

Adding recombinant AAVs to the cancer therapeutics mix

Patrick L. Mulcrone,^{1,2} Roland W. Herzog,¹ and Weidong Xiao¹

¹Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN 46202, USA; ²Department of Pediatrics, Indiana University, Indianapolis, IN 46202, USA

Gene therapy is a powerful biological tool that is reshaping therapeutic landscapes for several diseases. Researchers are using both non-viral and viral-based gene therapy methods with success in the lab and the clinic. In the cancer biology field, gene therapies are expanding treatment options and the possibility of favorable outcomes for patients. While cellular immunotherapies and oncolytic virotherapies have paved the way in cancer treatments based on genetic engineering, recombinant adeno-associated virus (rAAV), a viral-based module, is also emerging as a potential cancer therapeutic through its malleability, specificity, and broad application to common as well as rare tumor types, tumor microenvironments, and metastatic disease. A wide range of AAV serotypes, promoters, and transgenes have been successful at reducing tumor growth and burden in preclinical studies, suggesting more groundbreaking advances using rAAVs in cancer are on the horizon.

INTRODUCTION

Cancer is a collection of diseases caused by uncontrolled growth of damaged cells and is the second leading cause of death behind heart disease in the USA for women aged 40–79 years and men aged 60–79 years.¹ It is estimated that in 2022, 1.9 million new cases of cancer will be diagnosed, and more than 609,000 people will succumb to the disease.¹ Worldwide, cancer is a main cause of death, with estimates predicting case numbers around 29 million with 16 million deaths by 2040.^{1–4} These numbers demonstrate the societal, personal, and economical impact cancer has on the world population. Overall, cancer diagnoses have increased, yet early detection and improved genetic analyses and targeted therapies have met this challenge with improved survival outcomes for several tumor types. Nevertheless, once cancers metastasize, the 5-year survival rate of patients dramatically drops, and cancers become more difficult to treat. Therefore, new, and more effective treatments are needed to continue to improve outcomes for patients with cancer. Moving forward, it is imperative for researchers to develop effective and affordable treatments that target and prevent cancer development early, and in the case of later-stage disease, we need therapeutics that are potent, specific, and able to prevent metastasis and recurrent cancer development. Gene therapy treatments designed to target mutated DNA sequences and correct them to restore proper function are being explored and are having a positive impact on multiple disease outcomes, including cancer.^{5–11} In this review, we provide a brief overview of the cancer

therapeutics landscape before focusing on the various types of gene therapy yielding encouraging results in preclinical and clinical settings for cancer, including advantages and limitations of these therapies. Finally, we concentrate on the biology and application of adeno-associated viruses (AAVs), one type of viral gene therapy with the potential to make a major impact on the cancer field due to their versatility, low immunogenicity, and long-lasting effects.¹²

Current state of cancer therapeutics

Genomic sequencing of tumor biopsies has transformed cancer care and drug development. In the past, chemotherapies, radiation, and surgery were the best options for cancer patients, treatments that while effective to a certain degree, were potent and broad, causing side effects and potentially negatively affecting healthy cells as well as cancer cells (Table 1). With genomic sequencing, physicians and researchers are able to identify driver mutations and druggable targets in specific patients' tumors that allow for targeted therapies for cancers, improving responses and extending survival rates.^{32,33} As described in Table 1, the research into driver mutations and specific oncogenes has manifested clinically in a range of targeted therapies, such as small molecule inhibitors, antibodies, and immunotherapy that block these oncogenic pathways, as well as the various gene therapies being tested in myriad tumor types.

As a major trend in the cancer therapeutics field and a result of tumor sequencing, cancer immune therapy has made strong inroads in improving treatments and outcomes of patients. Currently, the main types of immune therapy that are making a great impact are the cellular immunotherapy, tumor-infiltrating lymphocytes (TIL), and immune checkpoint inhibitors (ICIs).²⁵ TIL therapy involves isolating lymphocytes that are present in a tumor mass and identifying *in vitro* which subsets of lymphocytes are best at targeting tumor cells via recognition of tumor-specific antigens.^{34,35} Almost 100 clinical trials involving TILs and a wide assortment of solid tumors are currently in phase II or III, with documented success in treating melanoma and non-small cell lung cancer (NSCLC).^{21,34,35} ICIs are a class of therapy that aims to activate anti-tumor T cells to prevent

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Correspondence: Weidong Xiao, Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN 46202, USA.

E-mail: xiaow@iu.edu



Table 1. Therapeutics for cancer treatment

Drug family	Subtypes	Examples	Mechanism of action	Disadvantages
Chemotherapy ¹³⁻¹⁷	platinum agents, alkylating agents, topoisomerase inhibitors, antimetabolites, taxanes	carboplatin and cisplatin, cyclophosphamide, etoposide and doxorubicin, 5-fluorouracil and gemcitabine, paclitaxel and docetaxel	intercalations into DNA, inhibit opening of DNA for replication, metabolite mimics block replication, inhibition of microtubules	targets non-transformed rapidly dividing cells, non-specific non-targeted, many reported side effects
Small molecule inhibitors ¹⁸⁻²⁰	receptor tyrosine kinase, JAK/STAT, HDAC serine/threonine kinase	imatinib, erlotinib, sunitinib, sorafenib, and cabozantinib, ruxolitinib panobinostat verulam and selumetinib	block enzymatic activity, blocks phosphorylation and signal cascades	can target receptors and proteins not driving tumor growth
Antibodies ²¹⁻²⁴	neutralizing, decoy receptor	bevacizumab, cetuximab, and trastuzumab afibercept	binds receptor to prevent signaling, intercepts ligand to prevent binding to receptor	CRS, allergic reactions, may be less effective in tumors driven by many oncogenes
Immunotherapy ^{7,21,25-28}	cell therapy, checkpoint inhibitors, monoclonal antibodies	CAR-T cells and TILs, <i>PD-1</i> , <i>PDL-1</i> & <i>CTLA-4</i> inhibitors, rituximab and blinatumomab	T cell engagement of tumor via antigen or surface protein, inhibition of T cell inhibition or death signal cascades, bind tumor cells and mark for T cell destruction	efficacy varies by cancer type, antigen loss, CRS, possible overactivation of immune system
Subtype specific	SERMs, ²⁹ proteasome inhibitors ³⁰	tamoxifen, ER + breast cancer bortezomib and carfilzomib, multiple myeloma	<i>ER</i> antagonist, bind subunit of 26S proteasome preventing its protein breakdown	ineffective if tumors mutate and/or recur
Physical	debulking excision, radiotherapy ^{1,2,4,31}	surgery, external beam radiation and internal radiation	physical removal of tumor mass, DNA damage of tumor cells	possibility of incomplete targeting and removal of all tumor mass

HDAC, histone deacetylase; CRS, cytokine release syndrome; SERM, selective estrogen receptor modulator; JAK/STAT, Janus kinase/signal transducer and activator of transcription.

the cancer from avoiding immune destruction.⁷ The main targets of ICIs are the cell surface proteins cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death-1 (PD-1), and PD-1's ligand, PDL-1.^{25-27,36} Inhibition of these three proteins blocks the inhibitory signal or death signal being induced by the cancer cells, which then facilitates T cell-mediated destruction of the cancer. Ipilimumab, pembrolizumab, and avelumab are some examples of FDA-approved ICIs having a positive impact in various solid tumors.^{26,27,36}

