), and anti-β-actin (mouse monoclonal; 1:40000, #A5441; Sigma-Aldrich). After three washes in PBS-0.1% Tween20, the membranes were hybridized with horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit or mouse; Biorad, CA, USA). Detection of signal bands was performed with Clarity Western ECL substrate (#1705061; Biorad). Images of membranes were acquired with a Uvitec Fire reader (Cambridge, UK) and analysed with Alliance Uvitec software (Cambridge, UK).

8. Expression of primary adenovirus receptors in cell lines

For the evaluation of CD46 and CAR cell surface levels, 2.0E+05 or 3.0E+05 cells (MRC5, HUVEC, A549, NCI-H1299, NCI-H1975, NCI-H727, U87MG, GCh6, GCh14, MIAPACA 2, PANC-1) were collected and incubated with anti-human CD46 (1:50; mouse monoclonal; #12239-MM05, Sino Biological) or with anti-human CAR (1:50; rabbit monoclonal; #10799-R271, Sino

Biological) for 30 minutes at 4°C. Cells were then washed with PBS and incubated for 30 minutes at 4°C in the dark with anti-mouse or anti-rabbit AlexaFluor-488 conjugated secondary antibodies (1:300; #A11070, #A11017, Thermo-Fisher). Cells were analyzed using a FACS Canto II cytometer (BD Biosciences).

Results

1. GRAd Wild-Type Characterization as Oncolytic Adenoviruses

ReiThera has never focused on exploring the potential use of its viruses in cancer therapy, which is why we decided to investigate whether the GRAd platform could be a viable alternative not only in the vaccine field but also for oncolytic virotherapy.

To do this, we used six replication-competent gorilla-derived adenoviruses (GRAd), two of which belonged to group C (C1 and C2) and four to group B (B1, B2, B3, and B4). We selected a panel of both tumor and non-tumor cell lines (Table 3) to conduct in vitro tests and proceeded with characterization tests following the natural infectious cycle of the virus as described below (Figure 13):

- **MTS Assay:** A cell proliferation assay that provided information on the effect of viruses on cellular metabolic activity.
- **Infectivity Assay:** An immunostaining assay targeting the hexon, a structural protein expressed only after viral DNA replication.
- **Productivity Assay:** A ddPCR-based assay to quantify the amount of mature virus produced.

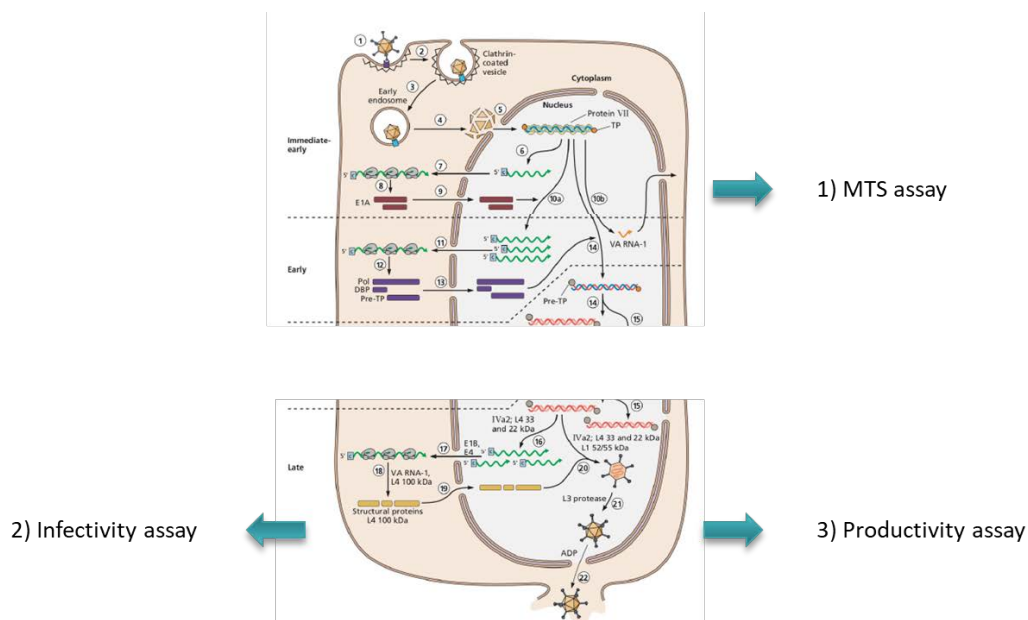


Figure 13 Adenovirus replication cycle and associated assays.

The key stages of the Adenovirus replication cycle. The Immediate-early and Early phases include viral entry, DNA replication, and early gene expression. During these phases, the MTS Assay was used to assess the impact of viruses on cell proliferation. The Late phase, characterized by the production of viral structural proteins, was monitored using the Infectivity Assay, which detects the expression of the hexon protein, indicating viral

replication. Finally, the Productivity Assay, based on ddPCR, was employed to quantify the production of mature viral particles, which occurs at the end of the replication cycle.

All experiments were conducted by testing the various GRAd viruses, with HAdV5 used as a control. For the MTS assay, cells were infected with GRAds and HAdV5 at different MOIs (1000, 100, 10, 1) and monitored at two different time points: 3 and 8 days post-infection (Figure 14). The desired outcome, if the viruses exhibit oncolytic properties, is a negative impact on the cellular metabolism of tumor cells (a percentage reduction in cellular metabolic activity) and no impact on normal cells. What was observed is that, in general, group B GRAd viruses had a more pronounced effect on all tumor cell lines compared to GRAdC, showing effects comparable to or even better than HAdV5. Moreover, the impact increased over time and with higher MOIs. However, the same effect was also observed in normal cells, with the exception of mesenchymal cells. In contrast, GRAdC viruses only affected the cellular metabolism of two lung tumor cell lines (NCI-H727 and A549) and had no effect on normal cell lines, even at higher MOIs, suggesting an initial indication of potential oncolytic properties.

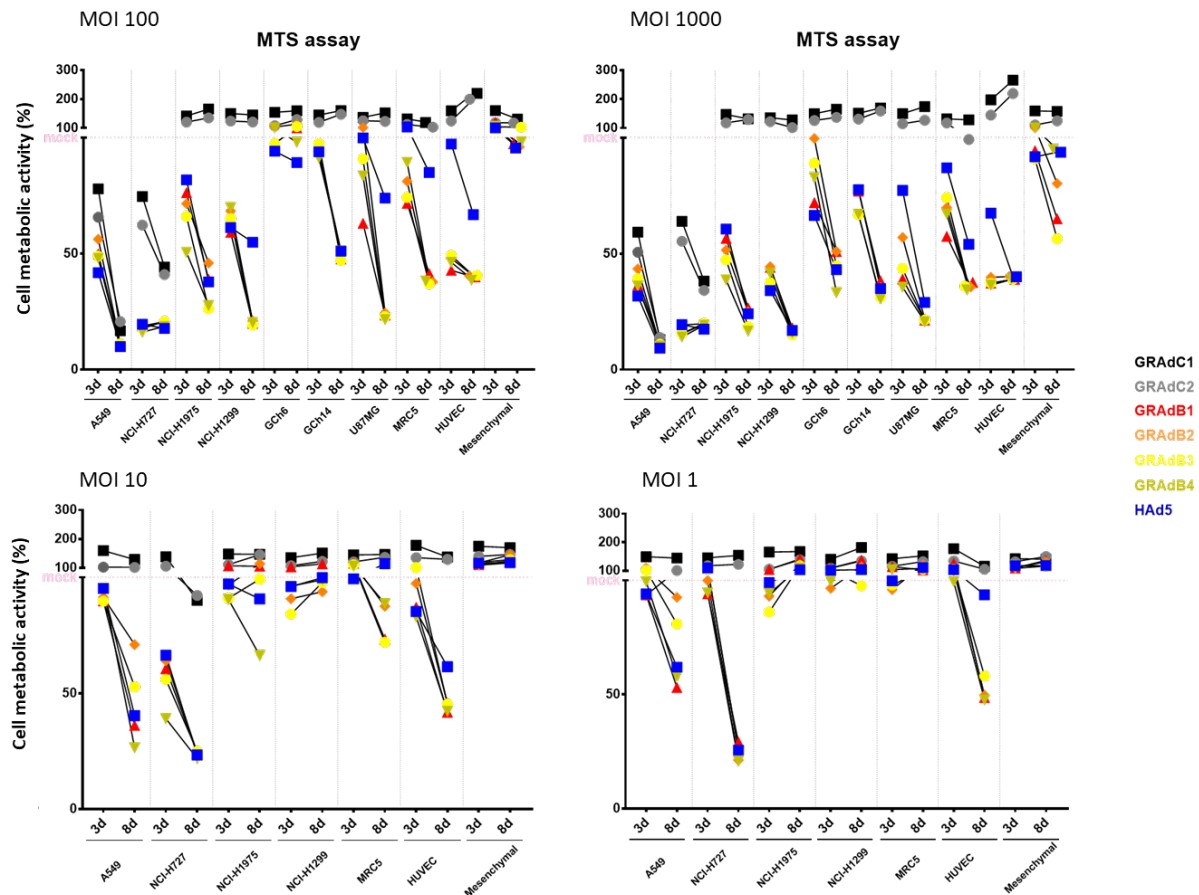
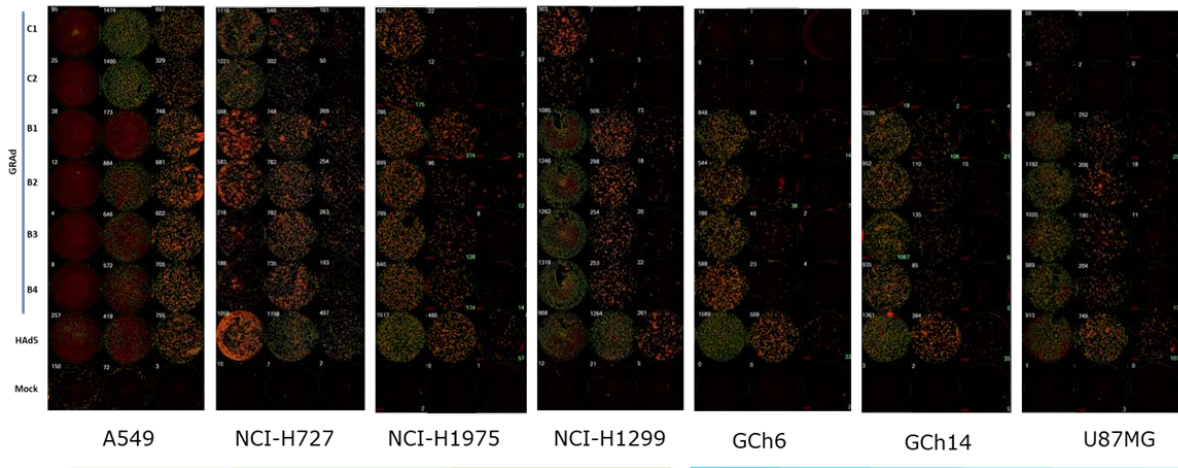


Figure 14 *Metabolic activity of tumor and normal cell lines infected by replication-competent adenoviruses.* A panel of tumor and normal cell lines has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAd C) and group B (GRAd B) and human adenovirus 5 (HAd5) at a Multiplicity of Infection = 100, 1000, 10, 1. The impact of two GRAd C (C1 and C2) and four GRAd B (B1, B2, B3 and B4) is shown. Cell lines and development time point on the x-axis: A549, NCI-H727, NCI-H1975, NCI-H1299, GCh6, GCh14, U87MG, MRC5, HUVEC and Mesenchymal; 3 and 8 days post infection. The symbols on the graph indicate the remaining metabolic activity upon infection, compared to the metabolic activity of the non-infected corresponding cell line (mock).

The same panel of cells was used for the infectivity assay (Figure 15). Here again, GRAd and HAdV5 viruses were used to infect cells at different MOIs (100, 10, 1), and the assay was developed 48 hours post-infection. The goal was to assess the viruses' replication potential in various cell lines, measured by the number of spots corresponding to the hexon, which indicates viral replication. The highest number of spots was observed with GRAdB infection, particularly in lung tumor lines, where the highest MOI caused CPE and a saturation phenomenon, preventing the instrument from assigning an exact spot count. Similarly, glioblastoma cell lines infected with GRAdB showed a high number of spots, although slightly fewer than those infected with HAdV5. Interestingly, in normal cell lines, GRAdB produced a

similar or even higher number of spots than HAdV5. As for GRAdC, a high number of spots was observed only in lung tumor lines, with a few spots in glioblastoma lines and none in normal cell lines, further supporting the potential oncolytic properties of these viruses.

