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# Adeno-Associated Virus (AAV) Vectors in Cancer Gene Therapy

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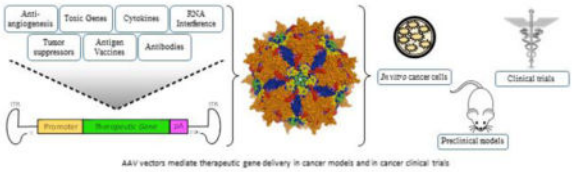
## Abstract

Gene delivery vectors based on adeno-associated virus (AAV) have been utilized in a large number of gene therapy clinical trials, which have demonstrated their strong safety profile and increasingly their therapeutic efficacy for treating monogenic diseases. For cancer applications, AAV vectors have been harnessed for delivery of an extensive repertoire of transgenes to preclinical models and, more recently, clinical trials involving certain cancers. This review describes the applications of AAV vectors to cancer models and presents developments in vector engineering and payload design aimed at tailoring AAV vectors for transduction and treatment of cancer cells. We also discuss the current status of AAV clinical development in oncology and future directions for AAV in this field.

## Graphical abstract




### Keywords

Gene therapy; gene delivery; adeno-associated virus; AAV; gene delivery vectors; cancer

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*Conflict of interest statement*. DVS and JLSO are inventors on patents involving AAV directed evolution, and DS is the co-founder of an AAV gene therapy company.

## INTRODUCTION

Cancer, a large group of diseases characterized by the unregulated proliferation and spread or metastasis of abnormal cells, collectively represents a major worldwide healthcare problem. In the U.S. alone more than 1.5 million cases are diagnosed each year, and cancer overall has a 5-year relative survival rate of 68%, making it the second leading cause of death after heart disease [1]. Standard treatments include surgery, chemotherapy, and radiotherapy; however, these are often incapable of completely eradicating a malignancy [2] and can be accompanied by serious side effects [3]. Thus, there is a strong unmet medical need for the development of novel therapies that offer improved clinical efficiency and longer survival times in patients afflicted with disease.

Gene therapy, defined as the introduction of genetic material into a target cell for therapeutic benefit, is a very promising treatment for many diseases, including monogenic diseases, cancer, cardiovascular disease, and neurodegenerative diseases. To date, more than 2,000 clinical trials employing gene transfer have taken place and in general have established that a number of vehicles or vectors are safe [4, 5]. Furthermore, the majority (64%, n=1,415 [6]) of gene therapy clinical trials to date have targeted cancer – including lung, skin, neurological, and gastrointestinal tumors – and have utilized a variety of therapeutic strategies such as anti-angiogenic factors, tumor suppressors, immunostimulation, and oncolytic viruses. In 2015, the first recombinant viral therapy – an oncolytic herpesvirus for the treatment of melanoma – received regulatory approval in the U.S. [7].

For cancer gene therapies to be increasingly successful, however, a major hurdle must be overcome: the development of gene delivery vectors that can safely, efficiently, and specifically deliver genetic material to the target cells. Non-viral vectors can be easily produced at a large scale and are readily amenable to engineering or enhancement of their functional properties via chemical modifications; however, they suffer from a low delivery efficiency and in some cases cell toxicity [8]. On the other hand, viral vectors harness the highly evolved mechanisms that the parental viruses have developed to efficiently recognize and infect cells and offer several advantages, which make them suitable for both therapeutic application and as tools for biological studies; however, their delivery properties can be challenging to engineer and improve. That said, viral vectors have been used in the majority (over 68% [6]) of gene therapy clinical trials, and the most frequently used have been based on adenovirus, retrovirus, vaccinia virus, herpesvirus, and AAV [9].

AAV vectors in particular have been increasingly successful due to their gene delivery efficacy, lack of pathogenicity, and strong safety profile [10]. As a result of these properties, AAV vectors have enabled clinical successes in a number of recent clinical trials that have established the promise of gene therapy in general, including for the treatment of diseases such as Leber’s congenital amaurosis (LCA) [11, 12], where over four Phase I and I/II clinical trials have demonstrated safety and long-term (over five years) improvement in retinal and visual function; hemophilia B, targeted in several Phase I and Phase I/II clinical trials that have shown long-term efficacy and no toxic effects [5, 13]; and the Sanfilippo B syndrome, where gene expression and consequently improved cognitive development have been sustained for at least a year and are still ongoing (Pasteur Institute Phase I/II trial,

unpublished). Moreover, alipogene tiparvovec (Glybera; uniQure), a gene therapy for lipoprotein lipase deficiency (LPLD) that employs an AAV vector, received regulatory approval by the European Medicines Agency in 2012 [14]. AAV vectors may also offer a strong potential for the treatment of cancer, and as presented in this review, their excellent gene delivery properties have been harnessed for *in vitro* cancer studies, *in vivo* pre-clinical cancer models, and more recently cancer clinical trials under development.

## ADENO-ASSOCIATED VIRUS (AAV) AND AAV VECTORS

### AAV Biology

AAV is a single-stranded DNA parvovirus with a 4.7 kb genome (Figure 1A) composed of the *rep* and *cap* genes flanked by inverted terminal repeats (ITRs) [15]. The *rep* gene encodes non-structural proteins involved in viral replication, packaging, and genomic integration, whereas the *cap* gene codes for structural proteins (VP1, VP2, VP3) that assemble to form the viral capsid, which serves as the viral gene delivery vehicle. Additionally, an alternative open reading frame nested within the *cap* gene encodes the assembly activating protein (AAP), involved in the targeting and assembly of capsid proteins [16]. Following cellular entry through cell surface receptor-mediated endocytosis, endosomal escape, trafficking to the nucleus, uncoating, and second DNA strand synthesis, the virus can enter its replication cycle in the presence of a helper virus [17]. In the absence of a helper, however, AAV genomes can establish latency and persist as episomes [18] or in some cases integrate into host chromosomal DNA [19].

### AAV-Based Vectors: Properties and Clinical Success

Recombinant AAV vectors can be generated by replacing the endogenous *rep* and *cap* genes with an expression cassette consisting of a promoter driving a transgene of interest and a poly(A) tail (Figure 1B). The *rep* and *cap* genes are then provided *in trans* as helper packaging plasmids together with adenoviral helper genes needed for AAV replication [10]. Over 100 natural AAV variants have been isolated, and variations in amino acid sequences result in somewhat different tropisms (the range of cells and tissues a virus can infect) [20], though none are pathogens [21]. Recombinant vectors have been generated from a number of these serotypes [10], though vectors based on AAV-serotype 2 (AAV2) have been the most widely studied and used in preclinical models and clinical trials to date. In general, vectors based on natural AAV variants have desirable gene delivery properties: a lack of pathogenicity and immunotoxicity, which grants them a strong safety profile [21]; the ability to infect dividing and non-dividing cells with reasonable efficiency [22]; the ability to mediate stable, long-term gene expression following delivery [20]; a ~5 kb genome that can carry a broad range of cargoes [23]; access to faster expression kinetics when using self-complementary, double stranded DNA forms of the vector genome [24]; and importantly the potential for engineering and optimizing the viral capsid and thus vector delivery properties [15]. Accordingly, AAV-based vectors have been harnessed in an increasing number of clinical trials (>130 to date) for tissue targets including liver, lung, brain, eye, and muscle [10, 25]. As a result of its properties, as mentioned above, AAV has enabled clinical efficacy in an increasing number of trials for monogenic diseases [5, 26–29].

For oncology applications, AAV vectors can transduce a wide variety of cancer primary cells and cell lines [30–32] and have the capacity to carry highly potent therapeutic payloads for cancer including anti-angiogenesis genes, suicide genes, immunostimulatory genes, and DNA encoding smaller nucleic acids (e.g. shRNAs, siRNAs) for post-transcriptional regulation of oncogenes [33]. AAVs therefore offer a strong potential as gene delivery vehicles for cancer gene therapy and have consequently been employed in numerous preclinical cancer models and in early stage clinical trials for cancer.

## ENGINEERING AAV VECTORS FOR CANCER GENE THERAPY

### Gene Delivery Challenges of AAV Vectors

Natural variants of AAV have enabled increasing success in human clinical trials, which have in turn provided strong momentum to the gene therapy field as a whole. That said, natural AAV serotypes have some shortcomings that render this success challenging to extend to the majority of human diseases, including cancer. As has been reviewed [10], barriers for AAV and other vectors include: prior exposure of most people to natural AAVs leading to anti-AAV neutralizing antibodies that can reduce vector delivery efficiency by orders of magnitude *in vivo*, poor vector biodistribution to important tissue targets, limited spread within those tissues, an inability to target specific cells, and limited efficiency for many therapeutically relevant target cells. These concerns have motivated the engineering of AAV capsids that can more efficiently traffic to and transduce cells, as well as the engineering of genetic cargos for higher potency and selective expression.