This process of genomic sequencing has also advanced subtyping of tumors, another detail that guides physicians and scientists in selecting treatment regimens and designing studies to expand cancer therapeutics research. For example, a monumental impact has been made in treating lung cancers with genomic sequencing. Meador and colleagues spent years identifying a subtype of NSCLC driven by EGFR (epidermal growth factor receptor).^{18,19} Even within this subtype, different mutations in the EGFR gene occurred in certain tumors in response to first-generation EGFR tyrosine kinase inhibitors (RTKi), illuminating mechanisms of targeted drug resistance and methods of treating patients most effectively with this NSCLC subtype. While several new RTKi's and monoclonal antibody therapeutics are being explored and having some success in treating drug-resistant NSCLC, many of these tumors develop new mutations that confer resistance. The majority of genetic aberrations that are driving these documented resistances occur in exon 19 as a deletion,

an L858R point mutation in exon 21, and various point mutations as well as an in-frame insertion in exon 20 of the EGFR gene, suggesting targeting the EGFR DNA directly with gene therapy or in combination with standard therapeutics might be the best way to treat these NSCLCs.^{18,19,34}

One of the most well-known and frustrating oncogenes in the cancer field is KRAS. Deemed an undruggable target decades ago, this gene has been the subject of multitudes of research, and targeted therapies directed at this protein are being studied for use in several tumor types, such as lung cancers, pancreatic cancer, and colorectal cancer.³⁷ Another tumor type that has benefited greatly from genomic sequencing to employ and guide targeted therapies is T cell acute lymphoblastic leukemia (T-ALL). With the identification of multiple subtypes of T-ALL by genomic analyses, each was shown to rely on distinct driver mutations and activated signaling pathways.^{13,38} These genomic subclassifications shaped how patients needed to be treated in the clinic. Indeed, drugs targeting specific pathways such as NOTCH, Jak/Stat, and PI3K are being researched and tested in T-ALL patients.¹⁴ In these few cancer examples, a common thread is that the oncogenic pathways driving these diseases are highly mutagenic, have been difficult to target effectively with current drug types, and they influence several other pro-oncogenic pathways. New and specific therapies that correct the mutations at the DNA level such as viral-based gene therapies could be incredibly impactful as a treatment. Indeed, reported therapeutic regimens being tested

pre-clinically that contain a gene therapy like AAV and established drugs like immunotherapy or chemotherapy are showing promise in cancers such as ovarian, hepatocellular carcinoma, and triple-negative breast cancer.^{5,7,9,26}

Additionally, drug repurposing for cancer treatment has been explored for several reasons: cost effectiveness, FDA approval already given for the drug, and immediate availability for patients.³⁹ One well-known example is thalidomide, an immunomodulatory drug whose original purpose was to treat skin pathologies like leprosy. Now, this drug is used to treat multiple myeloma usually in combination with the proteasome inhibitor bortezomib and/or the steroid dexamethasone.³⁰ Some success has been achieved with other classes of drugs, such as the anti-obesity drug orlistat, and some of the avermectin-type drugs.^{40,41} [Clinicaltrials.gov](https://www.clinicaltrials.gov) reports drugs such as chlorpromazine, metformin, and mebendazole being tested in patients for glioblastoma, prostate cancer, and hepatocellular carcinoma (HCC), respectively. While these drugs being explored for repurposing in cancer would be beneficial, their multi-targeting characteristics carry the risk of off-target effects and side effects that could be problematic for patients. Even with these improvements in the field, a percentage of patients still experience recurrent disease or have cancers that mutate and no longer respond to treatment regimens that were previously successful. Moreover, once cancers metastasize, spread to distant sites in the body, some treatment options are no longer effective at preventing growth.¹ Therefore, new types of drugs and treatment regimens are needed to continue to reduce the cancer death rate, prevent metastatic disease, and provide hope for those diagnosed with any type of cancer. Hence, gene therapy is being explored as a viable, long-term corrective option for treating several cancer types. It is true that non-viral-based therapies, such as cell therapy and nucleic acid-based therapies, have shown success in certain tumor types, especially hematological cancers.^{15,25,42–44}

Non-AAV gene therapies for cancer

As previously stated, gene therapy is defined as treatment of a disease using techniques that alter the genes of cells with the hope of preventing or stopping disease progression.⁴⁵ This type of therapy aims to replace defective genes or mutated cells that are the cause of a disease or add in a new gene that can help fight the disease and improve the patient's quality of life.⁴⁶ There are several types of gene therapy that have been used in the lab to study myriad of pathologies, and more of these gene therapies are being tested in clinical trials and approved for treating patients. Non-viral therapies, such as cell therapies and interfering RNA, as well as viral-based therapies are showing promising results in targeting not only rare disease but cancers as well.

Cell therapy

Cell therapy is a non-viral type of gene therapy that utilizes healthy cells or cells engineered *ex vivo* to perform a certain function and/or express certain proteins to combat cancer.²⁵ The most well-known version of cell therapy for cancer now is a T cell transfer therapy in the form of chimeric antigen receptor T cell (CAR-T) therapy. CAR-T cells are T cells that are removed from a patient's body and manipu-

lated in culture to better bind tumor cells and attack the cancer in the hopes of killing it. Several CAR-T therapies have been FDA approved to treat hematological cancers. One example, Kymriah (tisagenlecleucel), is used to treat B cell ALL and diffuse large B cell lymphoma (DLBCL). This CAR-T therapy recognizes CD19, a surface protein expressed at high levels on cancerous B cells and facilitates T cell expansion upon binding in order to kill the tumor.^{42,47,48} In March of 2021, the CAR-T Abecma (idecabtagene vicleucel) was approved to treat refractory or relapsed multiple myeloma (MM), as it targets the elevated expression of B cell maturation antigen (BCMA) present on MM tumor cells.⁴⁹ In addition to hematological cancers, CAR-T therapy is being explored for uses in solid tumors like breast cancer and brain cancers. Two main drawbacks to CAR-T therapy relate to the specificity of the target and the potential for the side effect known as cytokine release syndrome (CRS). In the examples of Kymriah and Abecma, their targets, CD19 and BCMA, are not tumor-specific proteins. These surface proteins are also expressed, although at lower levels, on healthy B cells and plasma cells, implying that healthy cells could also be targeted and killed by the therapy. CRS is a potentially severe inflammatory response that is reported to occur in some patients that receive CD19 CAR-T therapy.⁵⁰ Fever, abnormal heart-beat, and hypotension are common signs, with most severe cases leading to capillary leak syndrome, low blood pressure, organ failure, and death.⁵¹ A range of interleukins and interferon-gamma are just some of the cytokines that are activated in CRS.^{51,52}

Nucleic acid-based cancer therapies

Along with cellular therapies, researchers and physicians are testing other non-viral gene therapies for their efficacy against several cancer types. A large class of these therapies are based on nucleic acid structures and are designed to target RNA to prevent translation or to introduce new DNA into tumor cells to prevent growth or induce a host immune response. A key component of these nucleic acid-based gene therapies is the vehicle in which the therapy is encapsulated to facilitate stability and successful delivery to the target. Nanoparticles, liposomes, and microbubbles are just a few of the carriers being researched for gene therapy delivery.^{15,43,44} Groups report experimental success of DNA delivery of melanoma-specific antigens and IL-12 to treat melanoma lung metastases and colorectal cancer, respectively.^{53,54} Similar methods have been tested with the cargos being interfering RNA molecules (RNAi): small interfering RNA (siRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), and antisense oligonucleotides (ASO). These RNAi molecules each enact their inhibition in slightly different ways through base pairing at distinct stages of RNA processing, with the goal of silencing gene expression.^{55,56} siRNAs are double-stranded RNA molecules designed to be 100% complementary to the gene target. Therefore, siRNA is highly specific and potent. miRNAs are single-stranded natural RNA molecules that are produced by cells in the body. miRNAs are somewhat promiscuous as certain miRNAs can regulate expression of several genes. In cancer research, many groups have identified miRNAs that are tumor suppressive and those that promote cancer development, which has guided researchers in using miRNAs as therapy.^{57,58} shRNAs are double-stranded RNA molecules that are designed to undergo the