MOI 100 - 10 - 1



MOI 100 - 10 - 1

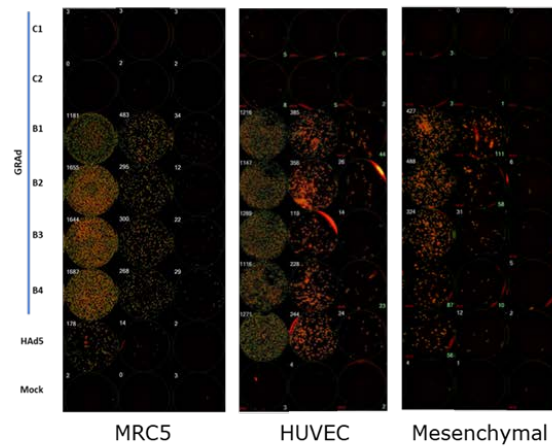


Figure 15 *Adenovirus replication in tumor and normal cells.*

A panel of lung cancer (A549, NCI-H727, NCI-H1975, NCI-H1299), brain cancer (GCh6, GCh14 and U87MG) and normal cell lines (MRC-5, HUVEC and Mesenchymal) has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (C1 and C2) and B (B1, B2, B3 and B4) and human adenovirus 5 (HAd5) at a Multiplicity of Infection (MOI) = 100-10-1 (from left to right). Each number overlaid on the circle with spots indicates the number of infected cells. It is expected to observe, per cell line and adenovirus combination, a decrease in the overlaid number moving from left to right; for example, when MRC-5 are infected with adenovirus GRAd B1, there are 1183, 483 and 34 spots when the infection is performed at MOI 100, 10 and 1, respectively. In some cell line/adenovirus combination the MOI of 100 is too high, causing the detachment of most cells, that are then only observed at lower MOIs; for example, when A549 are infected with adenovirus GRAd C1, there are 95, 1474 and 657 spots when the infection is performed at MOI 100, 10 and 1, respectively, indicating that MOI 100 had killed most cells.

After confirming the viruses' replication, the missing information was the presence and quantity of mature virus. To address this, a productivity assay was conducted using ddPCR to

detect viral DNA after DNase treatment to remove unencapsidated DNA at 3 and 8 days post-infection, using an MOI of 100 (Figure 16 a, b, c, d, e, f). As expected from previous experiments, the highest productivity levels (measured as viral particles produced per cell: Vp/cell) were observed with GRAdB and HAdV5 in all tumor and non-tumor cell lines tested. Specifically, after 8 days of infection in tumor cells, all viruses reached Vp/cell levels ranging from $1.0\text{E}+04$ to $4.4\text{E}+05$, with GRAdB and HAdV5 showing a productivity advantage over GRAdC. However, the most surprising result came from normal cell lines, where after 8 days, GRAdB and HAdV5 exhibited productivity between $4.7\text{E}+04$ and $2.2\text{E}+05$, while GRAdC remained undetectable.

This initial set of experiments demonstrated that we had viruses with potential application in oncolytic therapy, comparable to HAdV5, which is already widely used in this field with appropriate genetic modifications. Therefore, we decided to investigate whether it would be possible to engineer GRAd viruses to enhance their oncolytic capabilities, particularly by increasing the selectivity of GRAdB for tumor cells and expanding the tropism of GRAdC.



Figure 16 Adenovirus production in tumor and normal cells.

A panel of tumor (Lung cancer L1-L4 in 1) and 2) and Brain cancer B1-B3 in 3) and 4)) and normal cell lines (MRC-5, HUVEC and Mesenchymal in 5) and 6)) has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAdC1 and GRAdC2) and group B (GRAdB1, GRAdB2, GRAdB3 and GRAdB4) and

human adenovirus 5 (HAd5) at a Multiplicity of Infection (MOI) = 100. Lung cancer L1: A549; L2: NCI-H727; L3: NCI-H1975; Lung cancer 4: NCI-H1299; Brain cancer B1: GCh6; B2: GCh14; B3: U87MG. Panels 1), 3) and 5) indicate infections collected 3 days post infection; Panels 2), 4) and 6) indicate infections collected 8 days post infection. Per each cell line the table below the graph shows the productivity, calculated as nuclease-resistant viral genome obtained per cell, when infection has been performed at MOI 100.

2. Targeted Genetic Modifications in GRAdB to Enhance Tumor Selectivity

From preliminary experiments, no significant differences were observed in the behavior of the various GRAdB viruses. Therefore, we selected one of the four, specifically GRAdB3 (from now on referred to simply as GRAdB), for which we already had a vectorized version of the virus. The aim was to introduce the most commonly reported genetic modifications in the literature for HAdV5, to improve its selectivity. As a first step, we decided to focus on the early genes, particularly E1 and E3 (Figure 17).

We hypothesized that we could increase GRAdB's reliance on cell proliferation by modifying one of the Rb-binding domains within the E1A gene. The pRb plays a key role as a cell cycle inhibitor and is a critical regulator of the transition from the G1 to the S-phase. In the case of HAdV5, it has been previously demonstrated that the deletion of 24 bp in an Rb-binding domain of the E1A gene produces a mutant adenovirus with selective replication in tumor cells. Based on this, we analyzed the E1A gene of GRAdB for potential Rb-binding sites, and we identified the region most likely corresponding to the same site in HAdV5. We then deleted 24 nucleotides in the CR2 region on the plasmid, which corresponded to the LHCYEEGF amino acid sequence, confirmed the deletion via sequencing, and rescued the virus in A549 cells, generating GRAdB Δ 24 (Figure 17.1).

Similarly, we decided to target the E3 region. The deletion of the E3 region in OAdVs plays a crucial role in modulating the immune response and restricting viral replication to tumor cells. Specifically, the E3 proteins are involved in helping the virus evade the host's immune system. By deleting the E3 region, the virus becomes more susceptible to the host's immune response, which limits its ability to spread in normal, healthy cells, thus restricting its replication primarily to tumor environments where immune responses are often compromised. Therefore, we decided to delete E3, leaving only the upstream E3 12.5K region and the downstream E3 14.7K region—a configuration already familiar to us as it had been used to generate various replication-defective GRAdC backbones—thereby creating GRAdB Δ E3

(Figure 17.2). These two deletions were combined within the same virus to generate GRAdB $\Delta 24\Delta E3$ (Figure 17.1 and 17.2).

A sequence alignment comparing the E3 regions of GRAdB and GRAdC revealed the presence of an additional ORF in GRAdB, absent in GRAdC, called the CR1 δ region. To rule out any potential role this difference might play in the varying selectivity of the two viruses, we decided to include an additional modification: replacing the native ORFs CR1 β , CR1 γ , and CR1 δ in GRAdB with the CR1 β and CR1 γ ORFs from GRAdC, generating GRAdB with GRAdC E3 CR1 $\beta\gamma$ (Figure 17.3). As with the previous modifications, the changes in the E3 region were made on the plasmid, verified through sequencing, and the viruses were then rescued in A549 cells for subsequent experiments.

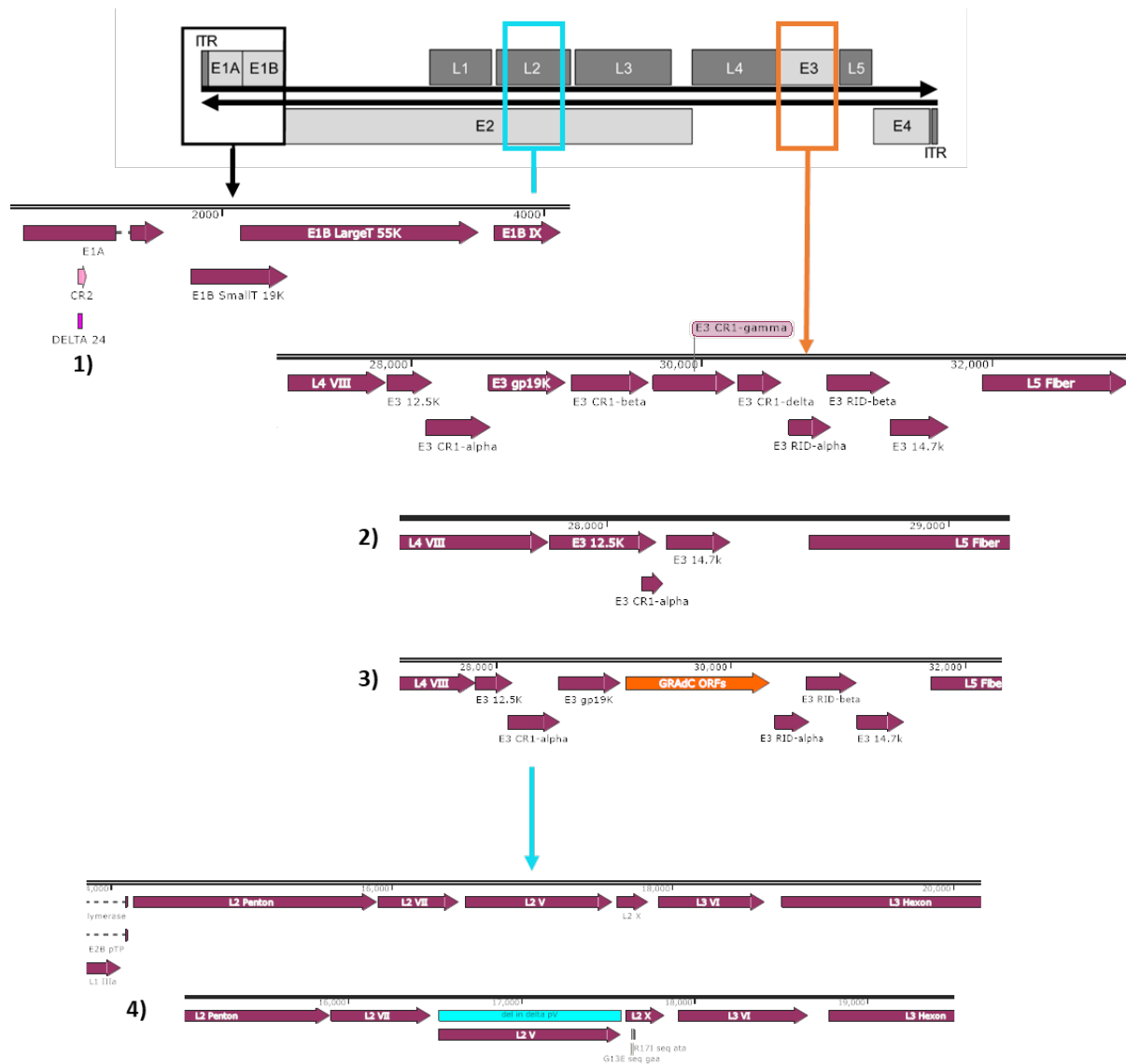


Figure 17 Genetic Modifications to GRAdB to Enhance Selectivity and Tumor-Targeted Replication.

The series of genetic modifications performed on the GRAdB adenoviral genome, aimed at enhancing the virus's selectivity for tumor cells while restricting replication in normal cells. 5.1: Modifications to the E1A gene. The deletion of 24 base pairs in the Rb-binding domain (CR2 region) of E1A is designed to increase GRAdB's reliance on tumor cell proliferation. This mirrors a mutation previously shown in HAd5 to promote selective replication in tumor cells. 5.2: The modification of the E3 region. The deletion of most of the E3 region reduces the virus's ability to evade the immune system in normal cells, thereby restricting replication to tumor environments where the immune response is typically weakened. 5.3: The replacement of CR1β, CR1γ, and CR1δ in GRAdB with the CR1β and CR1γ ORFs from GRAdC. This chimeric construct was created to test whether these regions contribute to the differential selectivity observed between GRAdB and GRAdC, with the goal of optimizing tumor-specific replication. 5.4: An attempt to modify the L2 gene, specifically deleting the core protein V region, which plays a role in viral replication and assembly. This modification aimed to impair the translocation of NPM1 from the nucleoli to the nucleoplasm in normal cells, reducing replication in non-tumor cells.

All the modified GRAdB viruses, as previously described, were used to infect normal cells (MRC5 and HUVEC) at MOIs of 100 and 1000, alongside the wild-type GRAdB and GRAdC, which were used as controls. The first readout of this experiment was the evaluation of the

CPE of these viruses on normal cells. The wild-type GRAdB represented the starting point, showing significant CPE in normal cell lines, while GRAdC represented the desired outcome—no effect on normal cells, comparable to the phenotype of uninfected (mock) cells. To assess this, microscope images were taken 8 days post-infection to provide an overall view of the behavior of these viruses after the introduced modifications (Figure 18). None of the tested modifications restored the selectivity of GRAdB to the level typical of GRAdC, as all the images showed high mortality in cells infected with the modified GRAdB, comparable to that of wild-type GRAdB. The only observable difference was in the infection of HUVEC cells at MOI 100 with GRAdB $\Delta 24\Delta E3$ and GRAdB with GRAdC E3 CR1 $\beta\gamma$, where a less pronounced CPE was observed compared to wild-type GRAdB, though still insufficient for our purposes.