### General Developments in AAV Vector Engineering

The amino acid sequence of the proteins that constitute the viral capsid (Figure 2A) determines an AAV vector's delivery properties, including interactions with tissue and vasculature, humoral and cellular components of the immune system, specific receptors on the target cell surface, the endosomal network following receptor-mediated internalization, the cytosol after the viral phospholipase domain enables endosomal escape, and ultimately the nucleus. Thus, engineering the AAV capsid can generate novel AAV variants with novel and enhanced delivery properties [10]. Such vector engineering efforts can be grouped into two categories: rational design, where structure-function relationships are used to guide specific capsid modifications, and directed evolution, where libraries of AAV capsids are generated using a range of mutagenesis techniques and then subjected to a selective pressure for properties of interest.

The AAV capsid has been rationally engineered in several ways. For example, capsid surface-exposed tyrosine residues, whose phosphorylation targets the virion for ubiquitination and subsequent proteasomal degradation, have been modified via site-directed mutagenesis to phenylalanine residues to generate variants with reduced proteasomal degradation and subsequent higher gene expression [34]. Structural capsid information has also been used to generate variants with some resistance to pre-existing neutralizing antibodies by mutating surface residues that may interact with Immunoglobulin G antibodies (IgG's) [35]. As reviewed below, rational design has also been employed to generate variants with enhanced transduction (infection and transgene expression) in tumor cells by using site-

directed mutagenesis and inserting peptides with motifs that bind to receptors highly expressed in cancer cells.

In general, however, the AAV delivery pathway from point of administration until the particle arrives in the nuclei of target cells is extremely complex, and there is often insufficient knowledge of viral structure-function relationships to enable rational design efforts. Therefore, another approach is based on the idea that evolution can generate novel and useful biological function even in the absence of detailed mechanistic knowledge. Specifically, directed evolution has been developed and implemented to generate greatly enhanced AAV variants for a variety of applications. In this approach, the AAV *cap* gene is genetically diversified to create large libraries of novel AAV variants (~$10^4$ – $10^8$) utilizing a range of molecular approaches including DNA shuffling, random point mutagenesis, insertional mutagenesis, random peptide insertions, and most recently ancestral reconstructions [36–45]. The libraries are then subjected to a selective pressure to acquire specific, advantageous delivery properties [10], and after a suitable number of selection rounds individual AAV variants are isolated, validated, and harnessed for therapeutic gene delivery in disease models. Directed evolution has been applied [15] to create novel, optimized AAV vectors with enhanced delivery to non-permissive cells such as human airway epithelium [46], neural stem cells [47], human pluripotent stem cells [48], retinal cells [49], and other tissues *in vitro* and *in vivo* [36, 37, 49–53]. AAV vectors have also been evolved for *in vivo* enhanced tissue spread and infection of non-permissive cell types [49, 50]. Thus, *in vivo* directed evolution strategies could potentially be extended to engineer novel AAV vectors for enhanced gene delivery to tumors.

## Rational Design for the Engineering of Cancer-Specific Transduction

Changes in protein expression patterns [54] and subsequent presentation of tumor-specific antigens [55, 56] in cancer tissues may enable the preferential targeting of AAV gene delivery vehicles to tumor tissues [57, 58]. For example, Grifman *et al.* [59] targeted aminopeptidase N (or CD13), a membrane-bound enzyme that is highly expressed in cancerous tissue and vessels and has consequently been explored for targeted cancer therapies [60]. Specifically, they modified the AAV2 capsid, whose primary receptor for cellular entry is heparin sulfate proteoglycan (HSPG), by introducing a NGR peptide motif, which binds to CD13 [57], either in replacement of antigenic loops of AAV2 (residues T448-T455 and N587-A591) or after residues 449 and 588 of the capsid (Figures 2B, 2C). Mutant viruses with the NGR motif exhibited reduced affinity for heparin, suggesting a tropism different from AAV2, and transduced the sarcoma cell lines KS1767 and RD (which express CD13 at high levels) 10- to 20-fold better than wild-type AAV2, demonstrating the selectivity of these vectors.

Integrins, which contribute to tumor progression and metastasis, are also highly expressed in tumor cells and tumor vasculature [61] and have accordingly also been harnessed for selective tumor transduction. AAV2 vectors have been modified by inserting a 4C-RGD peptide [62], whose RGD motif selectively binds to αvβ3 and αvβ5 integrins [57], into different sites of the *cap* gene. Mutant vectors with the RGD insertion after residues 584 and 588 of VP3 (Figures 2B, 2C) retained infectivity, and the A5884C-RGD mutant was shown

to bind to integrin and to mediate increased *in vitro* and *in vivo* gene delivery to integrin-expressing tumor cells. A5884C-RGD- mediated gene delivery was 40-fold higher on K562 human chronic myelogenous leukemia cells, 13-fold higher on Raji human lymphoblast-like cells, and 6-fold higher on SKOV-3 human ovarian adenocarcinoma cells, compared to wild-type AAV2.

Additionally, designed ankyrin repeat proteins (DARPins) targeting cancer-associated receptors have been fused to AAV2 capsid proteins for enhanced selectivity to cancer cells [63]. DARPin 9.29, which specifically binds to HER2/*neu*, a receptor overexpressed in cancer cells, was fused to VP2 and then used to package AAV2 particles whose affinity for HSPG had been ablated through site-directed mutagenesis. The resulting vectors (Her2-AAV) transduced cells in a HER2-dependent manner, showing selectivity for HER2-expressing cells and only weakly transducing cells not expressing the receptor. Moreover, systemically administered Her2-AAV vectors localized to subcutaneous tumors of $HER2^+$ SK-OV-3 cells in mice, compared to a lack of tumor cell transduction of AAV2 vectors, which instead localized primarily to the liver.

AAV5 has also been engineered by inserting homing peptides for integrins, sialyl Lewis X (sLex), and tenascin C (TnC), which are overexpressed in many cancer tumors [64]. Mutants with RGD peptides infected integrin-expressing cells 5-fold better than AAV5, and both integrin- and TnC-targeting AAVs preferentially transduced cells presenting these molecules while showing very low transduction of cells negative for these antigens. Mutants with the sLex-targeting peptide did not transduce sLex-expressing cells.

Cheng *et al.* [65] studied the effects of mutating surface-exposed tyrosine residues to phenylalanines on AAV3. Three of the single-residue mutants – Y701F, Y705F, and Y731F (corresponding residues in AAV2 depicted in Figures 2B, 2C) – showed 1.5-, 2.2-, and 8.8-fold enhanced transduction, respectively, of Huh7 hepatocellular carcinoma (HCC) cells and 2.3-, 3.3-, and 9.1-fold enhanced transduction, respectively, of Hep293TT human hepatoblastoma tumor cells compared to wild-type AAV3. A double mutant, Y705F+Y731F, showed further increased transduction (11-fold higher) on Huh7 cells. This double mutant, when administered intra-tumorally or systemically to a mouse xenograft model of human liver tumors, also showed enhanced transduction, as reported by fluorescence microscopy, compared to AAV3. Additional studies with AAV3 have mutagenized serine, threonine, and lysine residues to valine, glutamate, and arginine residues, respectively, in addition to changing tyrosine residues to phenylalanines (corresponding residues in AAV2 depicted in Figures 2B, 2C) [66]. Mutants S663V+T492V+K533R, S663V+T492V+K533R, and S663V +T492V had transduction efficiencies over 10-fold higher than wild-type AAV3 on Huh7 cells. Some of these mutants also showed 2- and 8-fold enhanced transduction on HepG2 and Hep293TT cells, respectively, and the S663V+T492V mutant showed 2-fold higher transduction than the Y705+731F mutant in mouse xenografts of these cells lines.

There has also been a strong interest in AAV delivery to immune cells. In particular, given that cancers can develop resistance mechanisms against both drugs and the immune system, cancer research efforts have also focused on stimulating the adaptive immune system to mount a T cell-mediated anti-tumor response. Immunotherapy offers important potential

advantages over traditional therapies, including selectivity for tumor cells and the generation of memory T cells that protect against recurring tumors [67]. This has motivated the engineering of viral vectors with enhanced infectivity for dendritic cells (DCs), antigen-presenting cells that can prime T cells and generate an anti-tumor cytotoxic T lymphocyte (CTL) immune response. Surface-exposed serine and threonine residues in AAV6 (corresponding residues in AAV2 depicted in Figures 2B, 2C) have been mutated to valine residues for enhanced *in vitro* transduction efficiency on monocyte-derived DCs (moDCs) [68]. Mutants T492V, S663V, and T492V+S663V showed enhanced infectivity, with the double mutant showing 5-fold higher transduction. This T492V+S663V mutant was used to transduce moDCs with human prostate-specific antigen (hPSA), which led to and a 3-fold higher hPSA expression in moDCs and a 1.3-fold stronger CTL response against human prostate adenocarcinoma cells compared to wild-type AAV6 gene delivery, underscoring the utility of enhanced AAV vectors that can be used for cancer immunotherapy, particularly if delivery can be achieved *in vivo*.