Table 2. Abbreviated list of clinically approved gene therapies

Various clinically approved gene therapies			
Drug	Gene therapy	Disease	Mechanism
Luxturna	rAAV2	<i>Leber's congenital amaurosis, retinitis pigmentosa</i>	AAV2 contains functional <i>RPE65</i> and targets retinal pigment epithelium ⁷³
Zolgensma	rAAV9	<i>spinal muscular atrophy</i>	AAV9 of <i>SMN1</i> to motor neurons ⁷⁴
Yescarta	CAR-T Cells	<i>DLBCL, non-Hodgkin's lymphoma, follicular lymphoma</i>	CAR-T cells bind cancer via <i>CD19</i> targeting, leads to CAR-T activation, then targeted destruction of tumor cells ⁷⁵
Kymriah	CAR-T Cells	<i>ALL, DLBCL</i>	autologous T cell therapy directed by <i>CD19</i> binding, then targeted destruction of <i>CD19</i> -expressing tumor cells ^{42,47,48}
Strimvelis (EU)	γ -retrovirus	<i>adenosine deaminase deficiency</i>	patient <i>CD34+</i> cells transduced with corrected <i>ADA</i> sequence then infused back into patient ⁷⁶
Abecma	CAR-T Cells	<i>relapsed/refractory multiple myeloma</i>	CAR-T cells recognize MM via <i>BCMA</i> targeting, leads to CAR-T activation, then targeted destruction of MM ⁴⁹
Glybera (EU)	rAAV1	<i>lipoprotein lipase deficiency</i>	AAV1 drives expression of <i>LPL</i> in muscle cells ^{77,78}
Imlygic	HSV-1 oncolytic virus	<i>melanoma</i>	Host immune response via GM-CSF production in melanoma cells ⁷⁹

DLBCL, diffuse large B cell lymphoma; *ALL*, acute lymphoblastic leukemia; *HSV*, herpes simplex virus.

biological processing to siRNA using cell machinery. shRNAs also allow for a greater chance of stable expression and suppression of target gene activity. ASOs are single-stranded RNA or DNA constructs designed to bind mRNA via complementary base pairing to regulate genes at the transcription level through physical steric blockade of producing mature mRNA or inducing RNase-H-mediated processing of the mRNA.^{59–61} Typically, ASOs are 15–30 bps and contain chemically modified backbones to improve stability, protection from nucleases, and enhance efficacy.⁵⁹ In the context of cancer biology, ASOs have been tested with moderate success in oral squamous cell carcinoma, Kaposi's sarcoma, and targeting a variety of angiogenic oncogenes; yet, no ASO is FDA approved to treat cancer to date.⁶⁰ Difficulties and concerns with RNAi therapies are mostly related to dosing, efficacy of vector, low delivery of RNA molecule, renal clearance, and off-target effects due to the selected carrier.^{7,59–61}

Oncolytic viruses

Another group of gene therapy vectors being researched for their efficacy against cancer are a group of viruses deemed oncolytic viruses. This class of viral-based gene therapy encompasses a range

of different virus families.⁶² The common thread among them is that they preferentially infect tumor cells, replicate within the cells, arrest their growth, and induce some form of tumor destruction. Depending on the design of the engineered virus or the biological properties of the unmodified virus, tumor destruction manifests most often as apoptosis, a focused immune response, or a reduction in tumor vasculature. Adenovirus, herpesvirus, measles virus, and vaccinia virus are among the types being tested in preclinical and clinical research with documented success in solid tumors such as HCC, melanoma, and glioblastoma. Excitingly, ovarian cancer and neuroendocrine tumors (NENs) are newer focuses for the field. The oncolytic adenovirus *d1922-947* is reported to synergize with the beta-blocker carvedilol and increases survival in a mouse patient-derived xenograft (PDX) model of ovarian cancer.⁶³ Inoue and colleagues report treatment with the vaccinia virus mpjX-594 enhances anti-PD1 immunotherapy in pancreatic NENs.⁶⁴ Additionally, rodent protoparvoviruses (PVs) are also being explored for their efficacy against many types of cancers. Specifically, H-1 parvovirus (H-1PV) and minute virus of mice have shown promise both translationally and at early clinical stages of research. These PVs contain a ssDNA genome and are reported to preferentially infect tumor cells.⁶⁵ They kill tumor cells mainly through activating DNA damage responses, causing reactive oxygen species, and inducing apoptosis.^{66–68} These mechanisms are observed in cancers that are very aggressive and difficult to treat: glioblastoma, gliomas, and pancreatic ductal adenocarcinoma.^{69–72}

As a group, oncolytic viruses are quite promising, as evidenced by the FDA approval of Imlygic, a weakened herpes simplex virus (HSV-1) used to treat melanoma present on skin or in lymph nodes (Table 2). While the exact mechanism of action is not fully understood, this HSV-1 is injected directly into the tumor mass and induces a localized immune response to the tumor via GM-CSF production and tumor lysis.⁷⁹ Yet, oncolytic viruses as a therapy also present with certain disadvantages. As they are a diverse group of vectors, production details and purity will depend on which carrier is used. Due to this, high titer and details in protocol issues can arise in moving to clinical studies.^{80,81} Given the mechanism of action of these viruses, off-target effects or amplified immune responses to the treatment are a concern. Flu-like symptoms and injection site issues such as pain have been reported.^{81–83} This reinforces the importance of design and tumor specificity. Also, activation of the immune response to some of these viruses reduces the amount of oncolytic virus (OV) that can target the tumor, thereby compromising the efficacy of the OV therapy.⁸³ Some reported mechanisms include macrophages engaging and clearing viral carriers, innate immune activation via Toll-like receptors caused by viral RNA or CpG islands, as well as the humoral immune activation through production of neutralizing antibodies toward the viral capsid.^{62,65,81,84,85} Currently, most oncolytic viruses are injected intratumorally, which excludes hematological cancers or cancers in early stage of disease that have not formed a mass.^{62,81–83} Of note, Reolysin, a reovirus-based oncolytic viral gene therapy, is being tested for efficacy against MM among many solid tumors (NCT02514382).