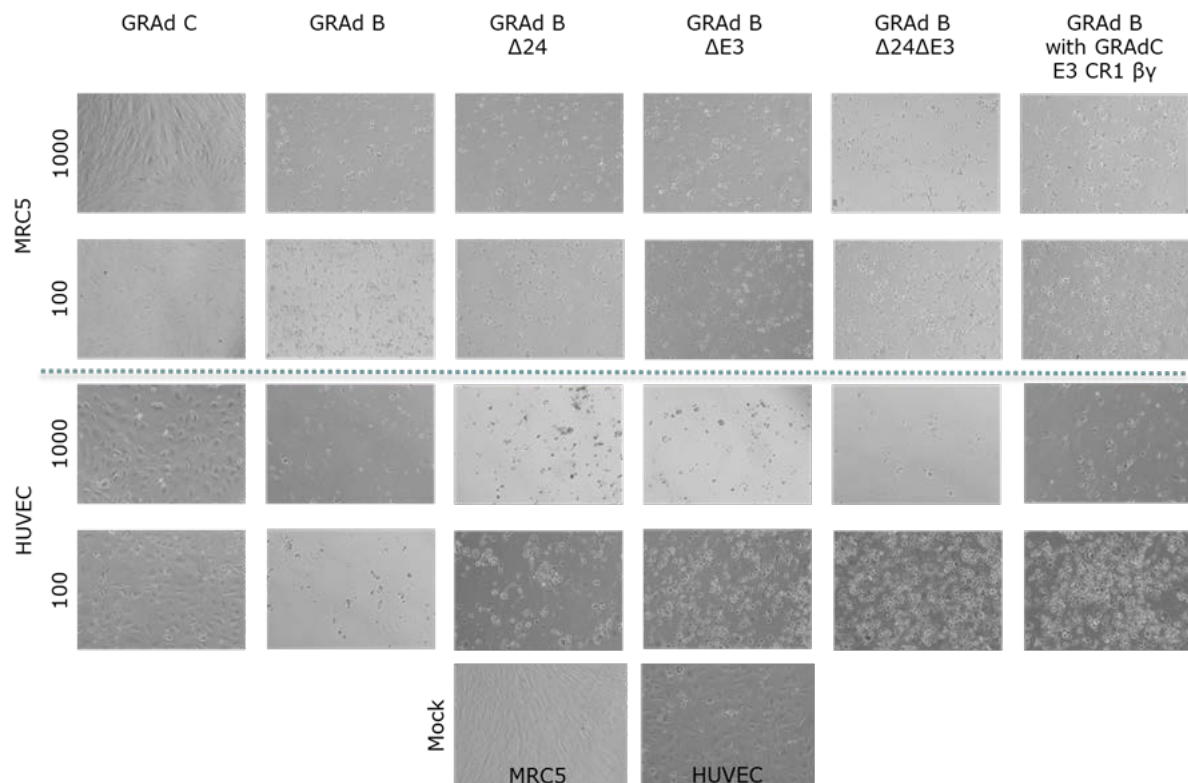


Figure 18 Evaluation of Cytopathic Effect (CPE) of GRAdB Variants on Normal Cells.

Cytopathic effects (CPE) of various modified GRAdB viruses on MRC5 (lung fibroblasts) and HUVEC (human umbilical vein endothelial cells) at MOIs of 100 and 1000. The images were taken 8 days post-infection and compare the effects of wild-type GRAdB, GRAdC (as a control), and the modified GRAdB viruses.

To further confirm the results, the productivity of the various viruses in MRC5 and HUVEC was also evaluated. The infections were harvested, and the virus produced was quantified by ddPCR. The Vp/cell values obtained were comparable across all GRAdB viruses for both MOIs and cell lines (Figure 19). The only undetectable samples were those from the GRAdC

infection, as previously demonstrated. These results provided sufficient evidence that none of the tested modifications led to any improvement in terms of increased selectivity for GRAdB, and thus, no further experiments were carried out.

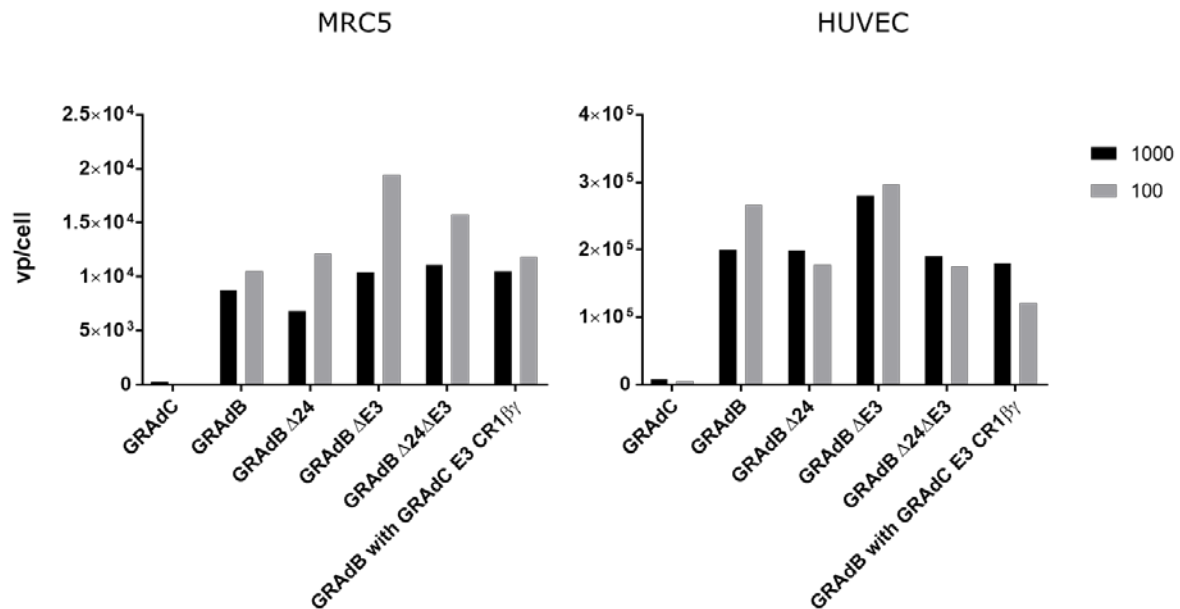


Figure 19 Quantification of Viral Productivity in MRC5 and HUVEC Cells.

Productivity of the different GRAdB viral constructs in MRC5 and HUVEC cells at two different MOIs (multiplicity of infection; MOI1000: black bars; MOI100: gray bars). The viral production was quantified using ddPCR (droplet digital PCR), with results expressed as viral particles per cell (Vp/cell).

We wanted to explore one final attempt by targeting a late gene, specifically the L2 gene, creating a mutant by deleting the core protein gene V region (Figure 17.4). We were inspired by an article where it was demonstrated that, by analyzing a pV-deletion mutant, Ad5-dV/TSB, the authors showed that pV promotes the translocation of Nucleophosmin-1 (NPM1) from the nucleoli to the nucleoplasm in normal cells, and this translocation is correlated with adenoviral replication[81]. In fact, the lack of pV causes a dramatic reduction in adenoviral replication in normal cells, but not in cancer cells, and Ad5-dV/TSB was defective in viral assembly in normal cells.

Based on this, we made the same modifications to GRAdB as were made to Ad5-dV/TSB [81], attempting to identify the exact corresponding regions: a deletion from nucleotide 16331 to nucleotide 17381 and incorporating suppressor mutations at positions 17447-17448 (G to A and C to A; G13E) and 17459 (G to T; R17I) in the precursor region of Mu. Position 1 of the nucleotide sequence refers to the left end of the GRAdB genome. Unfortunately, all attempts to rescue this virus were unsuccessful, and we abandoned this approach.

Alternatively, still considering the different selectivity of the viruses at our disposal, we decided to replace the pV of GRAdB with that of GRAdC, hypothesizing that the pV of GRAdC might be less efficient in translocating NPM1 from the nucleoli to the nucleoplasm in normal cells, which would negatively impact replication and potentially confer better selectivity. Unlike the previous attempt, this virus, GRAdB pV^{GRAdC}, was successfully rescued in A549 cells and used to assess its CPE in normal cells (MRC5). The cells were infected at an MOI of 100 with GRAdB pVGRAdC and with GRAdB and GRAdC as controls, and monitored at 3 and 8 days post-infection. As shown in the images in Figure 20, the virus with the chimeric pV displayed the same CPE as the parental virus. We therefore concluded that replacing the GRAdB pV with the corresponding region of GRAdC did not reduce replication in MRC5 cells.

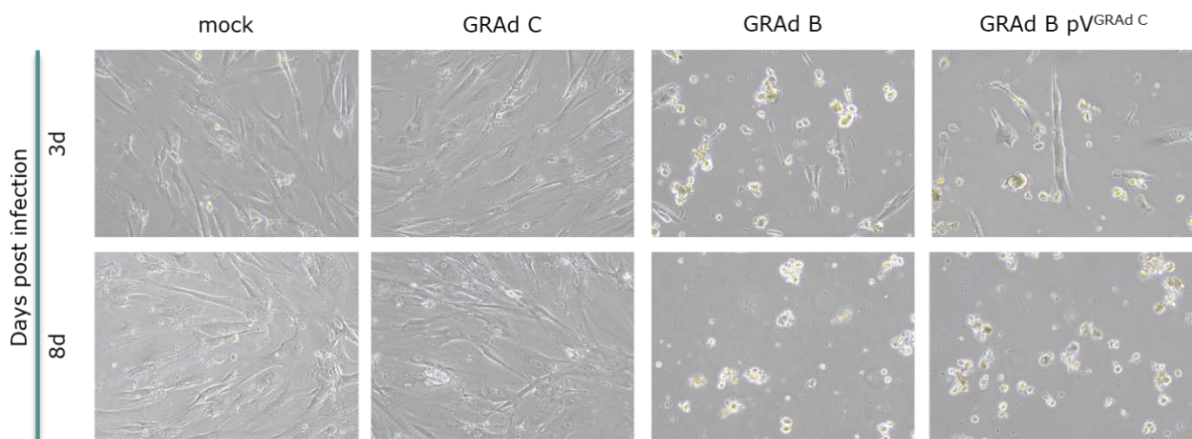


Figure 20 Assessment of Cytopathic Effect (CPE) of GRAdB pVGRAdC in MRC5 Cells.

This figure illustrates the cytopathic effect (CPE) of the GRAdB pVGRAdC virus, where the pV of GRAdB was replaced with the pV of GRAdC, on MRC5 cells. Cells were infected at a multiplicity of infection (MOI) of 100 and monitored at 3 days (top row) and 8 days (bottom row) post-infection.

3. Oncolytic GRAdB Vector as a Platform for Tumor-Specific EV20 Antibody Production

In recent years, innovative approaches have emerged for developing new biological therapies for cancer treatment, including the use of oncolytic viral vectors (OVVs) as vehicles to deliver therapeutic payloads. These vectors can be employed either alone or in combination with other treatments to enhance overall efficacy. Within this framework, we aimed to establish an initial proof of concept by testing whether our GRAdB virus could act as a “biofactory,” producing a therapeutic agent directly within tumor cells.

To explore this, we constructed a GRAdB virus encoding the humanized anti-HER3 antibody EV20, in both FL and SC formats (Figure 21). Specifically, we replaced almost the entire E3 region of the viral genome with the antibody sequences: in the FL version, the heavy and light

chain genes were connected via an IRES, while in the SC version, only the variable regions of the heavy and light chains were connected by a linker, along with the Fc domain. For both constructs, we did not introduce an external promoter; instead, we allowed expression to be driven by the endogenous E3 promoter and/or the MLP. After confirming the success of the cloning via sequencing, the plasmids were used to generate the viruses in A549 cells, leading to the production of GRAdB EV20 FL and GRAdB EV20 SC.



Figure 21 Construction of GRAdB Virus Encoding EV20 Antibody.

Design and construction of a GRAdB virus encoding the humanized anti-HER3 antibody EV20, both in its full-length (FL) and single-chain (SC) formats. The viral genome was modified by replacing a significant portion of the E3 region with the antibody sequences: in the full-length version, the heavy (HC) and light (LC) chain genes of EV20 are connected via an internal ribosome entry site (IRES), allowing both chains to be expressed from the same transcript; in the single-chain version, only the variable regions of the heavy and light chains are connected by a linker and followed by the Fc domain. In both constructs, the expression of EV20 is driven by the endogenous promoters.

The viruses were used to infect lung tumor cell lines (A549, NCI-H727, and NCI-H1975) at various MOIs (1, 10, 50, and 100). After 48 hours, supernatants from the infected cells were collected and subjected to separation on SDS-PAGE and analysed by western blot using an anti-human antibody to detect the presence of secreted EV20 antibodies. Both viral constructs successfully produced the antibody in all tested cell lines, with antibody levels increasing proportionally to the MOI (Figure 22.1).

The supernatants from infections at MOI 100 across the three tumor cell lines were heated to 56°C for 30 minutes to inactivate the virus, preparing them for further antibody

characterization experiments. Synthetic EV20, used as a control in subsequent experiments, was subjected to the same heat treatment. An ELISA assay comparing the binding kinetics of heat-treated versus non-heated EV20 revealed no significant differences in the binding capacity of the antibodies (Figure 22.2).

To evaluate the binding capacity of the viral-produced EV20, the supernatants were tested in both ELISA and FACS assays. The ELISA assessed the antibody's ability to bind HER3, while the FACS assay evaluated its ability to bind HER3 expressed on the surface of HER3-positive cells (A375m). The results, as shown in the Figure 22.3 and Figure 22.4, confirmed that EV20 produced by the GRAd viruses had proper folding and displayed excellent receptor-binding capabilities.

Next, we aimed to demonstrate whether the viral-produced EV20 could influence the HER3 activation pathway, particularly in the presence of its coactivator, NRG-1 β . The results, presented in the Figure 22.5, show that in the presence of EV20, there was a significant reduction in phosphorylated HER3 (p-HER3), which in turn led to a downstream reduction in phosphorylated Akt (p-Akt). A similar effect, albeit less pronounced, was observed with EV20 produced by the viruses in different tumor cell lines. This suggests that EV20 produced by viral expression has a similar inhibitory effect on the HER3/Akt pathway as the synthetic EV20. Specifically, both viral-produced and synthetic EV20 were shown to reduce p-Akt levels, indicating their comparable ability to inhibit HER3 activation and block the PI3K/Akt signaling pathway.