Additionally, AAV vectors have been modified by inserting protease recognition sequences on the capsid such that protease cleavage is required for complete viral transduction [69]. Specifically, short sequences encoding negatively charged amino acids, which serve as “locks” by interfering with virus-receptor interactions, were flanked by protease cleavage sites recognized by matrix metalloproteinases (MMPs) and genetically inserted into surface-exposed regions near the heparin binding domain of the AAV2 capsid. Inactivated or “locked” vectors had reduced heparin affinity and infectivity, and treatment with MMPs restored heparin binding and allowed efficient transduction. MMPs are highly expressed in most cancers compared to normal tissue [70], so such protease-responsive AAV vectors could provide enhanced selectivity towards cancerous tissues, as other studies that have exploited high levels of MMP expression for selective viral gene delivery have demonstrated [71].

## Directed Evolution for the Engineering of Cancer-Specific Transduction

As described above, directed evolution and library selection are alternative approaches that can generate highly efficient vectors, even in the absence of mechanistic knowledge underlying a particular gene delivery barrier. Michelfelder *et al.* [72] employed an *in vitro* selection scheme to identify variants from an AAV2-based random peptide insertion library that had high infectivity on tumor cells. In this case, analogous to the rational approach, the selected AAV variants shared the RGDXXXX amino acid motif and exhibited over 15-fold higher transduction than wild-type AAV2 vectors on PymT breast cancer cells. The investigators also performed an *in vivo* selection, in which they administered the AAV library to tumor-bearing mice, harvested tumor tissue, and then recovered the peptide sequences of viral particles that had successfully infected tumor cells. Selected mutants had peptide sequences rich in serine and glycine residues. Dominant clones displayed 40- to 200-fold higher *in vivo* transduction of breast tumor tissue compared to wild-type vectors and interestingly also showed enhanced cardiac tropism; however, the liver tropism of the mutants was comparable or only moderately lower than that of wild-type vectors.

A DNA shuffled library of AAV serotypes 1, 2, 5, 9, rh8, and rh10 was selected for transduction of U87 human glioma cells and generated infectious mutants after seven rounds of *in vitro* selection [73]. One of the selected clones, AAV-U87R7-C5, had a chimeric *cap* gene with contributions from serotypes 1, 2, rh.8 and rh.10. This mutant transduced U87 cells and a panel of other glioma cells moderately better than AAV2.

## Payload Engineering for Cancer-Specific Expression

In addition to tissue specificity and high transduction efficiency, AAV vectors for cancer gene delivery may also have regulatory elements that promote tissue-selective gene expression, a feature particularly important when off-target transduction with a cytotoxic or immunostimulatory factor is a potential risk. Promoters that are tissue-specific, tumor-specific, or tumor microenvironment-specific have been explored to restrict transcription of the delivered transgene to a cancerous tissue of interest [74]. For instance, the promoter for C-X-C chemokine receptor type 4 (CXCR4), which is overexpressed in many cancer tissues, was implemented to restrict AAV-mediated transgene expression to primary and metastatic breast cancer [75]. AAV2 vectors encoding firefly luciferase under the control of either the CMV promoter or the CXCR4 promoter were directly delivered to subcutaneous or liver-localized MCF-7 (a human breast cancer cell line) xenografts in mice, and the ratio of expression levels between tumor tissue and muscle was determined. Off-target luminescence in muscle from control CMV vectors was 4- to 21-fold higher than in tumor tissue. In contrast, expression from CXCR4 vectors was selective for tumor tissue, with off-target muscle luminescence levels of only 10%–50% relative to tumor luminescence. Systemic administration via a splenic port of CXCR4 vectors to tumor-bearing mice resulted in luminescence levels at the tumor site that were five-fold higher than in tumor-free mice, confirming selectivity for tumor tissue.

The human telomerase reverse transcriptase (hTERT) promoter has been widely used as a cancer-selective promoter, though its *in vivo* utility has sometimes been limited by its low strength [76]. In one study, it was combined with an advanced two-step transcriptional activation system (TSTA) to enhance cancer-specific gene expression in a panel of cancer cell lines *in vitro* and in an *in vivo* orthotopic liver tumor mouse model [77]. An intravenously delivered AAV2 vector carrying hTERT-driven firefly luciferase with the TSTA system led to liver tumor bioluminescence levels 18-fold higher than delivery of only hTERT-driven luciferase, and 16-fold higher than using the standard TSTA system.

As mentioned earlier, Pandya *et al.* generated an AAV6-based mutant with enhanced transduction of DCs [68]. They also developed a chimeric promoter sufficiently small to fit into AAV vectors by combining different functional modules of the CD11c promoter, which is specific to DCs. Gene expression using this chimeric promoter (chmCd11c) was restricted to DCs, and delivery of the hPSA gene under its control to DCs resulted in a CTL response against human prostate adenocarcinoma cells, albeit 50% lower than when using the stronger yet ubiquitous chicken beta-actin (CBA) promoter.

Recently AAV8's strong murine liver tropism was combined with a liver-specific promoter and post-transcriptional regulation based on miR-122a to restrict gene expression to cancer cells in a murine model of metastatic hepatocarcinoma (HCC) [78]. MiR-122a, which is

highly expressed in healthy liver tissue but downregulated in HCC, suppresses translation of mRNAs that harbor its target binding sequence. The HLP liver-specific promoter [79] was combined with tandem repeats of *miR-122a*-binding sequences inserted in the 3′ untranslated region of the expression cassette. Systemic delivery of the resulting construct encoding firefly luciferase led to 40-fold lower liver expression compared to control vector without *miR-122a* elements. Furthermore, vector administration to mice bearing a subcutaneous HCC xenograft of miR-122a-negative SK-Hep1 cells led to tumor-restricted bioluminescence, with no luminescence from surrounding healthy liver tissue, confirming the combinatorial tumor tissue specificity of this vector and expression cassette.

## AAV DELIVERY OF THERAPEUTIC PAYLOADS IN PRECLINICAL MODELS OF CANCER

In addition to efficiently transducing a variety of cancer cells *in vitro* [30–32], AAV has been increasingly employed to deliver therapeutic genes to *in vivo* preclinical tumor models. Over the last decade, the arsenal of delivered transgenes has greatly expanded, as have the types of cancer for which AAV vectors have been used. These transgenes can be divided into several categories: anti-angiogenesis genes, cytotoxic or suicide genes, cytokines for stimulating the immune system, tumor suppression and anti-tumor genes, DNA encoding small RNA's, antigens to stimulate antigen-presenting cells, and antibodies that block signaling.

### Anti-Angiogenesis Therapy

Angiogenesis, the formation of new blood vessels from existing ones, is an important process for tumor nourishment, growth, and metastasis [80]. Consequently, inhibiting angiogenesis in tumors to reduce their progression and capacity to metastasize is a longstanding anti-cancer strategy. Multiple gene delivery approaches have been used to inhibit vascular endothelial growth factor (VEGF), a potent angiogenic growth factor, including using decoy receptors, monoclonal antibodies, and a combination of gene delivery with small molecule inhibitors. Mahendra *et al.* [81] used AAV2 to deliver a soluble splice variant of VEGF-Receptor-1 (sFlt1), a decoy that competitively inhibits VEGF-A binding to its endogenous receptor. Intramuscular AAV2-sFlt1 injection of vector to mice harboring a subcutaneous human ovarian cancer cell line (SKOV3.ip1) xenograft resulted in reduced tumor volume and in 83% survival rate, compared to no survival of untreated mice, six weeks post tumor implantation. In an analogous study, intramuscular administration of AAV1-sFlt1 suppressed tumor growth and enhanced survival in a subcutaneous SHIN-3 ovarian cancer cell line xenograft model [82].

Soluble VEGFR1/R2, a chimeric VEGF receptor comprising domains of both VEGFR-1 and VEGFR-2, was combined with irradiated granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting tumor cell immunotherapy in mouse models of melanoma and colon carcinoma [83]. AAV8-sFVEGFR1/R2 was intravenously delivered to mice, followed by subcutaneous implantation of B16F10 melanoma tumor cells and later immunization with irradiated GM-CSF – secreting B16F10 (B16.GM) tumor cells. VEGR1/R2 delivery resulted in a mean survival time (MST) of 63 days, compared to 31 days for no treatment. This effect

was further enhanced by combining vector delivery with B16.GM vaccination (MST of 93 days), which also led to increased numbers of activated DCs and T cells.