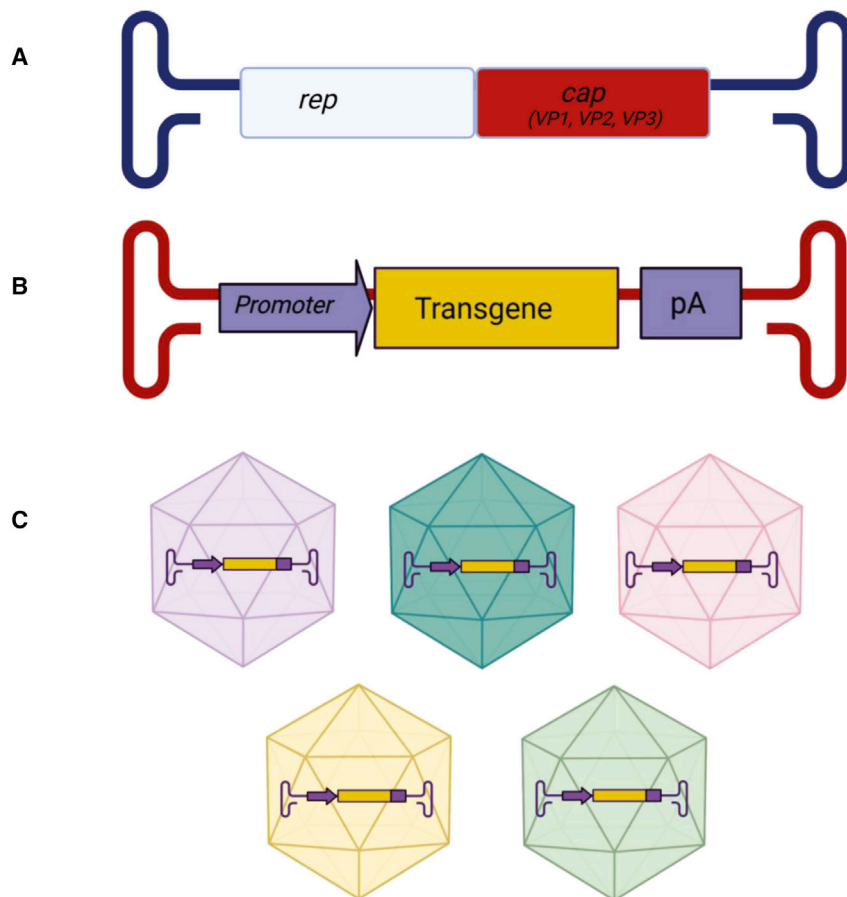


Figure 1. AAV and rAAV general structures

(A) WT AAV genome. Rep portion encodes genes for viral genome replication and packaging (*Rep78*, *Rep6*, *Rep52*, *Rep40*). Cap portion encodes genes for viral capsid proteins (*Vp1*, *Vp2*, *Vp3*). (B) General recombinant AAV (rAAV) genome structure. Constructed rAAVs consist of a promoter, transgene of interest, and a polyadenylation signal.^{9,86,88-92} (C) Representation of icosahedral AAV capsids containing rAAV genome. Different capsids, indicated by the various colors, can direct tissue tropism and targeted expression of a particular transgene driven by a specific promoter encoded in the rAAV genome.^{86,87,93-95}

promoters that can drive expression of the transgene of interest, rAAVs are minimally immunogenic and can infect dividing and non-dividing cells with the resulting genetic changes being long-term, making them widely applicable to many disease models. Also, AAV infection is not directly linked to causing any sort of pathogenesis, demonstrating a safety profile that suggests the virus would cause limited side effects. These details are emphasized by the increase in the number of clinical trials in the USA that include an AAV over the past 16 years. As highlighted by D. Kuzmin and co-authors, the various AAVs are now being used to target many different diseases through specific tissue tropism (Figure 1C).⁹⁵ On the other hand, the relatively small genome (4.7 kb), a rare possibility of rAAV integration into the host genome, and the

potential for people to have natural neutralizing antibodies against certain AAVs serotypes are some disadvantages of this gene therapy vector.^{88,89,96-98}

As previously stated, AAVs exhibit relatively low immunogenicity compared with other vectors and are not directly linked to causing any human pathology.^{7,9,99,100} These data suggest that several AAV serotypes are not inducing strong immune reactions in the host that could be detrimental to either the host's health or the efficacy of the rAAV-based therapy. As many rAAVs are being designed and tested in several cancers to cause a controlled, focused response to target the tumor, it is key that the AAV itself does not influence the host immune system in a way that could compromise the effects of the therapy.^{5,8,9,101-103} Even in reports of hemophilia A where a high dose of an rAAV driving expression of factor VIII is inducing immune responses, such as neutralizing antibodies toward the AAV capsid, strategies to dampen the immune response to the virus or enhance the efficacy of the rAAV are showing promise and could be broadly applicable.^{97,104-106} Other types of viral vectors and nucleic acid-based methods of gene therapy being explored do not consistently exhibit these characteristics of low immunogenicity or no links to human pathology, making AAVs an attractive vehicle for gene therapy. Moreover, rAAVs convey long-term expression of

RECOMBINANT AAV GENE THERAPY

AAV therapy, a viral gene therapy, has great possibility for improving therapeutics in the cancer biology field and effectively targeting several cancer types due to its low immunogenicity, minimal side effects, long-term expression of the transgene, adaptability, and no direct link to causing human diseases. AAVs belong to the parvoviridae family and are a single-stranded DNA virus with a genome of approximately 4.7kbs.^{86,87} The genome of the wild-type AAV encodes *Rep* and *Cap* genes, *Rep* encoding for viral replication, packaging and genomic integration, and *Cap* encoding for the structural proteins VP1, VP2, and VP3 that constitute the viral capsid (Figure 1A).⁹³ The AAV genome is buttressed by two inverted terminal repeat sequences (ITR), facilitating packing of the AAV genome into the AAV molecule.⁹⁴ If a helper virus is present, the AAV is able to replicate. With no helper virus, AAVs become latent and remain in episomal form. While rare, AAV genomic integration into host chromosomes has also been reported. The genome of AAVs can be manipulated and changed to create recombinant AAVs (rAAVs); this is the basis of AAV-based gene therapy. These rAAVs contain the two ITRs along with a specific promoter sequence, a gene of interest driven by that promoter, and a poly-A tail sequence that supports nuclear export of RNA and translation (Figure 1B). rAAVs are attractive for therapy for several reasons. Along with multiple tested and reliable