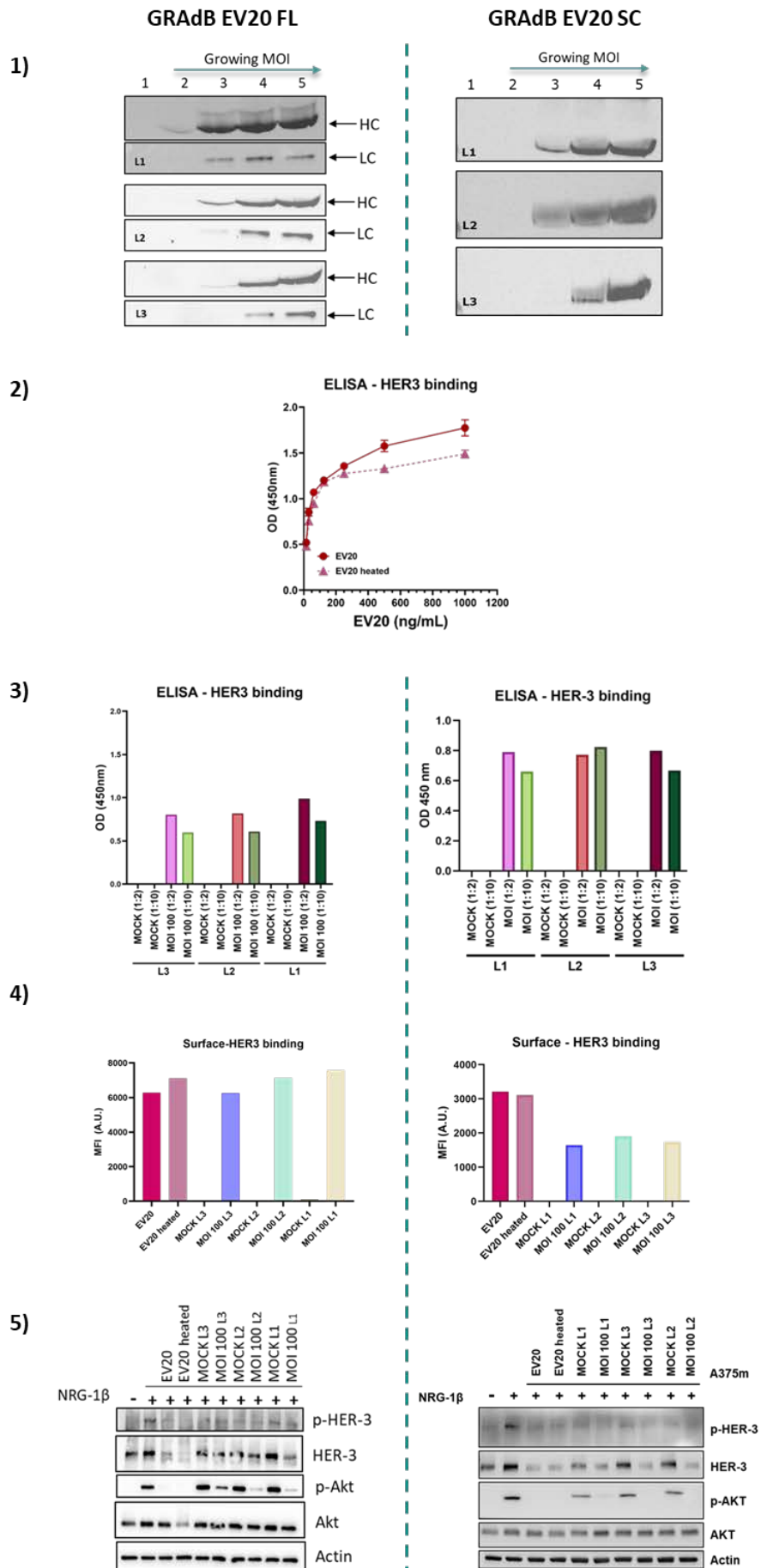


Figure 22 Production and Functional Assessment of EV20 Antibodies by GRAdB Viruses.

Results from multiple assays evaluating the production and functional capabilities of the EV20 antibody produced by GRAdB EV20 FL (full-length; left panel) and GRAdB EV20 SC (single-chain; right panel) constructs after infecting lung tumor cell lines L1=A549, L2=NCI-H727, and L3=NCI-H1975. 10.1: Western blot analysis shows that both viral constructs successfully produced EV20 antibodies in all tested cell lines, with the amount of secreted antibody increasing in proportion to the MOI. Loading order: 1=Mock (un-infected cells); 2=MOI1; 3=MOI10; 4=MOI50; 5=MOI100. 10.2: ELISA assay comparing heat-treated and non-heat-treated EV20 confirms that heating the viral supernatants to 56°C for 30 minutes did not affect the binding capacity of EV20, as no significant differences were observed. 10.3: ELISA results assessing the binding capacity of viral-produced EV20 to HER3, showing effective and specific binding to HER3, indicating proper folding of the viral-produced antibody. 10.4: FACS assay results demonstrating that the viral-produced EV20 can bind HER3 expressed on the surface of HER3-positive cells (A375m), confirming the antibody's binding capability in a cellular context. 10.5: Western blot analysis assessing the downstream effects of EV20 on the HER3/Akt signaling pathway. In the presence of EV20, there was a significant reduction in phosphorylated HER3 (p-HER3), leading to a downstream decrease in phosphorylated Akt (p-Akt). This effect was observed with both the synthetic and viral-produced EV20, indicating comparable abilities to inhibit HER3 activation and block the PI3K/Akt pathway.

4. Modifying GRAdC to improve tropism through chimeric fiber engineering

An alternative strategy we explored was to engineer the GRAdC virus, which had previously demonstrated excellent selectivity but, unlike GRAdB, could only replicate in a limited number of tumor cell lines. The primary limitation of GRAdC likely stems from the expression of its primary receptor, CAR, which the virus uses through its fiber to infect cells. Studies indicate that other receptors, such as CD46 (used by Group B adenoviruses) and $\alpha\beta3/\alpha\beta5$ transmembrane integrins (used by Group C adenoviruses via the RGD domain on the penton), are more widely expressed in tumor cells.

In fact, even when applying HAdV5 in oncolytic virotherapy, the virus is rarely used in its wild-type form. The most common modifications involve engineering the fiber to enhance cell entry. These include chimeric fibers combining HAdV5 with fibers from other adenoviruses in Group B (e.g., HAdV5/3, HAdV5/35, HAdV5/11), as well as modifications like the addition of an RGD domain to a loop in the fiber to enhance cell binding.

In light of these considerations, we conducted preliminary experiments to guide our vector engineering choices. First, we assessed the distribution of the two key receptors utilized by the viruses: CAR for GRAdC and CD46 for GRAdB. FACS analysis confirmed our hypothesis that CD46 was more uniformly expressed across both tumor and normal cell lines, while CAR displayed more variability in expression (Figure 23).

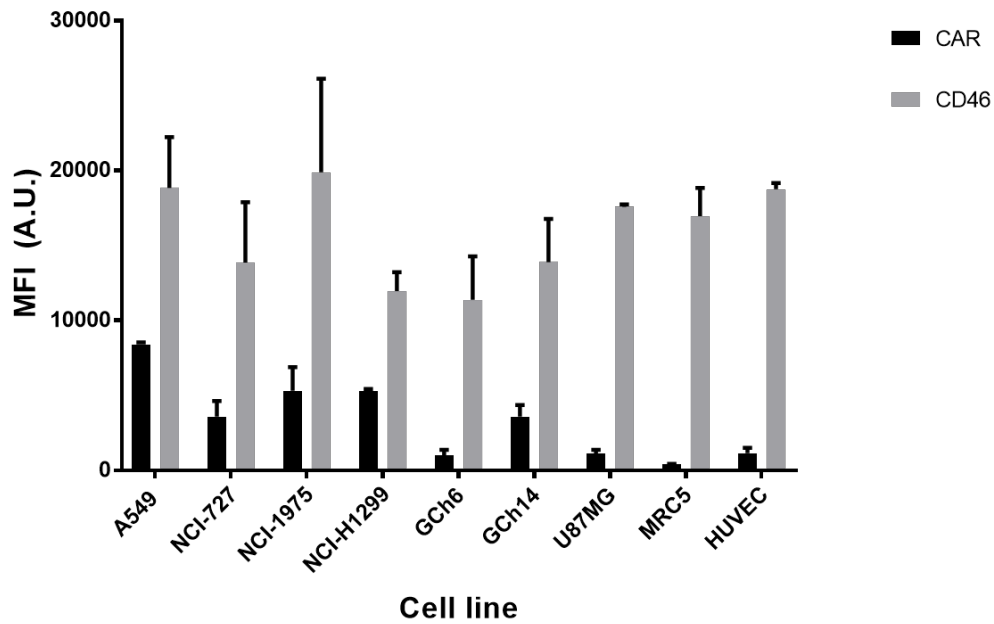


Figure 23 Adenovirus receptor expression.

Cell lines representative of lung cancer (A549; NCI-H727; NCI-H1975; NCI-H1299), brain cancer (GCh6; GCh14; U87MG) or normal tissue (MRC5, HUVEC) were stained with antibodies recognizing the CAR (black bar) or the CD46 (grey bar), representative of the receptors for adenoviruses of group C and B, respectively.

Next, we wanted to determine whether GRAdC could enter cells where no replication had previously been observed, such as MRC5 cells. We infected MRC5 and A549 cells (as a positive control) with a replication-defective GRAdC, where the E1 gene was replaced by GFP under the control of an exogenous constitutive promoter (GRAdC Δ 1 GFP). After 48 hours, images showed GFP expression (Figure 24), indicating that while GRAdC could not replicate in MRC5, it was still capable of infecting the cells.

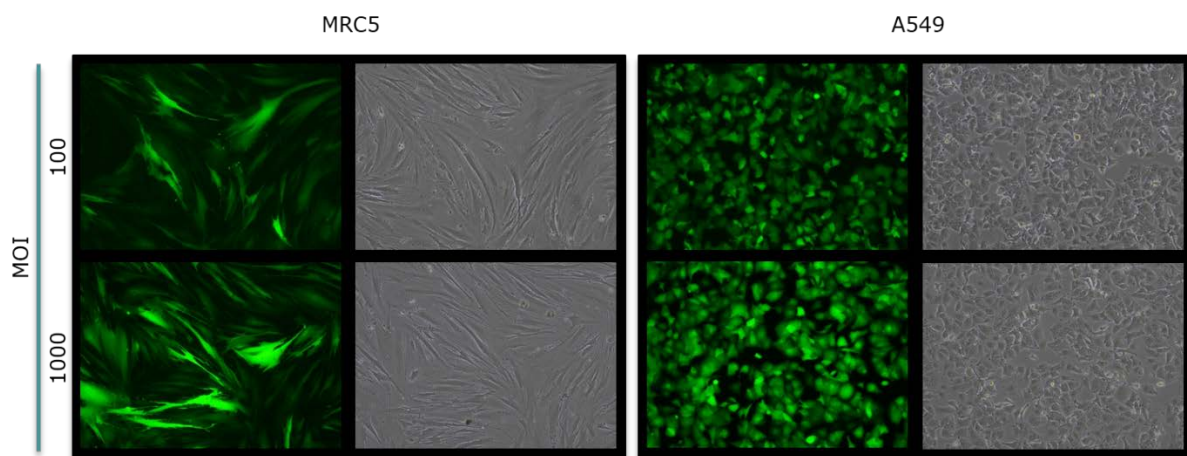


Figure 24 GRAdC infects MRC5.

MRC5 and A549 were infected with 100 or 1000 MOIs of GRAd C expressing GFP under the control of an exogenous constitutive promoter. Per each cell line, both the fluorescent and the brightfield signals are shown.

Given these findings, and noting that GRAdC had lower virus production than GRAdB in tumor cell lines, we hypothesized that improving GRAdC's entry efficiency by using the more widely expressed CD46 receptor might restore GRAdB-like replication while maintaining selectivity in normal cells.

To test this, we engineered a chimeric fiber for GRAdC, maintaining the native tail and shaft, but replacing the knob with that of GRAdB (Figure 25). This modification was introduced at the plasmid level, verified by sequencing, and the virus was successfully rescued in A549 cells, generating GRAdC FkB.

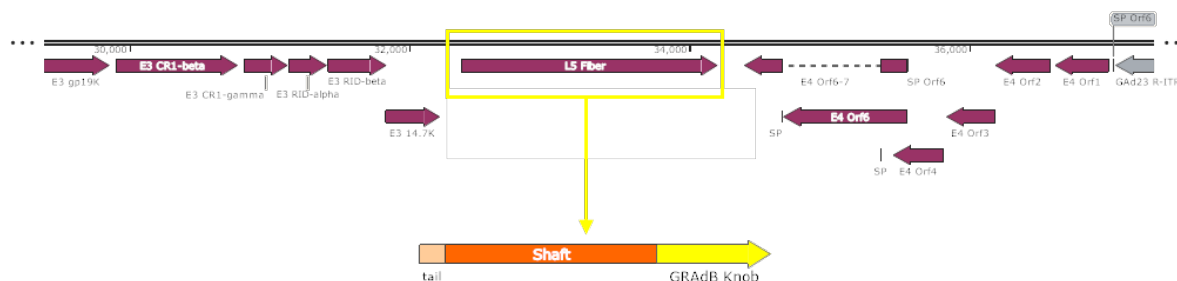


Figure 25 Engineering a Chimeric Fiber in GRAdC to Enhance Tumor Cell Tropism.