VEGF-C, which can mediate angiogenesis and metastasis to lymphatic vessels, has been targeted by delivering a secreted soluble VEGF-C decoy receptor, sVEGFR3-Fc, to melanoma and renal cell carcinoma mouse models [84]. Systemic portal vein administration of AAV2-sVEGFR3-Fc before subcutaneous implantation of A375-mln1 metastatic tumor cells had no effect on primary tumor growth, but inhibited metastasis to the lymph nodes, with only two out of seven mice developing lymph node metastases compared to all of nine mice in the control group. However, vector-mediated inhibition of metastasis to lung was less effective. Additionally, intramuscular injection of AAV2-sVEGFR3-Fc to the quadriceps inhibited lymph node metastasis in renal cell carcinoma (Caki-2) and prostate cancer models (PC-3) by 70% and 75%, respectively. Finally, intramuscular injection of AAV1-sVEGFR3 to mice bearing orthotopic endometrial cancer tumors (HEC1A) led to complete ablation of detectable lymph node and lung metastases compared to untreated mice [85].

In addition to VEGF receptors, other native inhibitors of angiogenesis have also been employed in anti-angiogenic gene delivery studies. Pigment epithelium-derived growth factor (PEDF) is a very potent inhibitor of angiogenesis that prevents the formation of new vessels from endothelial cells by interacting with VEGFR-1, without affecting the existent vasculature [86]. Intratumoral delivery of AAV2-PEDF to a mouse model of Lewis lung carcinoma (LCC) led to a 58% reduction in tumor size, reduced tumor microvessel density, increased tumor cell necrosis, and a 75% increase in MST compared to no treatment [87]. In a subsequent study, combining AAV2-PEDF with the chemotherapeutic drug cisplatin [88] prolonged survival by 150% and 50% compared to no treatment and either treatment alone, respectively. Investigators also reported greater tumor size reduction, tumor apoptosis, and tumor angiogenesis suppression compared to untreated mice or those receiving either treatment alone.

Monoclonal antibodies that block angiogenesis, widely used as a front-line treatment for many types of cancer, have been encoded into AAV vectors for sustained expression and therapeutic effects. Intravenous administration of AAV8-DC101 – encoding a neutralizing mAb against VEGFR2 – resulted in high antibody expression levels in serum, conferred protection against subcutaneous B16F10 melanoma and U87 glioblastoma tumors, and led to reduced tumor size (65% and 82% reduction, respectively) and long-term survival (10% and 80%, respectively) in both models [89]. Tumor angiogenesis was targeted in a DU 145 metastatic lung cancer model by delivering AAVrh.10 encoding a murine mAb with a VEGF-A antigen recognition site equivalent to that of the humanized VEGF-A antibody bevacizumab. Intrapleural vector administration led to high levels of anti-VEGF-A mAb expression, which resulted in reduced growth (76%), vascularization (63%), and proliferation (74%) of metastatic lung tumors and subsequent over 2-fold longer mean survival of treated mice [90]. In another study, the same group intraperitoneally delivered AAVrh.10 packaged with bevacizumab to intraperitoneal models of ovarian carcinomatosis based on A2780 or SK-OV3 cells, eliciting 90% reduced A2780 tumor growth, 82% lower A2780 tumor angiogenesis, and prolonged survival (1.6-fold and 1.2-fold higher mean

survival time in A2780 and SK-OV3 tumors, respectively) compared to untreated mice [91]. Additionally, the combination of vector delivery with chemotherapy drugs topotecan or paclitaxel generated further enhanced anti-tumor effects on A2780 xenografts (3.2-fold and 1.9-fold higher mean survival time, respectively, compared to untreated mice).

Endostatin and angiostatin, endogenous inhibitors of angiogenesis that prevent pro-angiogenic factors from interacting with endothelial cells [92], have been employed as protein therapies in clinical trials, motivating their use in gene therapies. Intratumoral delivery of AAV2-endostatin to mice carrying a subcutaneous human bladder cancer tumor (T24) yielded a 40% reduction in tumor volume and a 60% reduction in tumor angiogenesis, as well as enhanced tumor cell apoptosis [93]. The same group later combined endostatin with herpes simplex virus thymidine kinase (HSV-TK), a suicide gene that converts the pro-drug ganciclovir into a thymidine analog that is incorporated into and subsequently fragments DNA undergoing synthesis, for intratumor AAV2-mediated delivery to bladder cancer tumors [94]. This combination therapy led to threefold slower tumor progression and a 60% reduction in tumor size compared to untreated mice, with either therapy individually producing a 40% reduction in size. Another group [95] delivered AAV2-angiostatin to a mouse liver cancer model based on intraportally injected EL-4 tumor cells previously transduced *in vitro* with AAV2-B7.1, a molecule that stimulates T-cells. Delivery of AAV2-angiostatin to mice vaccinated with B7.1-transduced cells suppressed tumor growth by 87% and greatly increased survival rates, with six of ten treated, vaccinated mice surviving for over 100 days, compared to median survival rates of 33, 42, and 25 days in untreated vaccinated mice, angiostatin-treated unvaccinated mice, and untreated, unvaccinated mice.

Isayeva *et al.* [96] studied the effect of co-delivering endostatin and angiostatin in an intraperitoneal mouse model of ovarian cancer (SKOV3.ip1). Intramuscular injection of bicistronic AAV2-angiostatin-endostatin (AAV2-E+A) resulted in a 50% reduction in tumor size, increased tumor cell apoptosis, decreased tumor angiogenesis, and 30% of mice surviving for over 150 days compared to control mice surviving an average of 45 days. In a subsequent study [97], combining intraperitoneal AAV2-E+A delivery with the chemotherapy drug taxol led to complete survival of 90% of dually-treated mice and a 90% reduction in tumor size compared to untreated mice. The same group extended this combinatorial approach to the transgenic adenocarcinoma of mouse prostate (TRAMP) prostate cancer model by intramuscularly delivering bicistronic AAV6 encoding endostatin and angiostatin [98]. Mice receiving AAV6-E+A at an early age (5 or 10 weeks old) had low grade, smaller tumors, and 60% of them survived longer than 60 weeks, compared to median survival times of 30–35 weeks for untreated mice or those receiving AAV6-E+A at older ages (>18 weeks), respectively. Endostatin has also been used in conjunction with another angiogenic inhibitor, thrombospodin-1 (TSP-1), in a mouse orthotopic pancreatic cancer model (AsPC-1) [99]. Intramuscular delivery of either AAV2-endostatin or AAV2-3TSR (the antiangiogenic domain of TSP-1) prior to xenografting resulted in similar levels of protection, causing 45% lower tumor microvessel density and a 43% reduction in tumor size. Co-delivery of both vectors led to more marked effects in those parameters (65% and 62%, respectively) compared to either treatment alone.

Intramuscular delivery of AAV2-P125A-endostatin, an endostatin mutant with enhanced binding to endothelial cells and stronger anti-angiogenic effects, in an ovarian carcinoma mouse model (MA148) led to 72% smaller tumors and decreased angiogenesis, with a 46% reduction in the mean number of vessel nodes [100]. In a subsequent study [101], the same group delivered AAV2-P125A-endostatin in combination with the chemotherapy drug carboplatin to an orthotopic ovarian cancer model (MA148). This combination led to 25% higher median survival and 52% less vessel nodes compared to untreated mice.

A range of other antiangiogenic transgenes have been delivered with AAV vectors. AAV2 was used to deliver tissue factor pathway inhibitor (TFPI-2) – a suppressor of angiogenesis, tumor growth, and tumor cell invasiveness – to a glioblastoma mouse model (SNB19) [102]; kringle 5, a fragment of plasminogen with potent antiangiogenic properties, to a mouse model of ovarian cancer (MA148) [103]; and self-complementary cargoes encoding siRNAs against the unfolded protein response (UPR) proteins IRE1α, XBP-1, or ATF6 in a mouse breast cancer model (NeuT) with an AAV2 mutant with seven surface tyrosine to phenylalanine mutations [104]. Cai *et al.* [105] used AAV5 to deliver vasostatin – an endogenous inhibitor of angiogenesis – to a subcutaneous, orthotopic xenograft, and a spontaneous metastasis model of lung cancer (A549, LLC Lewis lung carcinoma, respectively). Finally, AAV8 encoding human plasminogen kringle 1–5, an inhibitor of angiogenesis, was administered to murine models of mouse melanoma (B16F1), mouse lung cancer (LLC), and human melanoma (A2058) [106]. Delivery of the described transgenes resulted in suppression of both angiogenesis and tumor growth.

## Delivery of Cytotoxic or Suicide Genes

Suicide gene therapy has been broadly investigated as an anti-cancer therapy [107]. The most utilized system is the herpes simplex virus type 1 thymidine kinase (HSV-TK), which converts ganciclovir (GCV) into the toxic metabolite GCV-triphosphate within cells expressing the enzyme and also induces bystander toxicity to neighboring tumor cells [108]. Intratumoral administration of AAV2-HSV-TK under Dox-inducible Tet-On regulation to a mouse model of breast cancer (MCF-7) resulted in 75% suppression of tumor growth with only moderate toxicity [109]. In a subsequent study, the same group confirmed these results and elaborated on the therapeutic mechanism of the HSV-TK system in MCF-7 cells [110]. AAV2-mediated delivery of sc39TK, a hyperactive variant of HSV-TK with enhanced affinity for GCV, to HeLa cells that were later implanted into mice to generate subcutaneous tumors enabled 70% tumor growth suppression upon GCV administration [111].