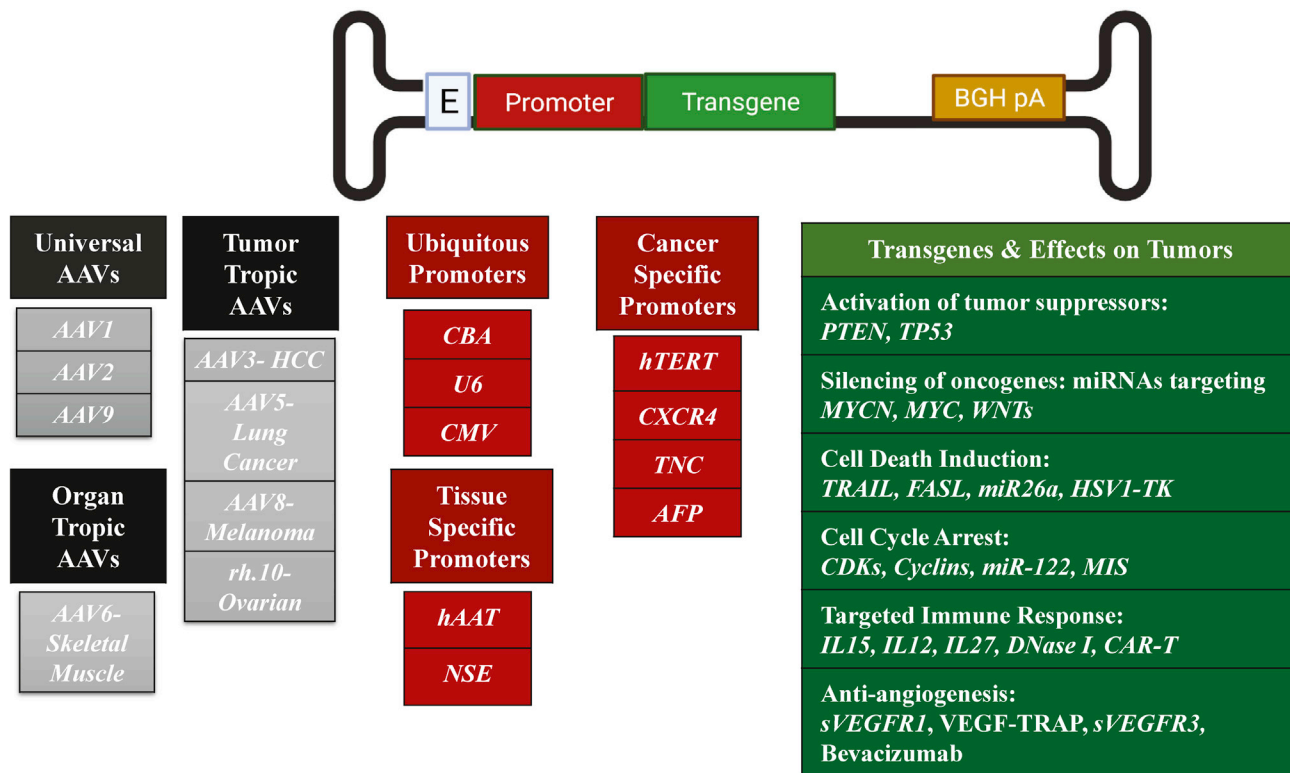


Figure 2. Design of rAAVs for various tumors

Reported AAV serotypes that target cancers include several common serotypes such as AAV2, 3, and 8. Some are used in specific tumor types, such as AAV8 for melanoma. The design of an rAAV for cancers consists of a serotype or promoter that targets tumor cells present in tissue by using a gene that is highly expressed in this cancer type; promoters will vary greatly based on genetic make-up of the cancer and the cancer type. Documented promoters used in various cancer research are listed in different categories. The transgene whose expression is controlled by the selected promoter will need to combat the tumor by inactivating a driver oncogene, activating a silenced tumor suppressor, inducing cell death or cell cycle arrest, preventing angiogenesis, or causing a targeted, localized immune response to the tumor. Reported and suggested transgenes are listed below each category. *E*, Enhancer sequence. *hAAT*, human alpha 1 antitrypsin. *hTERT*, human telomerase reverse transcriptase. *AFP*, alpha fetoprotein. *CBA*, chicken beta actin. *U6*, type III RNA polymerase III. *CMV*, cytomegalovirus. *CXCR4*, C-X-C motif chemokine receptor 4. *NSE*, neuron-specific enolase. *TnC*, tenascin-C. ^{5,6,8,9,53,55,58,61,101,102,115-126}

the transgene and are versatile as their genomes are easily manipulated and altered to meet the needs of specific studies and pathologies. One of the first AAV therapies approved for use in patients was Luxturna, an AAV2 used to treat Leber’s congenital amaurosis by inducing expression of the *RPE65* gene.^{73,107} Spinal muscular atrophy, another rare disease, is treated with an AAV9 gene therapy that facilitates alternative splicing of the *SMN* gene to produce the lacking SMN1 protein in patients with this disease.¹⁰⁸ Both AAV treatments are widely successful and have catalyzed the AAV therapeutics field now being explored in diseases such as hemophilia, Duchenne’s muscular dystrophy, and cancer.^{105,109-111} As cancer encompasses a heterogeneous group of diseases as well as single tumors containing intratumoral heterogeneity, the ability to tailor an AAV for a cancer type, specific tumor, or distinct cancer cell within a tumor is highly beneficial. Moreover, the long-term expression of the specific transgene conveyed by rAAV would provide a cancer therapeutic for patients with a longer half-life and could theoretically reduce frequency of hospital visits compared with regimens that contain other available treatments like chemotherapies, which require

several rounds of administration due to their short half-lives, periods of no therapeutic intervention, and clinic appointments (Table 1).^{1,4,15,31} Translational studies from multiple researchers show that several cancer subtypes, along with cancer-associated stroma and the supportive cancer microenvironment, are currently being targeted effectively with rAAVs.^{6,8,9,11,55,56,112-115}

rAAVs targeting primary tumors

rAAVs are a revolutionary therapeutic avenue being explored for specifically targeting primary tumors. These viral vectors are being designed to correct oncogene expression or restore tumor suppressor activity, to induce cell death, block angiogenesis, or to activate anti-tumor host immunity (Figure 2). Multiple AAV subtypes, including AAV6 and AAV2, have demonstrated success in infecting and reducing proliferation and growth of multiple cancer cell lines as well as the NeuT mouse mammary tumor model.^{55,116} M. Trepel et al. reported that an rAAV9 vector encoding HSV-TK, a suicide gene that creates a cytotoxic metabolite of the drug ganciclovir (GCV), along with a sequence that reduced cardiotoxicity, reduced mammary tumor growth and

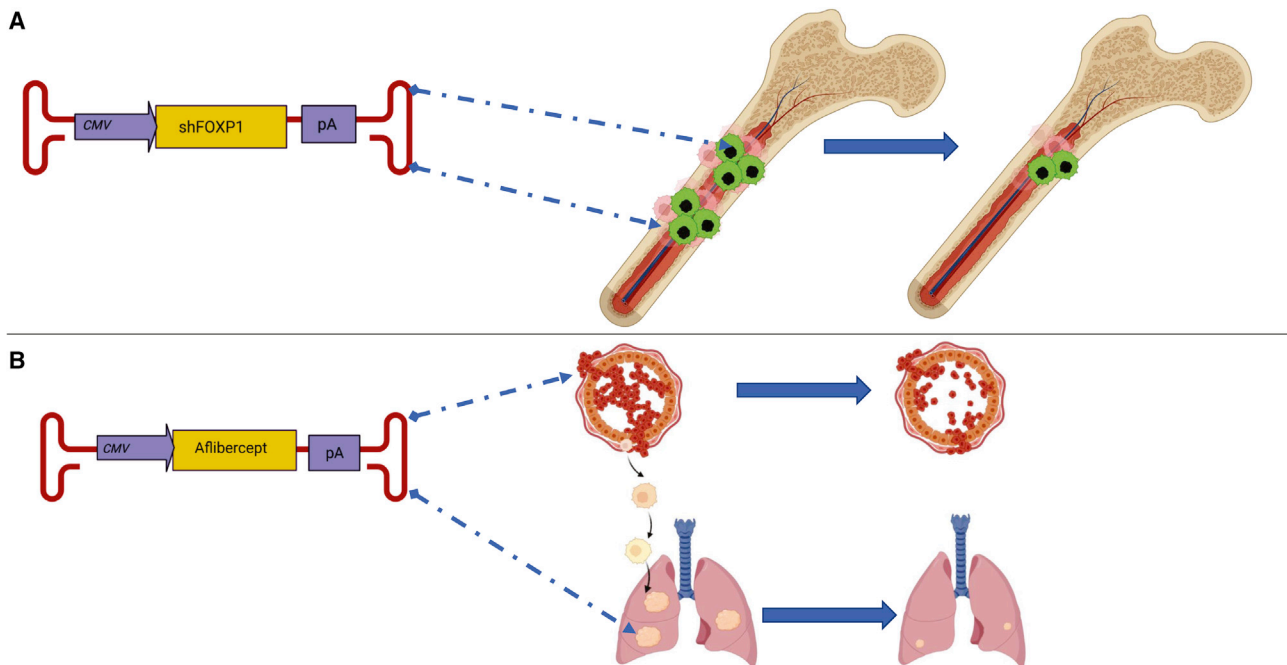


Figure 3. rAAVs targeting primary tumor and metastatic lesions

Basic designs of rAAVs used to target primary tumors and metastatic lesions. (A) rAAV2/9 drives expression of shRNA targeting the transcription factor FOXP1 in PDX osteosarcoma. Results demonstrated a shrinking of flank tumors in Balb/C-Nude mice by reducing human osteosarcoma growth.¹²⁷ (B) rAAV2 encoding expression of the VEGF-Trap antibody (aflibercept), a decoy receptor for vascular endothelial growth factor (VEGF), is successful at decreasing mammary tumor volume as well as the number of lung metastatic lesions in the syngeneic 4T1 murine mammary tumor model in Balb/C mice.¹²⁴