To improve GRAdC entry into tumor cells the viral fiber was engineered to contain a chimeric structure. The native tail and shaft of GRAdC were preserved, while the knob was replaced with that from GRAdB to leverage the more widely expressed CD46 receptor.

The newly engineered virus, GRAdC FkB, was characterized through a series of assays to evaluate its effect on cellular metabolism (MTS assay), replication (infectivity assay), and production (productivity assay) in lung cancer cells and MRC5 cells.

For the MTS assay, cells were infected at MOI 100 with GRAdC, GRAdB, and GRAdC FkB, and their metabolism was assessed at 3 and 8 days post-infection. GRAdC FkB showed a similar impact on the metabolism of tumor cells as GRAdB (significant reduction in the percentage of metabolic activity), while having no effect on the metabolism of MRC5 cells, just like the parental GRAdC (Figure 26). This demonstrated that the modification resulted in increased tropism toward tumor cells without affecting normal cells, as expected.

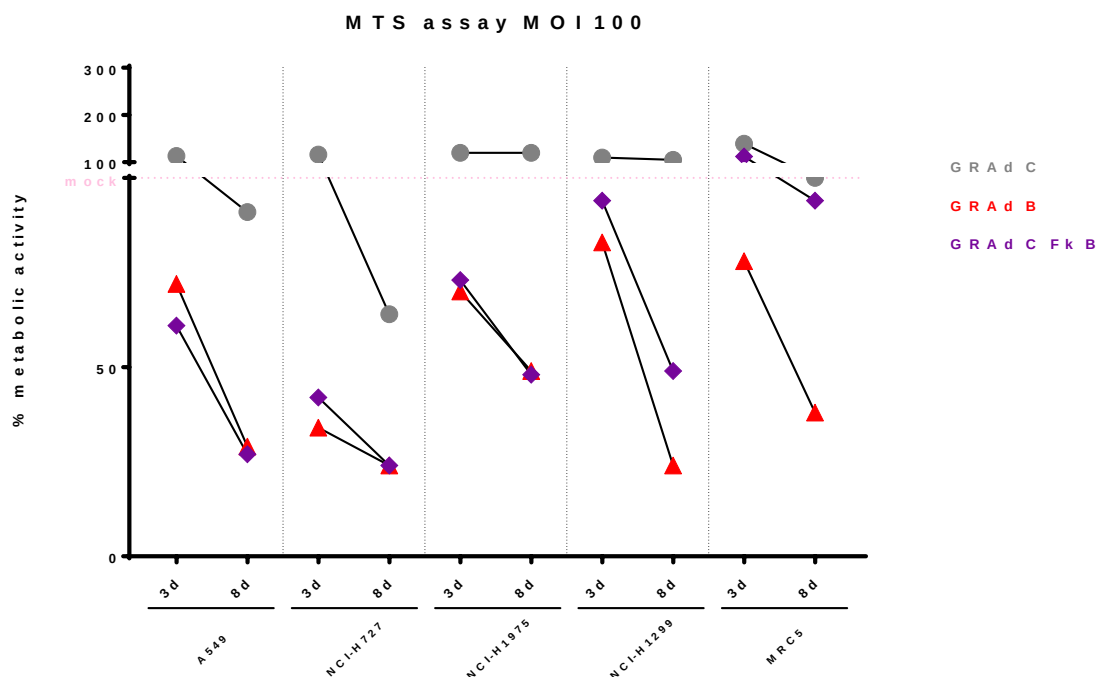


Figure 26 Metabolic activity of tumor and normal cell lines infected by GRAd wt and chimeric.

A panel of cell lines representative of tumor (A549, NCI-H727, NCI-H1975, NCI-H1299) or non-tumor (MRC5) tissue has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAd C), group B (GRAd B) and with a chimeric GRAd based on GRAd C but with the fiber knob replaced for the one of GRAd B (GRAd C Fk B) at a Multiplicity of Infection = 100. Cell lines and development time point on the x-axis. The symbols on the graph indicate the remaining metabolic activity upon infection, compared to the metabolic activity of the non-infected corresponding cell line (mock).

In the infectivity assay, lung tumor cell lines and MRC5 cells were infected at MOIs of 1, 10, and 100 with GRAdC, GRAdB, and GRAdC FkB, and the assay was conducted 48 hours post-infection. The number of infection "spots" observed with the chimeric GRAd was much closer to that produced by GRAdB than GRAdC in tumor cell lines (Figure 27). However, at higher MOIs, the number of spots did not decrease linearly with decreasing MOI due to the strong CPE caused by the more potent viruses and the detection limits of the instrument. In MRC5 cells, while GRAdB still showed a relatively high number of spots, GRAdC FkB had a profile more similar to GRAdC, showing only about 23 spots at the highest MOI (Figure 27). This indicated that the enhanced replication capacity of the chimeric GRAd was restricted to tumor cells, as intended.

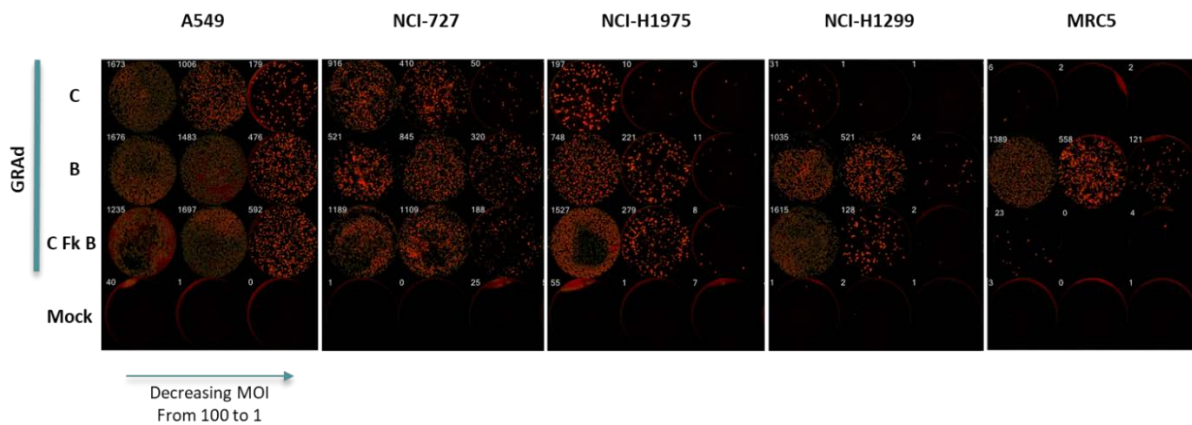


Figure 27 GRAds wt and chimeric replication in lung cancer and normal cells.

A panel of cell lines representative of tumor (A549, NCI-H727, NCI-H1975, NCI-H1299) or non-tumor (MRC5) tissue has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAd C), group B (GRAd B) and with a chimeric GRAd based on GRAd C but with the fiber knob replaced for the one of GRAd B (GRAd C Fk B) at a Multiplicity of Infection = 100-10-1 (from left to right). Each number overlaid on the circle with spots indicates the number of infected cells.

The final experiment, the productivity assay, involved infecting lung cancer and MRC5 cells at MOI 100 with GRAdC, GRAdB, and GRAdC FkB. Lysates were collected at 3 and 8 days post-infection and analyzed via ddPCR. At 3 days (Figure 28 - upper panel), GRAdB and GRAdC FkB showed the highest vp/cell values, but only in cell lines where these viruses had a weaker oncolytic effect (NCI-H1975 and NCI-H1299). In contrast, in cell lines where these viruses had a stronger oncolytic effect (A549 and NCI-H727), GRAdC showed the highest vp/cell values. This is due to the fact that the stronger and faster the CPE induced by the virus, the less newly synthesized virus is produced. By 8 days post-infection (Figure 28 - lower panel), GRAdC had the highest productivity, as it caused a more moderate CPE on the cells compared to GRAdB and GRAdC FkB. In MRC5 cells, once again, the only detectable signal came from GRAdB, confirming that the chimeric fiber modification did not affect the virus's replication in the only normal cell line tested.

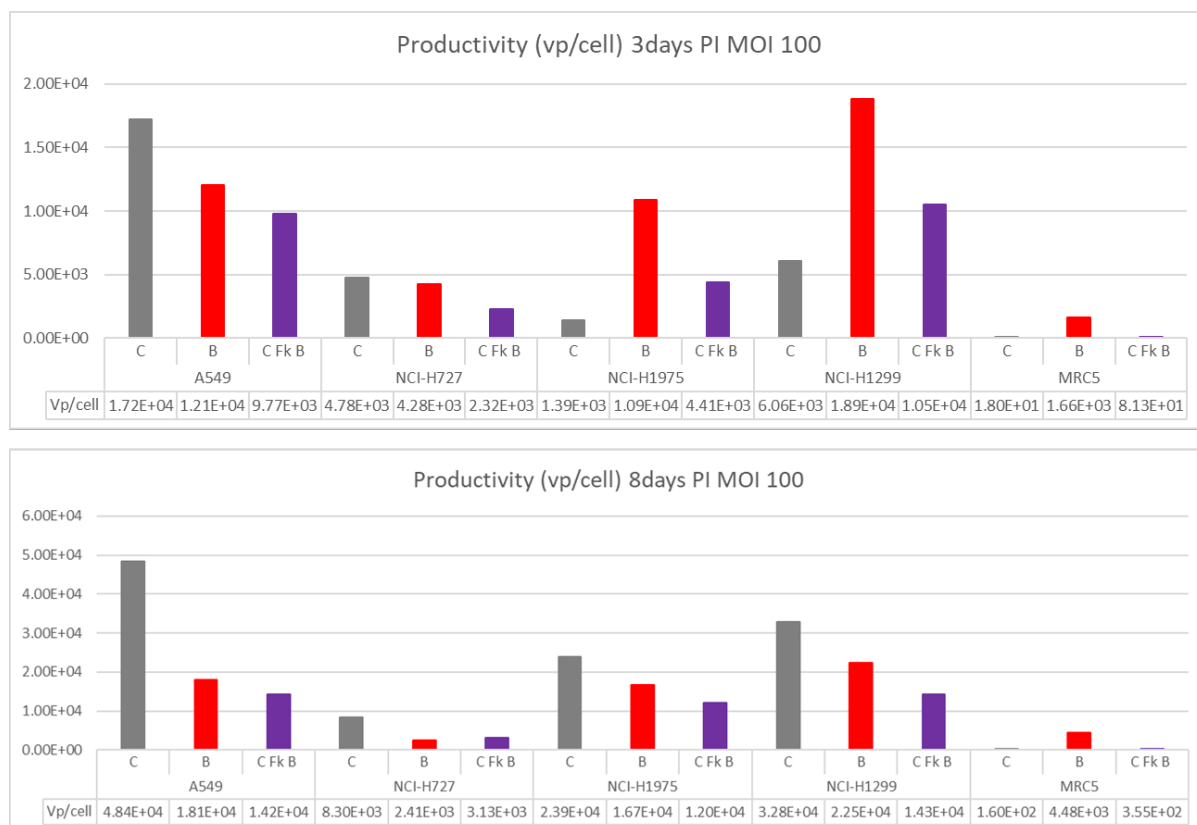


Figure 28 GRAd WT and chimeric production in tumor and normal cells.

A panel of tumor (Lung cancer A549, NCI-H727, NCI-H1975) and normal cell lines (MRC-5) has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAdC) and group B (GRAdB) and a chimeric GRAd based on GRAd C but with the fiber knob replaced for the one of GRAd B (GRAd C Fk B) at a Multiplicity of Infection (MOI) = 100. Upper Panel indicates infections collected 3 days post infection; Lower Panel indicates infections collected 8 days post infection. Per each cell line the table below the graph shows the productivity, calculated as nuclease-resistant viral genome obtained per cell, when infection has been performed at MOI 100.

To exclude the possibility that GRAdC FkB had replicated its genome without completing the maturation process, samples from MRC5 8 days post-infection with the three viruses were analyzed both with and without treatment using two different types of DNase (DNase and SAN) before virus decapsidation (Figure 29). The only virus showing significantly different results between the treated and untreated samples was GRAdB, for which viral DNA replication was observed to be one to two orders of magnitude higher depending on the DNase used, compared to mature virus production. This further demonstrated that GRAdC FkB was unable to efficiently replicate its genome in MRC5, preserving the selectivity of the parental GRAdC virus.