Other cytotoxic genes have also been employed in AAV-mediated delivery to cancer cells. Kohlschütter *et al.* [32] used AAV2 to deliver diphtheria toxin A (DTA) and p53 upregulated modulator of apoptosis (PUMA) to HeLa cells, SiHa cervical carcinoma cells, and RPMI 8226 myeloma cells *in vitro*. DTA exerted a cytotoxic effect on all cell lines, whereas PUMA delivery cause cytotoxicity in HeLa and RPMI cells. They also used an AAV2 mutant they had previously developed, RGDLGLS [72], to deliver DTA to mammary tumor cells from mice carrying the polyomavirus middle T antigen (PymT cells) and induced a 40% cytotoxicity.

### Immunomodulation through Delivery of Cytokines

The delivery of stimulatory molecules such as cytokines can elicit an enhanced immune response against tumors. A widely used cancer therapeutic is tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), which elicits a strong apoptosis effect primarily in tumor cells, but not in normal tissue, by binding to death receptors [112]. Intraportal administration of AAV2-TRAIL in an orthotopic mouse model of lymphoma (EL-4) suppressed tumor growth by 95% and enhanced median survival by 92%, an effect of induced apoptosis in the tumor cells metastasizing to the liver [113]. The same group subsequently delivered AAV2-sTRAIL (soluble TRAIL) to a subcutaneous mouse model of human liver cancer (SMMC-7721) [114]. Oral or intraperitoneal administration of AAV2-sTRAIL suppressed tumor growth by 88% and 82%, respectively. This group also observed 82% tumor size suppression and 52% enhanced median survival when administering sTRAIL, packaged in AAV5, to mice bearing subcutaneous or orthotopic A549 lung adenocarcinoma tumors [115]. Another group [116] also delivered sTRAIL, packaged in AAV2, to an A549-based subcutaneous lung adenocarcinoma tumor model. Intratumor vector delivery led to 62% reduced tumor size, and systemic vector delivery before implantation of A549 cells lowered the frequency of tumor occurrence to 43% compared to 100% in untreated mice.

Intratumoral delivery of TRAIL, packaged in AAV2 under the control of the cancer-specific hTERT promoter, to a subcutaneous SMMC7721 HCC xenograft led to a 70% suppression of tumor growth and long-term survival, compared to a survival of 78–105 days in untreated mice [117]. They subsequently combined AAV2-hTERT-TRAIL and cisplatin in a subcutaneous BEL7404 HCC model, which resulted in 94% reduction in tumor size and complete survival [118]. In another study, intratumoral AAV2-hTERT-TRAIL delivery to an HCC SMMC7221 mouse model was combined with administration of the chemotherapeutic 5-fluorouracil (5-FU) [119]. Combination therapy led to a strong anti-tumor effect, with an 83% reduction in tumor growth compared to treatment with vector or 5-FU only (60% and 16% reduction, respectively). The same group [120] extended intratumoral delivery of AAV2-TRAIL, combined with cisplatin, to a subcutaneous mouse model of head and neck squamous cell carcinoma (HNSCC), resulting in 40% smaller tumors. In a different study, intracranial delivery of AAV2-interleukin-12(IL-12), a potent immunostimulatory cytokine, in a RG2 rat model of glioblastoma led to enhanced TRAIL expression and microglia activation, 3.5-fold higher median survival time, and 30% reduced tumor volume [121].

Interferons (IFNs) are cytokines that induce antitumor effects that include interfering with cancer cell division and slowing tumor growth progression. Systemic administration of AAV8-hIFN-β to a mouse retroperitoneal xenograft of NB-1691 human neuroblastoma cells, by itself or combined with trichostatin, resulted in similar (90%) suppression of tumor growth relative to untreated or trichostatin-treated mice [122]. Maguire *et al.* [123] studied the protective effects of IFN-β expression against intracranial, orthotopic U87 xenografts of glioblastoma multiforme. Stereotactic delivery of AAVrh.8-IFN-β followed by cancer cell implantation prevented growth of glioma tumors, and even led to 100% survival against glioma challenge. Additionally, vector delivery into tumor-bearing mice led to regression of established tumors and subsequent complete survival. In another study, AAV2-IFN-β under

the control of the hTERT promoter reduced tumor growth by over 90% and enhanced survival in mouse models of colorectal cancer (SW620) and lung cancer (A549), with 87% and 83% long-term survival in treated mice, respectively [124]. Other interferons have been used in AAV-mediated delivery to cancer models, including intravenously administered AAV6-IFN-α to a B16F10 mouse model of metastatic melanoma, leading to a 60% reduction in the number of metastatic colonies and a modest enhancement in the survival of treated mice [125].

CD40-ligand (CD40L) is an immunostimulatory protein implicated in the activation of dendritic cells and induction of tumor cell apoptosis. Intratumor delivery of self-complementary AAV5-CD40L to subcutaneous A549 lung cancer tumors led to a 67% reduction in tumor size and 2.7-fold higher level of tumor cell apoptotic death [126]. Recently, self-complementary AAV5 vectors expressing a non-cleavable CD40L mutant were used to treat subcutaneous A549 lung cancer xenografts [127]. Intratumoral delivery of AAV5-CD40L and AAV5-CD40LM (i.e. mutant) reduced tumor size and increased apoptosis, with the CD40L mutant exhibiting greater effects than wild-type ligand (33% vs. 28% tumor reduction for wtCD40L).

Interleukins, another class of immunostimulatory molecules, have been employed in cancer gene therapies. Systemic delivery of AAV1 carrying melanoma differentiation-associated gene-7/interleukin-24 (mda-7/IL24), a cytokine capable of inducing tumor apoptosis and inhibiting tumor growth and metastasis, to a subcutaneous Ehrlich ascites tumor mouse model suppressed tumor growth by 63%, increased tumor cell apoptotic death, reduced tumor angiogenesis, and enhanced mean survival time by over 80% [128]. Likewise, intratumoral delivery of AAV2-IL24 to an orthotopic MHCC97-H HCC model increased apoptotic tumor cell death and inhibited tumor recurrence and metastasis in the liver and lung [129]. In another study, intramuscular delivery of AAV8-IL24 to a transgenic mouse model of mixed-lineage leukemia/AF4-positive acute lymphoblastic leukemia (MLL/AF4-positive ALL) reduced tumor angiogenesis by 57% [130].

Chang *et al.* [131] studied the delivery of IL-15, a cytokine capable of stimulating an immune response by inducing proliferation and activation of natural killer cells and T cells, to a BNL-h1 mouse model of metastatic HCC. Prophylactic intravenous delivery of AAV8 encoding an IL-15 superagonist followed by cancer cell implantation led to 82% lower tumor metastasis. Analogously, therapeutic delivery (vector delivered to tumor-bearing mice) led to both 80% lower tumor metastasis and enhanced mean survival by 41% with no apparent liver toxicity observed. Another group intramuscularly administered AAV2-IL15 to a transgenic model of SV40 T/t antigen-induced breast cancer prior to induction of breast cancer, which stimulated lymphokine-activated killer (LAK) cells, slowed tumor growth (tumor size reached 2500 $mm^3$ after 33 days vs. 20 days for control) and reduced final tumor size by 30% [132].

Other AAV-delivered immunostimulatory transgenes that have elicited anti-tumor effects include secondary lymphoid tissue chemokine (SLC), delivered preventatively and therapeutically by AAV2 to a Hepal-6 mouse liver cancer tumor model [133]; Nk4, delivered by AAV2 to a metastatic Lewis lung carcinoma (LLC) mouse model [134]; the cytokine

LIGHT, delivered by AAV2 to a TC-1 mouse cervical cancer model [135]; the granulocyte-macrophage colony stimulation factor (GM-CSF) cytokine, delivered by AAV1 to a 9L tumor model [136]; and TNF-α, delivered by AAV2 to a U251 human glioma xenograft mouse model [137].