eliminated negative effects on ejection fraction and cardiac damage of the polyoma middle tumor antigen mouse model.¹¹⁷ There is also documented success of rAAVs successfully targeting HCC. Dhungel and co-authors report multiple AAV-based therapies being explored to target HCC.⁶ Many of the early studies using rAAVs to target HCC drove expression of genes that caused apoptosis in these tumors. HSV-TK and TRAIL, a pro-apoptosis factor, are two of the most popular therapeutic genes studied in HCC.^{115,118,119} Upon treatment with GCV, tumor cells that express HSV-TK will undergo apoptosis once the metabolite GCV-3P is made. Neighboring tumor cells are affected, which leads to tumor death. Regarding AAV-driven TRAIL expression, the hTERT promoter has been shown to be specific in targeting tumor cells and causing apoptosis both *in vitro* and *in vivo* mouse models of HCC. In recent studies, scientists have expanded rAAV target genes to target HCC via other mechanisms. L. Yin and colleagues demonstrated that an AAV3 vector driving expression of either miR-26a or miR-122 was effective at reducing growth of Huh7-FLUC cells in NSG mice.⁵⁸ These miRNAs are known to target angiogenic pathways, Wnt/ β -catenin, and certain cyclins. Given the tropism of several AAVs to liver, HCC is a viable, strong cancer target for AAV gene therapy. Indeed, AAV2 was shown in two mouse models of HCC development as well as human precision-cut liver slice experiments to preferentially infect tumor tissue compared with nonmalignant liver area.¹²⁰ Additionally, using an AAV3 vector to drive expression of pyruvate dehydrogenase E1 α led to cell death in human HCC.¹²¹

Several other cancer types, including rare and aggressive tumors, are also being studied with rAAVs. H. Li and co-authors showed that an AAV2/9 vector controlling expression of an shRNA that silenced the oncogene FOXP1 greatly reduced osteosarcoma growth in a PDX model over a 5-week time course (Figure 3A).¹²⁷ As this tumor type is rare and treatment options are limited, rAAV therapy could be a great new resource for effective therapies. Moreover, modifications of capsid proteins and the addition of specific conjugates to certain AAVs, like AAV6, changed its transduction capabilities in infecting myriad of cancer cell lines including PC3 prostate cancer cells and T47D ductal carcinoma cells.¹¹⁶ Prostate (PCa) and breast cancer (BCa) are the most diagnosed cancers in men and women, respectively.¹ In an innovative study, Cripe and colleagues utilized rAAV8 and AAVrh74 to create enhanced, AAV-based versions of blinatumomab, a bispecific CAR-T antibody approved to treat CD19+ ALL.^{28,128} Their rAAVs extended the half-life of the antibody as well as converted the drug to be inducible in one iteration of the therapy; each modification resulted in improved survival of NSG immunocompromised mice injected with Raji lymphoma cells.²⁸ This report adds to the plethora of applications for rAAVs in cancer biology. Regarding rarer cancers, S. Lee and H-J. Ahn demonstrated that modifying AAV2 to interact with EpCAM, a surface protein highly expressed in ovarian cancer, was effective at delivering an shRNA targeting EGFR signaling, significantly reducing ovarian tumor growth in Balb/C-Nude mice.¹²⁹ AAV2 without this EpCAM

modification was ineffective at blocking tumor growth, demonstrating the wide range of ways to make AAVs effective in targeting tumors at the primary site. Similar to early research with HCC, many of the gene therapy studies regarding these tumor types have centered on driving expression of suicide genes by promoters like GRP78 and PSA for PCa, and ErbB2 and MUC1 for BCa.^{10,11,56,61,113} An AAV9 construct using either the non-specific chicken beta actin promoter or the neuron-specific enolase promoter driving expression of soluble TRAIL demonstrated a retardation of a PDX orthotopic glioblastoma tumor model.¹²² The breadth of cancers that are being successfully targeting in translational studies, especially those used in PDX models, suggests an exciting and hopeful future in cancer research.

rAAVs targeting metastatic lesions

The documented translational research successes of rAAVs in cancer studies infer that this viral gene therapy could be groundbreaking in treating a catastrophic event that occurs during many patients' battles with the disease, metastasis. Regardless of tumor type, once metastasis occurs and the primary tumor spreads to distant secondary sites, the 5-year survival rate of the patient dramatically decreases, emphasizing the need for early effective treatments and metastasis-specific therapies that prolong survival.^{1,4,10,22,130–135} The cells of a tumor that metastasize to a secondary organ express a genetic profile that is different from the primary tumor, a characteristic of tumor heterogeneity that in some ways makes metastatic cancer its own distinct disease.^{130,132,134,136} For example, several groups report specific secretome and genetic profiles of metastatic clones of cancer cell lines compared with the parental line, a biological trait that can be exploited by the engineering versatility of AAVs to specifically and effectively target subgroups of cancers located in either a primary or secondary site.^{54,132,137} In breast cancer and lung cancer, COL6A1 is reportedly expressed at higher levels in metastatic tissue compared with non-metastatic tissue.¹³⁸ Work published by M.A. Blanco and colleagues identified plasminogen and other tissue repair genes compiling a bone metastatic profile of the triple-negative breast cancer (TNBC) MDA-MB-231 cells and the murine mammary tumor line 4T1s. Forced expression of these plasminogen genes in the non-bony metastatic SCP28 breast cancer cell line caused these cells to invade bone and produce osteolytic lesions.¹³⁹ Prostate cancer cells that express high levels of ESL-1, a protein involved in diapedesis and endothelial adhesion, exhibited enhanced bone metastasis.¹³⁷

Encouragingly, rAAVs being synthesized to target metastatic lesions are showing promise in many metastatic tumor models. For example, Y. Xia and associates demonstrated that treating mice with the Anc80L65 AAV encoding DNaseI under control of the human alpha-1 antitrypsin promoter significantly reduced colonization of circulating MC38 CRC cells in the liver of C57Bl/6 mice through induction of a host immune response.¹²³ Increased expression of this enzyme induced a CD45+ immune response in the metastatic liver lesions and reduced the amount of tumor-supportive neutrophil extracellular traps (NETs) in the liver tumor microenvironment. Consequently, this rAAV treatment reduced colonization of circulating