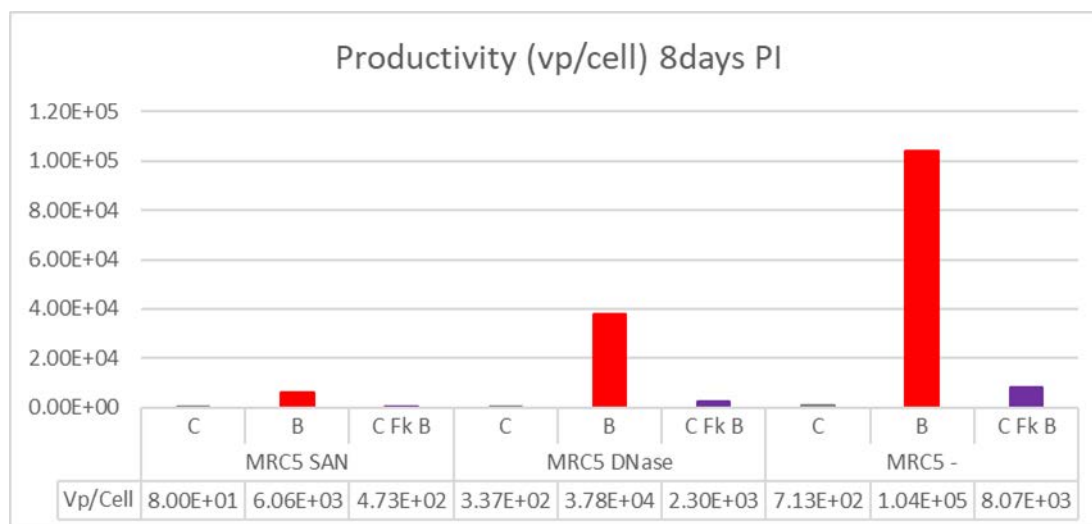


Figure 29 GRAd WT and chimeric production in MRC5 with and without DNase or SAN treatment.
Productivity of viral particles (vp/cell) 8 days post-infection (PI) in MRC5 cells, showing the comparison of the productivity levels of GRAdC, GRAdB, and GRAdC FkB with and without DNase or SAN treatment.

A category of modifications in the use of HAdV5 as an oncolytic agent, which we have not yet discussed in this section, involves alterations to the E1 region. This region is also manipulated to enable the use of the virus in oncolytic virotherapy. The modifications generally aim to restrict viral replication to tumor environments. Among the most well-known modifications are the replacement of the endogenous E1 promoter with a tumor-specific promoter (e.g., E2F, hTERT) or the deletion of 24 nucleotides in the E1A gene, which affects its binding to the pRb.

We had previously attempted to leverage this deletion to improve the selectivity of GRAdB, without success, but given that this virus belongs to a different group, we decided to investigate whether this modification could enhance the already high selectivity of GRAdC. We deleted the region corresponding to the amino acid sequence LTCHEPGF in the E1A gene of the pGRAdC FkB vector, confirmed the deletion via sequencing, and successfully rescued the virus in A549 cells, creating GRAdC Δ 24 FkB.

The newly modified virus was tested in MRC5 cells alongside GRAdC, GRAdB, and GRAdC FkB for comparison. The first test assessed the CPE, and, as expected, the cells infected with GRAdC, GRAdC FkB, and GRAdC Δ 24 FkB showed similar appearance to mock-uninfected cells (Figure 30).

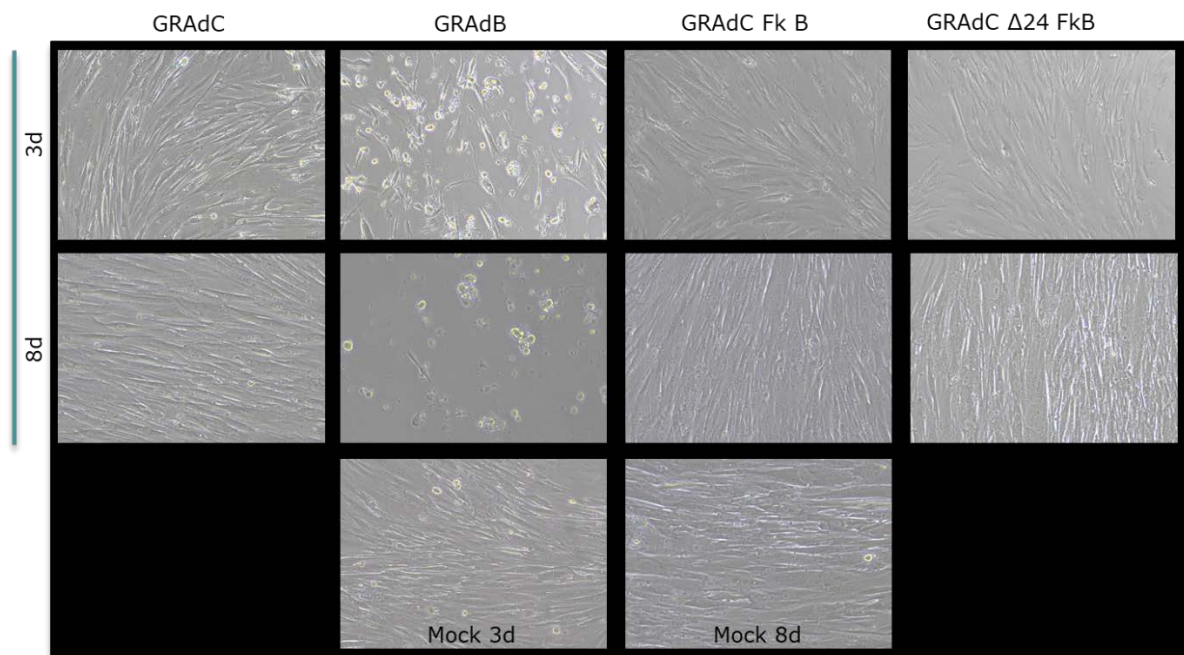


Figure 30 Assessment of Cytopathic Effect (CPE) of GRAd WT, GRAdC FkB and GRAdC Δ24 FkB in MRC5 Cells. *MRC5* has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAdC) and group B (GRAdB) and a chimeric GRAd based on GRAd C but with the fiber knob replaced for the one of GRAd B with or without 24 nucleotides pRb deletion (GRAdC Δ24 FkB and GRAd C Fk B) at a Multiplicity of Infection (MOI) = 100. CPE was observed in MRC5 cells infected with GRAdC, GRAdB, GRAdC FkB and GRAdC Δ24 FkB compared to uninfected cells (mock; lower row) at 3 (upper row) and 8 (middle row) days post-infection.

Both the MTS assay and the productivity assay were conducted to complete the analysis, but neither provided additional information on the performance of GRAdC FkB. Specifically, GRAdC Δ24 FkB did not impact cellular metabolism, nor did it produce new viral particles or replicate viral DNA in MRC5 cells (Figure 31.1 and 31.2).

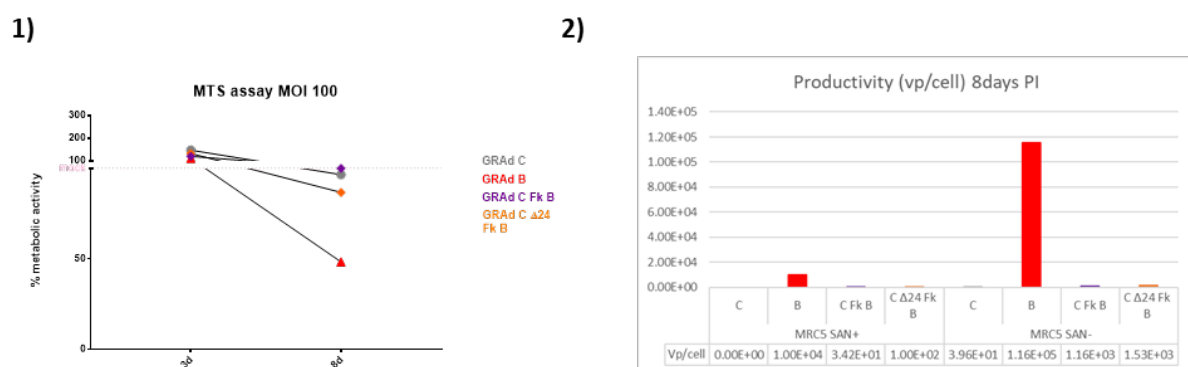


Figure 31 Comparison between GRAdC FkB and GRAdC Δ24 FkB.

1) Metabolic activity of MRC5 infected by GRAd WT, GRAdC FkB and GRAdC Δ24 FkB. Non-tumor (MRC5) tissue has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAd C), group B (GRAd B) and with a chimeric GRAd based on GRAd C but with the fiber knob replaced for the one of GRAd B with or without 24 nucleotides pRb deletion (GRAdC Δ24 FkB and GRAd C Fk B) at a Multiplicity of Infection = 100. Development time point on the x-axis: 3 and 8 days post-infection. The symbols on the graph indicate the remaining metabolic activity upon infection, compared to the metabolic activity of the non-infected

corresponding cell line (mock). 2) GRAd WT, GRAdC FkB and GRAdC Δ 24 FkB production in MRC5 with and without DNase or SAN treatment. Productivity of viral particles (vp/cell) 8 days post-infection (PI) in MRC5 cells, showing the comparison of the productivity levels of GRAdC, GRAdB, GRAdC FkB and GRAdC Δ 24 FkB with and without DNase or SAN treatment.

The only assay that could offer more sensitive insights was the infectivity assay, which could potentially reveal a reduction in the already limited number of spots produced by GRAdC FkB. However, as the image demonstrates (Figure 32), the two chimeric viruses—with and without the Δ 24 deletion—produced a comparable number of spots, indicating that this deletion did not add any significant benefit to the chimeric virus.

Based on these findings, we decided to use the GRAdC FkB chimeric virus, retaining the wild-type E1 region, for further experiments.

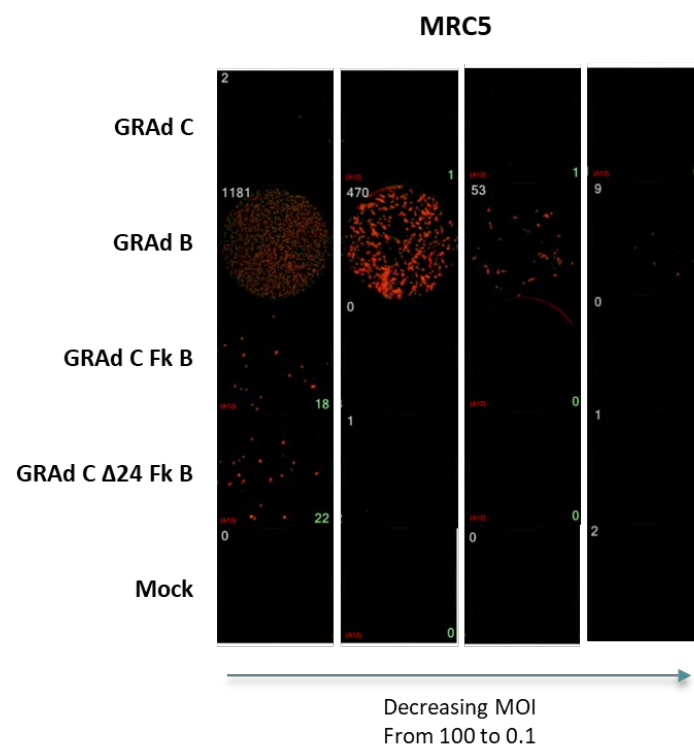


Figure 32 GRAds WT, GRAdC FkB and GRAdC Δ 24 FkB replication in MRC5.

MRC5 non-tumor tissue has been infected by replication-competent gorilla-derived adenoviruses (GRAd) group C (GRAd C), group B (GRAd B) and with a chimeric GRAd based on GRAd C but with the fiber knob replaced for the one of GRAd B with or without 24 nucleotides pRb deletion (GRAdC Δ 24 FkB and GRAd C FkB) at a Multiplicity of Infection = 100-10-1-0.1 (from left to right). Each number overlaid on the circle with spots indicates the number of infected cells.

5. Oncolytic GRAdC FkB: Targeted Antibody Delivery and Tumor Cell Selectivity
Engineering GRAdC with the chimeric fiber of GRAdB allowed us to create a virus with the characteristics of an effective OV. Compared to its parental form, GRAdC FkB demonstrated

broader tropism in tumor cells while preserving the selectivity of the parental GRAdC. We had previously shown with GRAdB that it was possible to express an anti-cancer antibody within tumor cells, so we applied the same approach to what we consider to be our improved OV, GRAdC FkB.

We decided to introduce one final modification by arming the virus with the EV20 SC antibody to test its in situ expression. The vector was constructed in the same configuration used for GRAdB EV20 SC, where the EV20 SC sequence was inserted into the E3 region of the plasmid, without the addition of an exogenous promoter. Cloning was verified by sequencing, and the virus GRAdC FkB EV20 SC was rescued in A549 cells before proceeding with characterization experiments.

Lung cancer cells (A549, NCI-H727, and NCI-H1975) were infected with the virus at MOIs of 1, 10, 50, and 100. The supernatants containing the antibody were collected 48 hours later, separated on SDS-PAGE, and detected by Western blot using an anti-human antibody. The antibody's presence was clearly visible at higher MOIs in all tested cell lines (Figure 33.1).