### Delivery of Tumor Suppression and Repair Genes

Another anti-cancer gene therapy strategy is the delivery of transgenes and nucleic acids that can elicit tumor suppression or down-regulation of tumorigenic proteins (i.e. those overexpressed in tumor cells). Several groups have delivered dominant negative mutants of survivin, an anti-apoptotic protein overexpressed in most types of cancer. Tu *et al.* [138] used AAV2 to deliver the C84A survivin mutant, capable of inducing apoptosis, to SW1116 and Colo205 colon cancer cells that were then subcutaneously injected in nude mice, and they observed suppressed tumorigenesis and reduced tumor growth by 80% and 55%, respectively. Alternatively, intratumor therapeutic delivery inhibited the growth of previously established tumors by 81% and prolonged mean survival of treated mice by 75%. The same group [139] subsequently delivered another dominant-negative survivin mutant capable of inducing apoptosis and reducing tumor growth, T34A, to an HCT-116 human colon cancer mouse model using AAV2 and obtained similar results, with further enhanced therapeutic effects when combining AAV with oxaliplatin (62% of mice showing complete survival). Two other groups [140, 141] also used AAV-mediated delivery of the C84A and T34A survivin mutants, respectively, for *in vivo* models of gastric cancer and observed reduced tumor growth, increased tumor cell apoptosis, and increased tumor sensitivity to 5-fluoracil.

The C-terminal fragment of the human telomerase reverse transcriptase (hTERTC27), which exerts an anti-tumor activity by inducing telomere dysfunction, was packaged in AAV2 and intratumorally delivered to a human glioblastoma multiforme U87-MG tumor xenograft mouse model [142]. The results were increased levels of tumor necrosis and apoptosis, increased infiltration of polymorphonuclear neutrophils into the tumor, reduced angiogenesis, and consequently an 83% reduction in tumor growth and two-fold higher median survival of treated mice. The same group then combined AAV2 and adenoviral vector delivery of hTERTC27 and obtained a synergistic therapeutic effect of 2.56-fold higher median survival compared to untreated mice [143].

A wide array of other anti-tumor transgenes have been delivered to preclinical cancer models using AAV vectors. These include AAV2-maspin to LNCaP and DU145 prostate cancer tumors [144]; AAV2-nm23H1 to SW626-M4 metastatic ovarian cancer tumors [145]; AAV2-HGFK1 (kringle 1 domain of human hepatocyte growth factor) to CT26 and Lovo colorectal carcinoma tumors [146]; AAV2-encoded anti-calcitonin ribozymes to an orthotopic implantation model and a transgenic model of prostate cancer [147]; AAV2-4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1) to a K-ras$^{LA1}$ lung cancer model [148]; AAV2-mediated delivery of the chemokine receptor CXC chemokine receptor 2 (CXCR2) C-tail sequence to an HPAC pancreatic tumor model [149]; AAV1 delivery of IL-24 and apoptotin to a HepG2 liver cancer model [150]; AAV2-TAP (alpha-tocopherol-associated protein) to PC-3 and LNCaP prostate cancer tumors [151]; delivery of trichosanthin, packaged in AAV3-S663V+T492V vectors, to HuH7

hepatocellular carcinoma (HCC) tumors [66]; AAV2-decorin to a U87MG glioblastoma multiforme model [152]; AAV2-cathelicidin to HT-29 colon cancer tumors [153]; AAV8-mediated delivery of Niemann-Pick type C2 (NPC2) to the N-methyltransferase knockout (Gnmt−/−) transgenic spontaneous HCC model [154]; and AAV9-mediated delivery of human Mullerian inhibiting substance (MIS, albumin leader Q425R MIS (LRMIS)) in a xenograft model of ovarian cancer [155]. Additionally, groups have delivered the p53 tumor suppressor gene, commonly mutated in cancerous cells, using AAV2 vectors to bladder cancer cells [156] and non-small cell lung cancer cells [157] *in vitro* and to an H358 bronchioalveolar carcinoma tumor model *in vivo* [158].

## AAV Vectors Encoding Small RNAs

AAVs encoding RNA cargoes are another anti-cancer modality that has been employed in a variety of tumor models. Prophylactic administration of AAV2 encoding small hairpin RNA (shRNA) targeting Epstein-Barr virus (EBV) latent membrane protein-1 (LMP-1) to a C666-1 nasopharyngeal carcinoma (NPC) mouse model led to a 47% inhibition of tumor metastasis, though no effects on tumor growth were observed [159]. AAV2 encoding antisense RNA targeting the E7 oncogene from human papilloma virus 16 (HPV16) present in CaSki cervical cancer cells and subsequent implantation of these cells reduced tumor formation by 80% and inhibited the size of formed tumors by 79% compared to transplantation of uninfected cells [160]. Sun *et al.* [161] delivered AAV2 encoding shRNA targeting the androgen receptor (AR) gene to 22Rv1 prostate cancer xenografts. Among the group of AR-shRNAs that they screened, AAV2-ARHP8 suppressed tumor growth by 88% when delivered intratumorally, and systemic delivery of AAV2-ARHP8 caused elimination of xenografts. Intratumoral AAV2 encoding siRNA against Snail, a transcription factor involved in anti-apoptotic and chemoresistance upregulated in pancreatic cancer, to a PANC-1 xenograft model of pancreatic cancer suppressed tumor growth by 76% [162]. Subsequently, the same group delivered AAV encoding siRNA targeting the Slug gene, a suppressor of apoptosis, to an orthotopic QBC939 model of cholangiocarcinoma, a type of liver cancer [163]. Intratumoral injection of AAV2-SlugsiRNA led to 51% reduced tumor growth alone and complete tumor regression when combined with radiation treatment. Delivery of AAV2 encoding shRNA against FHL2 (four and a half LIM-only protein 2), a putative oncogene involved in various cellular processes including proliferation and migration, to a LoVo colon cancer xenograft led to 66% reduced tumor volume, an effect that was enhanced to a 95% reduction with co-administration of 5-FU [164]

Other AAV serotypes have also been employed for delivery of small RNA-encoding payloads. Delivery of AAV1 encoding shRNA against Hec1 (Highly Expressed in Cancer 1) to a U251 glioma xenograft mouse model increased tumor cell apoptosis but did not ultimately reduce tumor growth [165]. Systemic delivery of self-complementary AAV8 vectors encoding microRNA miR-26a, which becomes downregulated in HCC cells, to the tet-o-MYC, LAP-tTA bi-transgenic HCC mouse model resulted in high expression levels of miR-26a in the liver, reduced tumor occurrence, and 65% smaller average tumor size without any observed toxicity [166].

### Delivery of Antigens for Stimulating Antigen-Presenting Cells (APCs)

Adeno-associated virus vectors have also been employed to deliver antigens to antigen-presenting cells and thereby elicit an immune response against tumor cells expressing that antigen, i.e. a tumor vaccine. An excellent example of AAV-mediated vaccination focused on antigens from human papilloma virus 16 (HPV16), which is associated with the development of cervical and anogenital cancer. Several groups have used AAV to express the HPV16 structural protein L1 and thereby induce anti-HPV neutralizing antibodies [167]. L1-based virus-like particles (VLPs), which are non-infectious and morphologically identical to HPV virions but do not carry any oncogenes, are safe vaccine agents that can elicit high titers of neutralizing antibodies. Liu *et al.* used intramuscularly delivered AAV2-HPV16L1 to elicit anti-L1 antibodies, then compared the resulting titers with those generated by either an AAV control vector, HPV16 VLPs composed of L1, naked DNA encoding L1, adenovirus coding for mGM-CSF, or a combination of AAV2 and adenovirus [167]. The antibody titer induced by AAV2-L1 delivery was 20% lower than that generated by VLPs; however, a single dose of combined AAV and adenovirus led to titers as high three doses of VLPs. AAV2-L1 delivery also led to accumulation of macrophages and DCs at levels comparable to VLP delivery.

Interestingly, a single dose of intranasal AAV5-mediated delivery of HPV16-L1 was sufficient to generate long-lasting, high titers of serum anti-L1 antibodies in mice, comparable to those generated by VLP delivery [168]. Additionally, vector delivery led to generation of mucosal antibodies in vaginal washes and to a long-term cellular immune response against HPV16. The same group subsequently investigated intranasal vaccination of mice by delivering an HPV16 L1/E7 fusion gene using AAV5, AAV8, or AAV9 vectors and showed serotypes 5 and 9 were most effective at generating neutralizing antibodies and a CTL response against HPV16 [169]. Moreover, the murine study was followed by successful intranasal vaccination of rhesus macaques using HP16 L1 delivered by AAV5 and AAV9 vectors, where the latter induced long lasting immunization [170]. Building upon their previous study of AAV-mediated vaccination against HPV16 [171], Liao *et al.* [172] developed vaccines against three HPV16 oncogenes – E5, E6, and E7 – that conferred immune protection against cervical cancer tumor growth. They used AAV2 to deliver a long peptide targeting HPV16 E5, E6, and E7 that induced an immune response against TC-1 cells, which express HPV16 proteins; vaccinated mice were protected from tumor growth for 300 days.