MC38 CRC cells in the liver. Focusing on a different hallmark of cancer, pulmonary metastatic lesions of the aggressive 4T1 mouse mammary carcinoma model were targeted with an AAV2 vector that drove expression of VEGF-Trap, an antiangiogenic chimeric protein that prevents angiogenesis by binding VEGF-A as a decoy receptor. Not only were the number and size of lung metastatic lesions reduced, but the primary tumor size was also decreased because of this VEGF-Trap gene therapy (Figure 3B).¹²⁴ Importantly, VEGF-Trap expression maintained throughout the *in vivo* studies after a single dose. A third example of successful application of rAAVs in targeting metastatic disease is reported in bone.¹³⁰ Bone metastases remain a devastating clinical outcome of several commonly diagnosed cancers including breast and prostate. Work from Y. Yao and colleagues reveals that AAVs could be an effective treatment in the future for these types of lesions.¹⁴⁰ They used an rAAV2 driving expression of Kringle 1 domain of human hepatocyte growth factor, a known antiangiogenic and antiproliferative protein, to prevent osteolytic lesion development *in vivo* using a bone-tropic MDA-MB-231 human TNBC cell line. As metastatic disease results in extremely worse prognoses for cancer patients regardless of tumor type, building upon results such as these will be critical in future AAV gene therapy work.

rAAVs targeting the tumor microenvironment

It is well-documented that non-cancerous cells, extracellular matrices, and physical properties of various organs and regions of the body facilitate tumor development and metastasis.^{21,130,131,133,135,141–151} Because of these data, several conventional cancer therapeutics indeed have been designed to block signaling of and interactions between stromal cells such as cancer-associated fibroblasts, vasculature, and pro-tumor immune cells, and tumor cells with varying successes.^{22,152–157} In the context of gene therapy, rAAVs are also being designed to target these types of tumor-supporting cells. As previously mentioned, the rAAV utilized by Y. Xia and co-authors to drive expression of DNase I in order to target liver metastatic lesions of the MC38 CRC cell line was successful via targeting NETs and increasing the population of anti-tumor CD8 T cells in the liver lesions (Figure 4).¹²³ rAAVs containing sPD-1 have been documented to alter the host immune response in mouse models of cancer. Elhag et al. showed increased anti-tumor cytotoxic T lymphocyte infiltration in a hepatoma mouse model, leading to increased survival.¹⁵⁸ Similarly, Z. Tan and colleagues published in a mesothelioma mouse model that an rAAV-DJ serotype driving sPD-1 fused with a common oncogene, TWIST1, and increased CD8+ T cells in the tumor mass, leading to increase survival.¹⁵⁹ rAAV2 has also been reported to infect cancer-associated vasculature in the NeuT mouse mammary tumor model, reducing vascular length and increasing apoptosis in tumor-associated "endothelial cells (ECs)."⁵⁵ Along with altering the immune response to tumors, A. Volak et al. showed that an rAAV from their group, deemed exo-AAV, was able to cross the blood-brain barrier and be modified to infect various stromal cells in an aggressive glioblastoma mouse model, including reactive astrocytes and tumor-associated microglia. When the exo-AAV was designed to target astrocytes using glial fibrillary acidic protein promoter, a modest survival increase was observed along with an increase in tumor mass

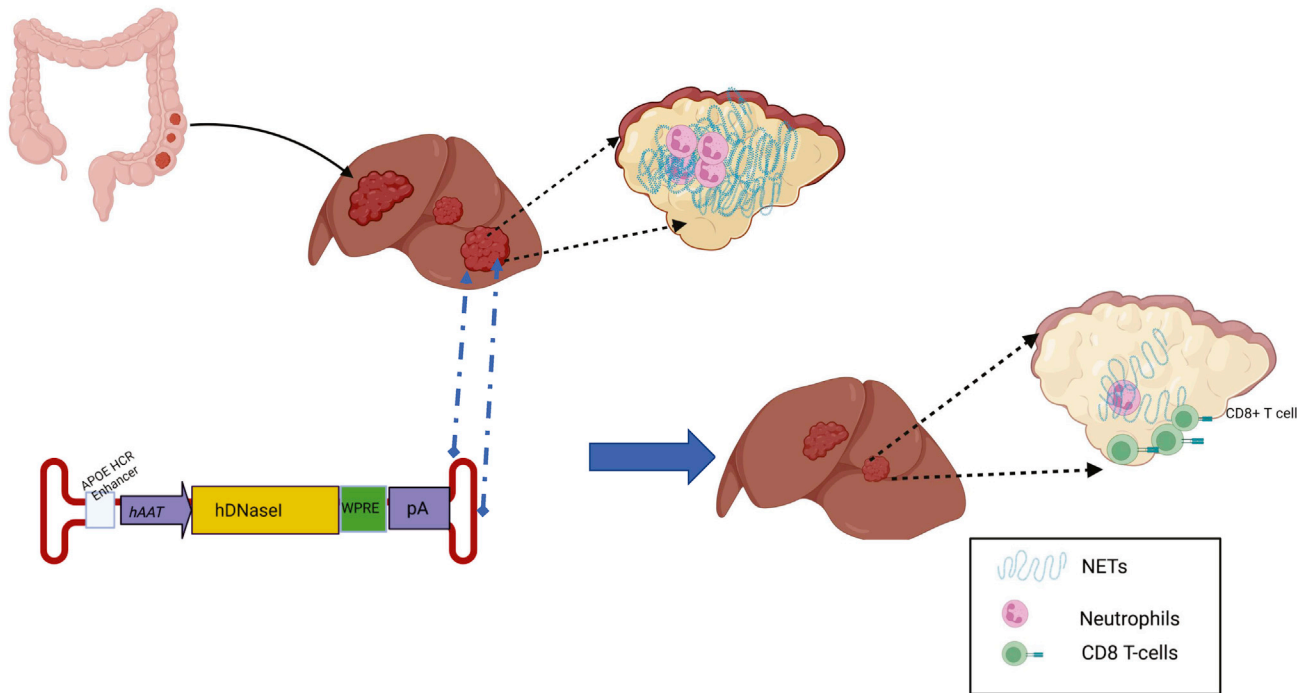


Figure 4. rAAV targeting the tumor microenvironment

rAAV2/Anc80L65 for DNase I is effective at treating colorectal cancer liver metastases by reducing the presence of neutrophil extracellular traps (NETs). Enhancing DNase I activity reduces NETs and neutrophil infiltration in colorectal cancer (CRC) liver lesions, ultimately reducing lesion size and increasing the presence of anti-tumor CD8+ T cells.¹²³ *APOE-HCR*, apolipoprotein E-hepatic control region. *WPRE*, woodchuck hepatitis virus posttranscriptional element.

death.¹⁰³ Indeed, these preclinical benefits of targeting the tumor microenvironment demonstrate even more possible applications for rAAV gene therapy to impact various cancers, especially those in need of new treatments, in the clinic.

Intriguingly, AAVs infect cells by initial contact with a range of cell-surface receptors and integrins. For example, AAV2 is known to interact with heparan sulfate proteoglycan, laminin receptor, MET, and integrins containing the α_v subunit.^{46,96,160–162} Overexpression of many of these proteins and their ligands is a driver of cancer growth and metastasis, as well as being expressed by myriad supporting cells that fuel cancer growth. These data infer that cancer cells and their supportive stroma are prime targets for rAAVs infection. The versatility and malleability of rAAVs via transgene expression, capsid serotype, and the promoter sequence that drives the transgene can be synthesized to effectively target cancer, specifically those that have not had much progress in therapeutics in recent history.

rAAV design and specificity in targeting cancers

The effectiveness of rAAVs in targeting cancer depends on multiple details: the specific AAV capsid protein, the encoded promoter, and the transgene being driven by the specific promoter.¹⁶³ These components of the AAV can be manipulated to create an adaptable range of effective AAVs to target many aspects of cancer development, growth, and metastasis in various tissues.