Additionally, GRAdC FkB EV20 SC and GRAdB EV20 SC were used to conduct the same experiment in MRC5 cells. In this case, clear antibody expression was observed only for GRAdB EV20 SC, whereas a faint band was detectable at MOI 100 for the chimeric virus (Figure 33.2). This can be explained by the fact that efficient antibody expression requires the virus to replicate its genome, and, as demonstrated in previous experiments, GRAdC FkB is unable to replicate in MRC5 cells. This finding provides further evidence of the virus's selectivity—when armed with an anti-tumor molecule, it will only express it in tumor cells.

Furthermore, the antibody expressed by GRAdC FkB demonstrated binding capacity to its receptor HER3, both in vitro and on the cell surface, and it was also able to inhibit the PI3K/Akt intracellular signaling pathway downstream (Figure 33.3-33.5).

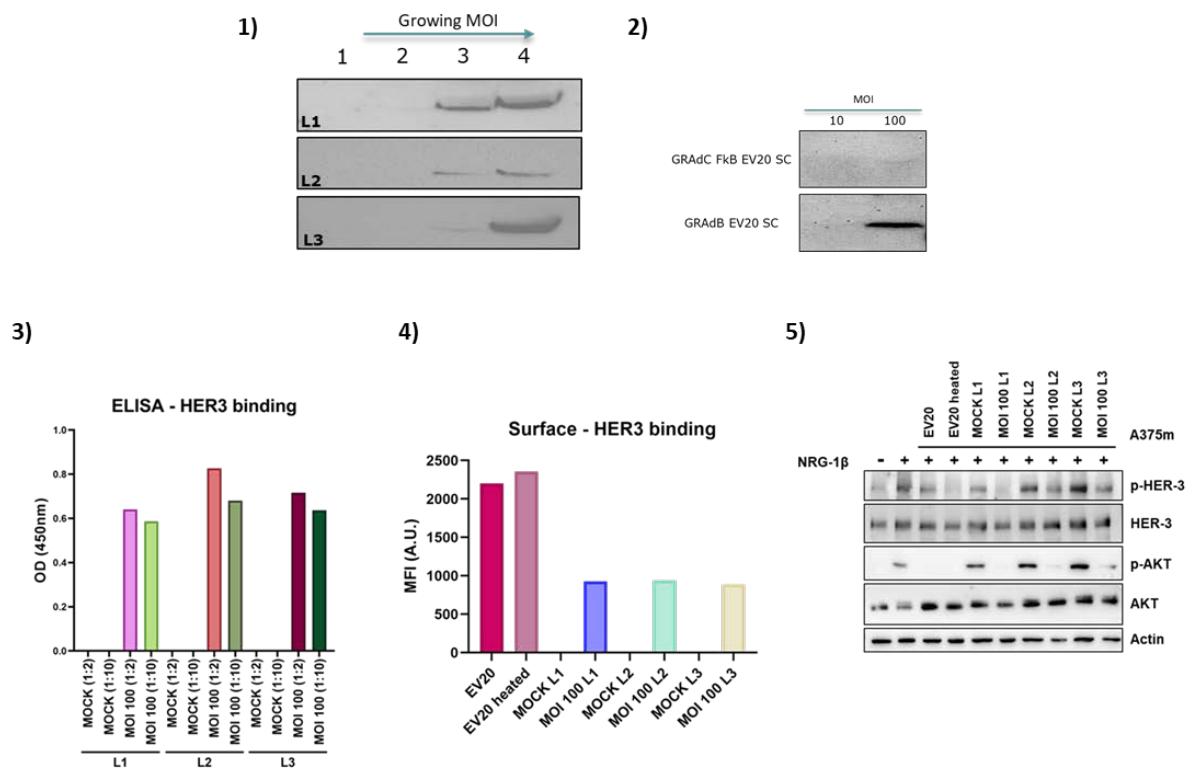


Figure 33 Expression and characterization of a therapeutic payload dependent on chimeric GRAd replication.

1) L1=A549, L2=NCI-H727 or L3=NCI-H1975 were infected with GRAd C Fk B EV20 SC expressing a single-chain antibody targeting HER3, based on the EV20 sequence. Infection was performed at MOI 1-10-50-100, and supernatant-containing antibodies were separated on SDS-PAGE and blotted with an anti-human antibody. 2) MRC5 cells were infected either with GRAd C Fk B expressing the SC, or with GRAd B expressing the SC at an MOI of 10 or 100. The supernatant-containing antibody were separated on SDS-PAGE and blotted with an anti-human antibody. 3-5) Supernatant derived from A549 (L1), NCI-H727 (L2) or NCI-H1975 (L3) infected with 100 MOI of GRAd C Fk B SC were tested for the ability of binding the HER3 on plates 3), on cells 4), and for the ability to affect downstream intracellular signaling 5). The adenoviruses contained in the supernatant were heat-inactivated for 30 minutes at 56°C; EV20 is the positive control, EV20 heated is the positive control treated for 30 minutes at 56°C.

Discussion and Conclusions

In recent years, significant progress has been made in the field of oncolytic virotherapy using HAdVs. However, one of the primary limitations of these vectors lies in the phenomenon of preexisting immunity, which has necessitated the exploration of various genomic engineering techniques to mitigate the issue. This limitation has also shifted attention toward less prevalent serotypes or even adenoviruses derived from other species, particularly nhpAds. As demonstrated in the context of vaccination, nhpAds are particularly appealing because they do not circulate in human populations and thus are less likely to encounter preexisting neutralizing immunity. Moreover, nhpAds exhibit high sequence similarity to HAdVs and share many functional characteristics, such as receptor usage and immune modulation capabilities[74]. Following the successful use of chimpanzee and gorilla adenoviral vectors in COVID-19 vaccination campaigns[73][75], nhpAds may also be leveraged as replicating OVVs in humans.

This study investigated the potential of gorilla-derived adenoviruses belonging to ReiThera's collection (GRAd) as candidates for oncolytic virotherapy, focusing on similarities and differences among themselves, and in comparison to HAdV5. The findings provide new insights into the role of GRAd viruses in cancer therapy while addressing critical challenges such as off-target effects and the need for selective tumor targeting.

Initial results revealed significant differences between GRAdB and GRAdC viruses. GRAdB viruses exhibited stronger oncolytic capabilities, particularly in lung and glioblastoma tumor cell lines, where they induced significant CPE. In some cases, these effects occurred with faster kinetics than those observed for GRAdC and HAdV5. However, their sustained replication in normal cells raised concerns about the feasibility of using them in their wild-type form. Nevertheless, similar challenges have been addressed for HAdV5, where solutions to restrict its replication have been proposed and are currently present in clinical vectors. This provided the rationale for exploring similar strategies to enhance the selectivity of GRAdB. By contrast, GRAdC viruses displayed a narrower tropism, selectively targeting specific lung tumor cell lines while sparing normal cells, even at high MOIs. This selective replication in certain cancer types highlighted GRAdC's promise as a potentially safer therapeutic vector.

Our efforts initially focused on enhancing the tumor selectivity of GRAdB viruses through various genetic modifications, such as deletions and substitutions in the E1A, E3, and pV

regions, as these were the regions whose modifications allowed the generations of HAdV5-derived oncolytic vectors with the desired restricted replication selectivity. However, these modifications produced only limited improvements.

The deletion of one of the Rb-binding domains in E1A, a well-established modification used in many oncolytic derivatives of HAdV5 (commonly referred to as delta-24 mutants), allows E1A to retain most of its primary functions, including its role in the transcription of viral early genes. While this approach did not yield the desired results in GRAdB, the development of Goravir [74] demonstrates that this modification can improve tumor specificity in group B gorilla adenoviruses. This difference is somehow puzzling, as the deletion applied in Goravir and GRAdB $\Delta 24$ is identical, both at the amino acid and nucleotide sequence level. However, it is worth noting that previous experiments with Goravir were conducted at much lower MOI values, ranging from 0.003 to 10 MOI, whereas we tested MOI values of 100 and 1000. Infection MOI can significantly impact experimental outcomes, as we also observed smaller differences between genomic modifications at MOI 100 that disappeared entirely at MOI 1000 (Figure 18). Thus, a direct comparison between Goravir and GRAdB $\Delta 24$ is not currently possible, and further experiments conducted at lower MOI values might reveal differences that have so far remained undetected.

The deletion of the E3 region, which plays a crucial role in modulating immune responses and restricting viral replication to tumor cells, was another strategy explored. Although this approach did not significantly improve GRAdB selectivity, we observed that E3 deletion or substitution conferred a selective advantage to the modified viruses compared to their wild-type counterparts, particularly in HUVEC cells (Figure 18). This could be attributed to the slower growth kinetics of the HUVEC cell line. Again, the MOI at which the experiment was conducted may have influenced the results, and testing lower MOIs could provide a more informative answer as to whether this strategy enhances virus selectivity. Noteworthy, the success of Enadenotucirev[40]—a group B human adenovirus with near-complete E3 deletion and inherent tumor selectivity—supports the potential utility of this modification, with the caveat that Enadenotucirev is a fully HAdV, leaving open the possibility that the impact of the modification of the E3 region is species-specific. It is important to note that Enadenotucirev also includes a 25-nucleotide deletion in the E4orf4 region, resulting in a frame shift. The impact of this mutation on the oncolytic activity remains to be determined, but it is

hypothesized that this deletion, in combination with the E3 deletion, attenuates the virus, thereby enhancing its tumor selectivity.

Attempts to disrupt GRAdB replication by removing the pV protein, which in HAdV5 promotes the translocation of NPM1 from the nucleoli to the nucleoplasm [81]—a process correlated with adenoviral replication and essential for effective viral assembly in normal cells—failed to produce a viable virus. This may be due to the fact that, although we carefully identified the regions to modify in GRAdB based on those altered in HAdV5, a perfect correspondence may not exist between the two, as they belong to different species and serotypes. Furthermore, in HAdV5, the modifications included not only the complete deletion of pV but also suppressor mutations in the precursor region of Mu. When these same modifications were applied to GRAdB, the virus could not be rescued, possibly because the changes affected the complex alternative splicing mechanisms to which adenoviruses are subjected.

We did make two interesting observations though, i.e. 1. that wild type GRAdB, but not GRAdC, promotes the relocalization of NPM1 from the nucleoli to the nucleoplasm in MRC5 cells and 2. that only co-infection with a replication-competent GRAdB, but not with a replication-competent GRAdC, enables sufficient genome replication of a replication-defective GRAdC-pIX-GFP and subsequent GFP accumulation (data not shown). While this last result suggests that the phenotype of GRAdB is dominant over the phenotype of GRAdC, and therefore that GRAdB can, at least partially, complement in trans the functions missing in GRAdC, it is still unclear whether this phenomenon is directly correlated with the translocation of NPM1, and further experiments aiming at this should be performed. Interestingly, work from Grummit and colleagues [82] discuss NPM1 mutants displaying a prominent nucleoplasmic but not nucleolar localization. Experiments overexpressing these mutants in MRC5 might help in shedding light on the role of NPM1 (re)localization in GRAdC replication in non-cancer cells.

Collectively, these observations prompted us replacing the native GRAdB pV protein with the one of GRAdC, to assess whether this modification would affect selectivity, i.e. replication in normal cells, which was not the case (Figure 20). These findings suggest that the translocation of NPM1 from the nucleoli to the nucleoplasm is not critical for GRAdB replication or, alternatively, that GRAdB utilizes alternative mechanisms to achieve this function.

The shortcoming of GRAdB modifications to replicate the selectivity of GRAdC highlights the need for alternative approaches to engineer viruses capable of achieving robust therapeutic outcomes with minimal off-target effects. Future experiments with optimized parameters, particularly at lower MOI values, will be critical to uncovering the true potential of these modifications and identifying more refined strategies for improving OV selectivity.

A significant breakthrough in this study was the development and characterization of GRAdC FkB, a chimeric virus combining GRAdC tail and shaft with GRAdB fiber knob. Through a series of experiments, we demonstrated that the chimeric GRAd construct successfully merged the advantageous properties of GRAdB and GRAdC, achieving broader tumor tropism while maintaining selectivity (Figure 26; Figure 27; Figure 28). The expanded infection range of this virus is likely linked to its receptor usage, as many HAdVB types are known to utilize the CD46 protein for cell entry. CD46 is not only ubiquitously expressed but is also upregulated in numerous tumor types, unlike CAR, which is often downregulated in several tumor types. Tumors with reduced CAR expression pose poor targets for CAR-binding oncolytic Ads. Loss of CAR expression is associated with poor prognosis and promotes metastasis due to decreased cell adhesion. This characteristic has been pivotal in the development of several oncolytic derivatives from the HAdVB species, including HAdV3, HAdV11, and, more recently, HAdV35 [58].

The classification of human adenoviruses within group B is more nuanced than it may initially appear. They can be further divided into three sub-serotypes: B1 adenoviruses primarily bind to CD46 (e.g., HAdV35), B2 adenoviruses preferentially bind to DSG2 (e.g., HAdV3), and B3 adenoviruses prefer CD46 but also bind DSG2 (e.g., HAdV11) [72]. Based on this, our first attempt to construct a GRAdC chimera involved replacing its knob with that of HAdV11 to exploit entry through both receptor types. Despite our efforts, we were unable to rescue the GRAdC Fk HAdV11 virus.