Various other antigens have been delivered using AAV vectors to preclinical cancer models for APC stimulation and antibody generation. AAV2 encoding a B-cell leukemia/lymphoma 1 (BCL1) idiotype led to the generation of anti-Id antibodies and protection against BCL1 cell-based tumors [173]. Intramuscular AAV2 packaged with the LMP2/1-hsp fusion gene, consisting of the Epstein-Barr virus latent membrane proteins 1 and 2 fused to heat shock protein as an adjuvant, to a tumor model based on SP2/0 cells expressing LMP2 led to a humoral and CTL response against LMP2-expressing tumor cells, 83% reduction in tumor growth, and long-term survival of 90% of treated animals [174]. In addition, intramuscular AAV6 encoding melanoma antigen Trp2 generated an antitumor response against B16.F10 tumors when combined with Toll-like receptor (TLR) agonists [175] The same group

subsequently co-administered TLR agonists and AAV2 encoding carcinoembryonic antigen (CEA) to vaccinate mice against colon cancer cells, resulting in antitumor response against MC38 cells expressing CEA [176]. In another study investigating neu-expressing TUBO breast cancer tumors [177], intramuscular vaccination with AAV5-neu or AAV6-neu resulted in humoral and cell-mediated immune response, leading to 50% and 100% long-term survival, respectively. Similarly, oral vaccination led to 80% and 100% long-term survival with AAV5 and AAV6, respectively. Additionally, oral AAV6-neu vaccination also protected against re-challenge with TUBO tumor cells 320 days after original tumor cell implantation. Han *et al.* [178] used AAV2 to systemically deliver a soluble form of B and T lymphocyte attenuator (BTLA) in combination with a heat shock protein (HSP70) vaccine to a B16F1 mouse melanoma pulmonary metastasis model, which initially reduced metastatic foci but did not prevent late-stage metastatic melanoma. Conversely, prophylactic treatment caused enhanced innate and adaptive immune responses against tumor cells, leading to 80% inhibition of tumor formation and long-term survival of 83% of treated mice. One general challenge with prophylactic tumor vaccination with AAV, however, is that a single administration can lead to long-term neutralizing antibodies that cross-react against multiple AAV serotypes, complicating subsequent AAV administrations to treat other conditions.

## Delivery of Antibodies to Block Signaling

A number of anti-cancer monoclonal antibodies (mAbs) therapeutics have been developed to target cancer cells for immune system processing and presentation, to inhibit cancer cell growth and tumor progression by blocking antigens involved in tumor cellular processes such as migration, and to inhibit immunosuppressive signaling molecules and thereby boost anti-tumor immune responses. Adeno-associated virus vectors have been employed to deliver genes encoding such monoclonal antibodies for long-term expression in preclinical models.

Ho *et al.* [179] delivered 14E1, a murine antihuman epidermal growth factor (EGFR) antibody, by intramuscular administration of AAV1 vectors in the A431 human vulvar carcinoma xenograft model. Administration of vector prior to tumor cell xenografting completely inhibited or reduced tumor growth by 93% when administered 28 days before and 1 day after tumor cell implantation, respectively. Gene delivery also led to long-term survival, with a majority of mice showing complete tumor regression. Intratumorally injected AAV2 encoding adximab, a mouse-human chimeric antibody against death-receptor 5 (DR5), to mouse models of human liver cancer (SMMC7221) and colon cancer (HCT116) reduced tumor growth (58% and 40% reduction, respectively) and increased tumor cell apoptotic death (2-fold and 2.6-fold higher, respectively) in both models [180]. Finally, administration of an AAV9 vector encoding a monoclonal antibody against the glycolytic enzyme alpha-enolase (ENO1) prior to xenografting orthotopic CFPAC-1 pancreatic ductal adenocarcinoma (PDAC) tumors led to high concentrations of anti-ENO1 antibody in serum and a 95% reduction in lung metastases [181].

## AAV VECTORS IN CANCER CLINICAL TRIALS

The promising results of adeno-associated virus vectors in preclinical models of cancer, coupled with their clinical successes for monogenic diseases [10], have motivated the translation of AAV vectors into oncology clinical trials.

A phase I trial performed between the Peking University School of Oncology and the Beijing Cancer Hospital and Institute [182] administered cytotoxic T lymphocytes (CTLs) that had been activated by dendritic cells (DCs) previously transduced with AAV2 vectors carrying carcinoembryonic antigen (CEA) to cancer patients who had failed to respond to standard treatments. From the 25 patients evaluated after treatment, 2 showed partial remission, 10 showed stable diseases, and 13 had progressive disease, with a resulting mean progression-free survival of 3.1 months. Importantly, no treatment-related serious adverse events were reported for any patients. Larger, randomized studies may follow.

A clinical trial (ClinicalTrials.gov Identifier:NCT02496273), led by Wu Changping, M.D. and Jiang Jingting, Ph.D. at The First People's Hospital of Changzhou in China, is investigating the clinical safety and efficacy of administering CEA-specific CTLs activated by DCs previously loaded with CEA via AAV2 transduction. This phase I clinical trial is focused on stage IV gastric cancer patients, will monitor T cell populations and tumor progression, and is projected to initiate in January, 2016.

Another clinical trial (ClinicalTrials.gov Identifier: NCT02602249), developed by Beijing Doing Biomedical Co., Ltd in China, is similarly investigating the clinical safety and efficacy of administering CTLs specific for the Mucin 1 (MUC1) antigen, whose overexpression can be associated with cancer, to stage IV gastric cancer patients. MUC1-specific CTLs will be activated by DCs either loaded with MUC1 via AAV2 transduction or directly pulsed with a MUC1 peptide. This study is projected to be completed in June, 2018.

Finally, although not targeting cancer cells *per se*, another phase I clinical trial (ClinicalTrials.gov Identifier: NCT02446249) led by John A Chiorini, Ph.D. at the NIH is investigating the safety of using an AAV2-aquaporin (AAV-hAQP1) gene therapy for patients with irradiation-induced parotid salivary hypofunction (xerostomia), a condition that can develop in patients with a history of radiation therapy for head and neck cancer. The investigators have already completed a separate phase I clinical trial (06-D-0206) using adenovirus, where they showed safety and therapeutic efficacy of hAQP1 delivery to a single parotid gland. The study initiated in April, 2015, and the investigators will monitor therapeutic efficacy of the treatment by measuring parotid salivary gland output as well as safety through traditional clinical and immunological measures.

## FUTURE PROSPECTS AND CONCLUSIONS

AAV vectors have enjoyed increasing clinical success as a result of their excellent safety profile and high gene delivery efficacy. To date, over 130 clinical trials [6] have employed AAV vectors to treat conditions in a wide range of tissues, including muscle, eye, liver, central nervous system, heart, and lung diseases [10]. The approval of Glybera in the European Union and recent reports on clinical trials, including those for Sanfilippo B

syndrome (Pasteur Institute, Phase I/II) and Leber's congenital amaurosis (Spark Therapeutics, Phase III), underscore the strong promise of AAV-mediated therapeutic gene delivery and foreshadow the development and approval of AAV gene therapies in the United States in the near future.

As presented in this review and summarized in Table I, AAV vectors, particularly AAV2, have been extensively used in a variety of preclinical models of cancer to deliver a wide array of transgenes, including anti-angiogenic factors, suicide genes, immunostimulatory genes and antigens, tumor suppressors, payloads encoding small interfering nucleic acids, and monoclonal antibodies. Despite the high prevalence of gene therapy clinical trials directed at cancer, AAV vectors have only recently entered this field, which has so far been focused on oncolytic viruses – including adenovirus, herpes simplex virus, and reovirus – and to a lesser extent, non-viral methods [183]. However, AAV vectors offer several complementary advantages that can be harnessed for anti-cancer therapies, including the potential for high efficiency transduction, the promise of vector engineering for targeted delivery, and gene expression in post-mitotic cells.

Due to their inability to replicate or efficiently integrate in transduced tumor cells, AAV vectors may have a limited potential for sustained oncolytic or pro-apoptotic effects, as evidenced by some *in vitro* and *in vivo* studies [184]. Instead, AAV vectors may be an excellent platform for eliciting protective anti-tumor effects via tumor suppression and immunostimulation – a strategy that ongoing AAV cancer clinical trials are currently employing. Delivery of tumor-suppressive or stimulatory payloads – such as anti-angiogenic factors, monoclonal antibodies, cytokines, antigens for loading APCs, and immunomodulatory factors - would harness AAV's excellent safety and delivery properties, and, more importantly, would not require transduction of all tumor cells to induce an efficient therapeutic effect.

In particular, AAV vectors can deliver tumor-specific antigens to generate tumor-specific humoral and T cell-mediated responses – in efforts to vaccinate against the tumor – and this approach has been explored in both murine and non-human primate preclinical models that have strongly suggested the vector's potential against conditions like cervical cancer (HPV-L1) and prostate cancer (hPSA). Antigen delivery can also be employed in the *ex vivo* transduction of antigen-presenting cells like dendritic cells (DCs), which after being stimulated, loaded with the antigen, and reinjected into the patient, can elicit a CTL response against the tumor. Dendritic cell vaccines are safe and effective, and as explained above, mark some of the first AAV clinical trials directed at treating a type of cancer; this approach could potentially be extended to other types of cancer previously treated with DC vaccines, including melanoma, colon cancer, and prostate cancer [185].