There are 13 natural serotypes of AAV, and each has distinct biological properties, including tissue tropism.^{89,90,164} For example, AAV7, AAV8, and AAV9 have a reported tropism for liver and are being used to target diseases like hemophilia.^{105,109} Other AAVs, such as AAV1, infect heart and skeletal muscle regularly, and AAV2 and AAV3 infect the retina as well as the liver.^{86,165} The receptors that certain AAVs interact with to enter cells varies, and this is related to the specificity seen in certain cells and tissue types. Scientists have also been able to engineer AAVs with specific capsids that contain a mixture of several natural AAVs, hybrid AAVs, modifications on surface proteins that make the capsids bind receptors with greater affinity, or AAVs that combine traits from different animal sources such as rats, mice, and goats.^{12,89,166,167} Interestingly, the AAV tropism of non-transformed tissues does not always match the tropism profile of AAV infection of tumor tissues. AAV3 is reported to infect liver cancer cells at a greater rate than healthy hepatocytes.^{58,121} This disparity is actually advantageous, as a key point of cancer therapy should be to spare as many healthy cells as possible and target only tumor cells. Moreover, our group is exploring chemical modifications of AAV capsid proteins, which is illuminating different profiles of AAV organ tropism and infection patterns.⁹¹

A review by Montaña-Samaniego and colleagues superbly explains how AAV design should be engineered with the promoter in mind to maximize efficiency.⁶¹ Genes of the specific tissue affected by the tumor, genes that are specific to cancers across types, and/or genes

specific to a particular tumor type or subtype can be used as the promoter encoded by the AAV to direct the virus. There are several promoters considered ubiquitous; this refers to the non-specific nature of the gene from which the promoter is derived and the potential for broad, high levels of transgene transcription and translation. Some examples include CMV, CB, EF1 α , and TBG. While reliable to induce expression of the transgene, the drawback in using these types of promoters is the potential in altering stromal, non-cancerous cells. This group of promoters contrasts with tissue-specific promoters used to induce more controlled, targeted expression of the transgene of interest. For example, rAAVs designed to target hemophilia, a set of hematological clotting disorders, contain the liver-specific transthyretin promoter.^{96,161–163} This distinct promoter relegates transgene expression of the absent clotting factor protein (*FVIII* for HemA or *FIX* for HemB) to hepatocytes and the organ where these clotting proteins are normally synthesized.^{98,161,168–170} This concept can be expanded to using cancer-specific promoters from genes that are generally overexpressed in several tumor types, as well as tumor-specific promoters, which would be a gene unique to the particular tumor target of the rAAV. Some effective promoters currently being explored include hTERT, AFP, and ERBB2.^{113,125,171} One of the goals of rAAV therapy is to be as targeted and specific to the cancer mass as possible, and the selection of the promoter is a key feature in achieving tumor specificity.

Equally important in engineering rAAVs for cancer is the transgene being driven by the promoter in the vector. As stated earlier, myriad genes are being explored for efficacy against several cancers. These transgenes demonstrate varying levels of effectiveness against primary tumor lesions as well as some metastatic growths that occur during progression. Pro-apoptotic genes such as TRAIL and miR26a, cytokines that stimulate anti-tumor immunity like IL-27 and IL-15, and antiangiogenic proteins are some examples of *in vitro* and translational research successes.^{101,102,124,172–174} The AAV serotype and the promoter will control the distribution and effects of the transgene in the target tumor and tissue, giving rise to a potential revolutionary set of tools to treat cancer and improve patients' lives. rAAVs that are specific for tumors or common metastatic sites could be designed as prophylactics for patients recovering from a primary tumor or those at high risk of developing cancer. Moreover, rAAVs can be used as a long-term treatment for established cancer lesions and preventing recurrence, as an attractive, beneficial feature of this developing therapy is the long-term effects observed after one dose observed in diseases such as Leber's congenital amaurosis, hemophilia, and spinal muscular atrophy.^{73,105,108,109}

Combination therapies with rAAVs for cancers

In addition to targeting the many cells and factors that comprise and influence cancer progression, approaches combining rAAVs with current cancer therapies are being explored pre-clinically.^{5,7} Many of the combinations include a chemotherapeutic agent such as platinum-based drugs, 5-fluorouracil, and doxorubicin that supplements the rAAV gene therapy that is targeting a specific aspect of the tumor.^{5,16} These combinations apply cell cycle stress on the tumor cells

that lead to toxicity and cell death. Inhibition of angiogenesis via encoding expression of bevacizumab or inducing cell death pathways through expression of pro-apoptotic gene TRAIL are some rAAV designs that have shown enhanced cytotoxic stress in tumors, leading to more death when combined with a standard chemotherapy.^{17,23,175} While more details on the specific mechanisms of action need to be determined for these combination treatments, the results are promising and add to the potential impact rAAVs could have in the cancer field.

Conclusion

Cancer is a devastating group of diseases that continues to have a massive negative global impact. Despite improved survival rates through early detection, better care, and improved therapies, millions will succumb to the disease. Gene therapy is having a positive impact on several diseases in the clinic. rAAVs, a virus-based gene delivery platform, could be a groundbreaking new group of effective therapies for targeting cancers of any type. rAAV's versatility, ability to be tailored to specific tissues and cells, and low immunogenicity are traits that epitomize the ideal cancer treatment: specific, potent, and with potentially limited side effects. With cancer patients living longer, and dormant secondary lesions having the ability to manifest months to years after an initial treatment, the long-term effects of a successful, targeted rAAV therapy could change the way cancers are treated, making them more of a manageable disease than an outright, eventual death sentence. The limitations and hurdles of using rAAVs, namely natural immunity to certain serotypes present in individual patients, a relatively small genome size (4.7–5 kba), documented production of neutralizing antibodies to various serotypes, low chance of genomic integration into the host genome, and possible off-target infection of certain tissues or cell types based on capsid serotype, promoter, or transgene, will need to be a top interest of scientists and clinicians as research testing of rAAVs and cancer expands. As there are many aspects of cancer that need to be addressed clinically, current results of rAAVs and cancer are encouraging in the hope that rAAVs will be used to target cancer, cancer metastatic disease, and the myriad comorbidities that arise specifically from the disease. Although more translational and preclinical research is warranted to ensure safety of the many different rAAVs that can be designed for cancers in the clinic, the possibilities of this viral gene therapy are limitless and could direct the future of cancer therapeutics, especially for metastatic cancers, residual and relapsed disease, and rare cancers.

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AUTHOR CONTRIBUTIONS

P.L.M. and W.X. conceived the concept for the review. P.L.M. wrote the manuscript and designed figures using [BioRender.com](https://www.biorender.com) licensed through IUSM Herman B Wells Center for Pediatric Research.

R.W.H. and W.X. edited the manuscript and provided scientific guidance for the final draft. P.L.M. conducted revisions for the review. All members approved the final draft.

DECLARATION OF INTERESTS

R.W.H. is editor-in-chief of Molecular Therapy and has served on scientific advisory boards for Regeneron Pharmaceuticals and BioMarin Pharmaceuticals. W.X. holds equity in the following: Ivygen Corporation and Nikegen LLC.

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