While the chimera of HAdV5 with the knob of HAdV11 has been successfully developed[83], the combination of our GRAdC with HAdV11 did not work. There is no obvious explanation for this outcome, but we hypothesize that constructing a chimeric fiber between adenoviruses from different species may not be feasible due to compatibility issues with other domains, particularly the shaft to which the knob is directly attached. Alternatively, more complex issues related to protein folding or trimerization may be involved. Nevertheless,

successfully generating the species-specific GRAdC-B chimeric fiber was a significant achievement, given the positive results it enabled us to obtain.

We cannot exclude the possibility that this success was not only due to the fact that both main components were derived from viruses of the same species but also because our GRAdB may belong to a serotype different from HAdV11, B1, or B2. It might, therefore, be possible to develop a chimera with the knob of HAdV3 or HAdV35. Assigning a more precise classification to our GRAdB would undoubtedly be valuable. This could be achieved through competition assays using antibodies specific to the primary receptors of group B adenoviruses (CD46 and DSG2) in cells expressing both receptors, or alternatively in distinct cell lines expressing only one receptor, to determine in which scenario virus entry is compromised and identify the primary receptor used by our virus.

Regarding the GRAdC FkB chimera, it was not immediately evident that enhancing the GRAd chimera's ability to enter normal cells—where CD46 expression levels were shown both in this study and in literature to be comparable to those of tumor cell lines and generally higher than CAR expression (Figure 23)—would allow the virus to retain its selectivity. This challenge was particularly important given that group C adenoviruses (HAdVC) are known to employ multiple pathways to infect cells, including exploiting the FX to bind the viral hexon. However, the high selectivity of the GRAd chimera, as well as GRAdC in general, appears to be driven by replication mechanisms that remain unresolved and are independent of the virus's entry into normal cells.

We did observe a slight reduction in selectivity during infectivity assays, as evidenced by the appearance of approximately 20 spots in MRC5 cells (Figure 27), indicating instances where the virus successfully replicated its genome. To determine whether deleting 24 nucleotides in the E1A gene could further enhance the chimeric GRAd's selectivity, we engineered an additional modification. However, the modified virus exhibited behavior in normal cells that was entirely comparable to the wild-type E1A GRAd chimera (Figure 31; Figure 32). This suggests that our level of selectivity may already be so high that it does not allow us to detect the subtle improvements that might result from the delta-24 deletion. The selectivity of Goravir [74] was evaluated as the ratio of cell viability between MRC5 and A549 cells following viral infection. Based on this, we can exclude the possibility that MRC5 cells are not a suitable model for assessing this type of modification. Moreover, the authors define a ratio greater

than 1 as indicative of tumor selectivity. In our case, calculating this ratio using data from the MTS assay yields a value of approximately 2 for the chimera with wild-type E1 (Figure 26). Unfortunately, the chimera with the E1 delta-24 deletion was screened only in MRC5 cells and not in tumor cells, so we are unable to calculate the same ratio for that virus. If we were to calculate this ratio based on the results of the infectivity assay, which quantifies the number of cells in which the virus successfully replicated its genome, we would obtain a much higher ratio, emphasizing the significant difference in replication between tumor and normal cells. For instance, considering the number of spots observed at an MOI of 100 for the GRAd chimera in A549 cells (1235 spots, a number already underestimated due to exceeding the detection limit of the instrument) and in MRC5 cells (23 spots), we would calculate a ratio of 54 (Figure 27). This indicates that the virus replicates 54 times more efficiently in the tumor cell line than in the normal cell line.

In both the MTS assay and the infectivity assay, the chimera, with or without the delta-24 deletion, showed no detectable impact on the metabolism of MRC5 cells. This demonstrates that the virus is inherently selective, making the additional delta-24 deletion unnecessary under these conditions. Further experiments with higher MOIs or longer infection times could explore subtle differences in selectivity.

This remarkable characteristic of GRAdC, which has not previously been observed or discussed in the literature, has prompted us to protect this innovation through a patent application, which is currently under review.

We also explored the ability of GRAdS to serve as biofactories for therapeutic agents, specifically by cloning the sequence of EV20, a humanized anti-HER3 antibody, in its FL and SC versions into the E3 region of the virus without adding an exogenous promoter. Using GRAdB, we demonstrated the possibility of expressing an anti-cancer antibody within tumor cells in both antibody formats. The expressed antibody showed binding capacity to its receptor HER3, both in vitro and on the cell surface, and was also able to inhibit the PI3K/Akt intracellular signaling pathway (Figure 22). This is particularly significant in tumors where the HER3/Akt pathway plays a critical role in promoting growth and resistance to conventional therapies.

We subsequently inserted the EV20 SC version into the chimeric GRAd virus in the same configuration as in GRAdB and obtained similar results. An additional experiment compared

EV20 expression between GRAdB and the chimeric GRAd in normal cells. A substantial difference emerged: while the antibody was clearly expressed by GRAdB in MRC5 cells, only a faint band was detectable at an MOI of 100 for the chimeric virus (Figure 33). This experiment provided further evidence of the benefits conferred by the selectivity of the chimeric GRAd. When armed with a therapeutic payload, the chimeric virus expresses the payload exclusively in tumor cells, sparing normal cells and thereby avoiding off-target effects.

The advantage lies in the insertion site of the antibody. Being located in the E3 region, the antibody can only be expressed after viral genome replication. Consequently, in cells where the virus does not replicate, there is no expression of the payload. The virus armed with EV20 provides a dual benefit: it not only exploits the lytic power of the virus but also combines it with the beneficial action of the antibody. This potentially recruits an immune response triggered both by the release of immunostimulatory molecules following virus-mediated cell destruction and by ADCC functions induced by EV20[80].

Our results demonstrate the feasibility of oncolytic adenovirus-mediated local antibody production within the TME, which could have numerous applications in cancer therapy. Local production offers the potential to improve efficacy-toxicity ratios in a cost-effective manner, particularly when considering the savings from avoiding synthesis of the antibody.

The success of the chimeric GRAd vector also underscores its advantages over traditional human adenovirus platforms such as HAdV5. While HAdV5 remains widely used due to its well-documented safety profile and genetic flexibility, its effectiveness is often compromised by the high prevalence of neutralizing antibodies in human populations. The chimeric GRAd, derived from NHP, offers a unique advantage in this regard. Its lower seroprevalence significantly reduces the impact of preexisting immunity, enhancing its efficacy (Figure 12.1). Preliminary biodistribution data suggest reduced hepatic sequestration, which opens the possibility for systemic administration (Figure 12.2). However, if more detailed biodistribution studies reveal that GRAdC is not as effectively detargeted from the liver as initially assumed, we could consider making modifications to the hexon protein. It is well-established that the association of adenoviral capsids with serum factor X underpins the clearance of vector particles by the reticuloendothelial system (RES). Therefore, disrupting this association can help reduce liver sequestration. In the case of HAdV5, this is achieved by replacing the HVR7

of the hexon with the corresponding domains from alternate human serotypes, such as HAdV3 [87] or HAdV48 [83]. For GRAdC, we could consider using GRAdB as a substitute for this purpose.

In conclusion, this study establishes GRAd viruses, particularly their chimeric derivatives, as innovative and versatile tools for oncolytic virotherapy. Their ability to combine targeted replication and payload delivery positions them as a significant advancement in cancer treatment strategies. These findings pave the way for safer and more effective therapies, offering hope for improved outcomes in patients with challenging cancer types.

Future Perspectives

The research conducted throughout this thesis has led to the development of a highly promising tool for oncolytic virotherapy: the GRAdC FkB chimeric virus. This construct combines the most advantageous characteristics of its two parental wild-type serotypes, merging the high selectivity of GRAdC with the enhanced tropism provided by the GRAdB knob. However, it is important to acknowledge that, at this stage, the study offers a proof of concept regarding its potential. Future research will be essential to fully explore and validate the capabilities of this chimeric virus.

One of the immediate future steps will be to expand the panel of cell lines used in in vitro studies to confirm and strengthen the promising results observed so far. It will be particularly important to include a broader range of both normal and tumor cells to ensure a comprehensive evaluation of the virus's selectivity and efficacy. Among the tumor types to be considered, glioblastoma multiforme (GBM) stands out as a compelling candidate. GRAdB has previously demonstrated a notable capacity for replication in GBM cell lines, suggesting that the chimeric GRAdC FkB, which benefits from the GRAdB knob's improved tropism, could achieve similar or even enhanced results. The success observed in lung cancer cell lines—where replacing the native GRAdC knob with that of GRAdB significantly improved replication—supports this hypothesis.

In addition to standard in vitro assays, co-culture experiments involving both normal and tumor cells could provide a more nuanced understanding of GRAdC FkB's selectivity. Based on current data, it is anticipated that the virus would selectively target and destroy tumor cells while sparing normal cells, a key requirement for clinical applications. Such co-culture systems would more closely mimic the complexity of the tumor microenvironment, offering insights into how the virus might behave in vivo.

Moving from in vitro to in vivo studies is a crucial next step in validating GRAdC FkB's therapeutic potential. Using the existing GRAdC FkB EV20 SC vector provides an immediate opportunity for in vivo experimentation. Initial studies could employ immune-competent mouse models to assess the virus's efficacy and safety within a living system. These studies would not only help confirm the therapeutic effects observed in vitro but also provide critical data on how the virus interacts with the host immune system within the complex tumor microenvironment.

A particularly promising approach for initial in vivo validation involves using a xenograft model where NCI-H1975 cells are implanted into mice to form tumors. These tumors could first be treated with Osimertinib to induce HER3 overexpression, thereby creating an ideal environment for the EV20-targeting component of the GRAdC FkB EV20 SC virus. Following this pre-treatment, the chimeric virus could be administered, allowing us to simultaneously evaluate the virus's lytic activity and the immune engagement promoted by the virus/antibody combination. Such experiments would provide valuable data on the dual-action potential of the GRAdC FkB chimera, combining direct tumor cell lysis with the targeted effects of the therapeutic antibody.

Beyond its current configuration, the GRAdC FkB platform holds significant potential for therapeutic customization. This study has already demonstrated the feasibility of arming GRAd vectors with therapeutic payloads, opening the door to a wide range of new applications. One particularly intriguing avenue for future research involves integrating cytotoxic agents or targeted therapeutics into the viral genome. This approach could enhance the virus's anti-tumor efficacy while minimizing systemic toxicity—a major challenge in conventional cancer therapies.

An exciting candidate for future exploration is Omomyc, an anti-MYC miniprotein that disrupts MYC dimerization with its obligate partner MAX and inhibits their interaction with the E-box DNA-binding site[84]. Omomyc has shown considerable promise in cancer models, and its safety has been recently confirmed in humans during a Phase I clinical trial (NCT04808362). Integrating Omomyc into a highly selective vector like GRAdC FkB could further enhance its clinical potential, ensuring targeted delivery directly to tumor cells and reducing the risk of off-target effects.

Additionally, recent studies have validated a novel immunoconjugate, EV20/Omomyc, which targets HER3-expressing, MYC-addicted neuroblastoma cells [85]. In this context, the development of a GRAd chimera armed with the single-chain (SC) version of EV20 replacing the knob and Omomyc inserted into the E3 region could provide a triple therapeutic benefit: high specificity for HER3-overexpressing tumors, the lytic action of the virus, and the therapeutic effect of Omomyc produced directly within the tumor environment.

However, constructing such complex chimeras presents significant technical challenges. Previous attempts to engineer GRAdC FkB variants with the SC version of EV20 replacing the

knob, using a truncated form of the bacteriophage T4 fibrin protein to substitute portions of the fiber shaft, were unsuccessful. While similar approaches have succeeded with HAdV5 [34], [86], the same strategy failed in the GRAd context, likely due to differences in fiber structure that affect stability and function. Despite these setbacks, this avenue remains worthy of future exploration. Engineering a suitable replacement that replicates the natural fiber's optimal length, flexibility, and trimerization remains a complex but potentially rewarding challenge in adenovirus vector development.

Another promising strategy for enhancing GRAdC FkB's therapeutic scope involves broadening its tumor tropism through receptor targeting. The virus already demonstrates the ability to bind alternative receptors like CD46, expanding its potential applications. However, targeting tumors such as GBM—a highly aggressive brain tumor—may require further modifications. The limited expression of the CAR receptor on glioma cells is a known barrier to efficient gene transfer using conventional adenoviral vectors. Incorporating the RGD-4C ligand into the fiber protein could address this limitation by enabling the virus to interact with cellular α_v integrins, significantly enhancing glioma transduction[88].

This integrin-targeting strategy, combined with transcriptional targeting via E1 gene mutations (such as the delta-24 deletion that disrupts pRb or p53 binding), has already proven effective in glioma treatment. DNX-2401 (Ad5-D24-RGD), a modified adenovirus employing this approach, has shown a favorable safety profile and therapeutic efficacy in both preclinical and clinical trials. While most studies have focused on adult glioma patients (NCT00805376, NCT01956734, NCT02197169, NCT02798406), recent research has expanded into pediatric gliomas, specifically pediatric high-grade glioma (pHGG) and diffuse intrinsic pontine gliomas (DIPG), leading to the initiation of a Phase I/II clinical trial for newly diagnosed DIPG (NCT03178032)[89].

The GRAdC FkB chimera could serve as a promising tool for exploring treatments for these challenging tumor types. Should the virus prove insufficiently effective in targeting certain tumors, further modifications—such as inserting the RGD-4C domain into the fiber protein—could be explored to improve its integrin-binding capability and enhance its specificity for gliomas.

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