Another important immunostimulatory strategy consists of augmenting anti-tumor cytotoxic T lymphocyte (CTL) responses by inhibiting negative immunoregulators such as CTLA-4 and PD-1 [186]. Blocking antibodies against CTLA-4, PD-1, and PD-1 Ligand 1 (PD-L1) have led to significant improvements in the treatment of various cancers (e.g. melanoma, renal cell carcinoma, lung cancer), and are either approved by the FDA (ipilimumab, anti-CTLA-4 approved for melanoma) or in advanced clinical trials [187]. AAV's gene transfer

properties have already been exploited for the delivery of recombinant anti-angiogenesis monoclonal antibodies, including bevacizumab, to preclinical cancer models. AAV vectors could therefore be used to stimulate a CTL response by local delivery of blocking antibodies against CTLA-4 or PD-1. Long-term, sustained expression of these or other therapeutic antibodies could reduce overall dosage while increasing local expression levels, and consequently improve treatments for multiple indications such as non-Hodgkin's lymphoma, breast cancer, and colorectal cancer.

As another future direction, AAV vectors can benefit from further developments that would make them even more suitable gene delivery vehicles for cancer therapies. Novel vectors with selective tropism towards the tissue of interest, low off-target transduction, and the capacity to evade pre-existing neutralizing antibodies would promote high levels of gene expression, a requirement for a strong therapeutic effect. Furthermore, AAV vectors would strongly benefit from engineering for localization to primary and secondary tumors as well as to tumor initiating cells (sometimes regarded as "cancer stem cells"), which are resistant to traditional therapies and greatly contribute to the poor prognosis and post-therapy relapse of many cancers [2]. Vectors may also be engineered for specific transduction following different routes of administration – for example, systemic delivery, localized injection to post-mitotic non-tumor tissue for sustained transgene expression and secretion, or intratumoral administration – which can in turn influence gene delivery efficacy [188]. As previously discussed, directed evolution – which has successfully been applied to enhance existing vector properties or engineer entirely new and optimized properties for delivery to normal tissues – similarly offers strong potential for engineering novel AAV vectors for cancer therapies. Capsid engineering efforts can additionally be combined with the development of innovative payloads that can provide tissue selectivity, strong expression, and maximization of genomic space for transgene delivery.

Finally, numerous studies presented in this review combined therapeutic gene delivery with traditional chemotherapy drugs or other therapeutic strategies to yield therapeutic effects greater than those elicited by either treatment alone. Thus, integrating AAV-mediated gene delivery with standard therapies (e.g. surgery, chemotherapy, radiotherapy) to develop novel anti-tumor treatment strategies offers strong promise in future cancer gene therapy studies.

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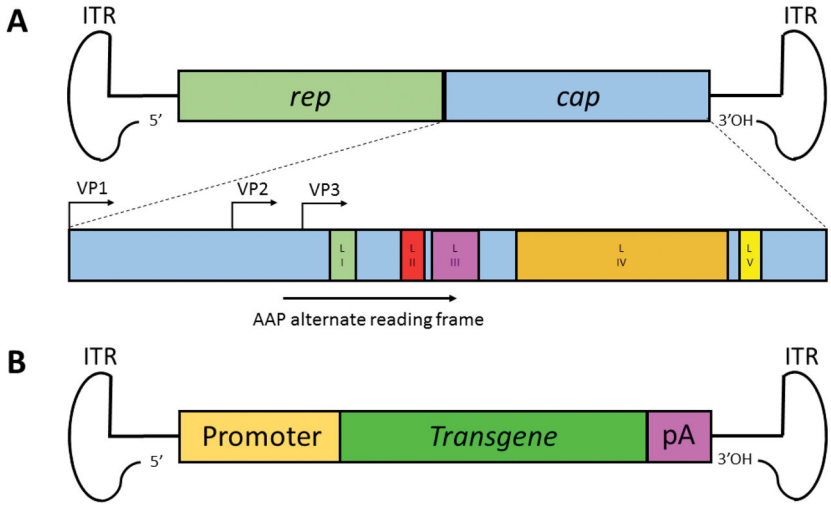


**Figure 1. Genomic structure of AAV and AAV vectors**

**(A)** The 4.7kb AAV genome is composed of the *rep* and *cap* genes flanked by inverted terminal repeats (ITRs). The *rep* gene codes for non-structural proteins involved in viral replication, packaging, and genomic integration, while the *cap* gene encodes the structural proteins VP1, VP2, and VP3 that assemble to form the viral capsid in a ratio of 1:1:10, respectively, in a total of 60 protein subunits. The assembly-activating protein (AAP) is translated from an alternate open reading frame. Also depicted are capsid loop domains I through V (LI-LV), which contain variable regions that influence gene delivery properties. **(B)** Recombinant AAV vectors are generated by replacing the *rep* and *cap* genes with a gene expression cassette (e.g. promoter, transgene, poly(A) tail) flanked by the ITRs. Vectors are then packaged by supplying the *rep* and *cap* genes *in trans* as well as adenoviral helper genes required for AAV replication.

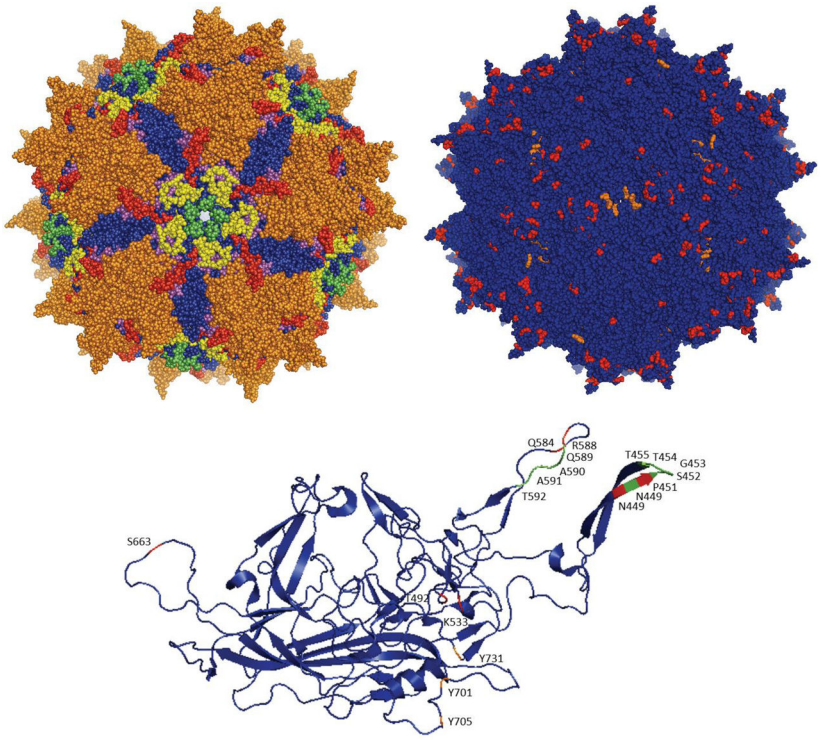


**Figure 2. Representation of AAV2 capsid structure and individual monomeric protein**

**(A)** Crystal structure of the AAV2 capsid [183], the most widely used and studied AAV serotype. Loop domains I through V are depicted following the same color scheme as in Figure 1A. **(B)** Residues that have been mutated to engineer vectors for transduction of cancer cells are mapped onto the AAV2 crystal structure and depicted in orange (tyrosine to phenylalanine mutations) or red (other mutations). **(C)** Mutated residues are similarly depicted in the individual VP3 monomer structure. Additionally, residues that have been removed to insert protein-binding peptides [58] are depicted in green. Images were produced with Pymol [184].

**Table 1**

AAV vectors employed in gene delivery to preclinical models of cancer.

| Therapeutic Modality | AAV Vector | Type of Cancer Model | Delivered Transgene | References |
|---|---|---|---|---|
| *Anti-angiogenesis* | AAV1 | Ovarian cancer | VEGFR-1 (sFlt1) | 82 |
| | | Endometrial cancer | Soluble VEGFR3 | 85 |
| | AAV2 | Ovarian cancer | VEGFR-1 (sFlt1) | 81 |
| | | Melanoma | Soluble VEGFR3-Fc | 84 |
| | | Renal cell carcinoma | Soluble VEGFR3-Fc | 84 |
| | | Prostate cancer | Soluble VEGFR3-Fc | 84 |
| | | Lewis lung carcinoma | PEDF | 87, 88 |
| | | Bladder cancer | Endostatin | 93, 94 |
| | | Liver cancer | Angiostatin | 95 |
| | | Ovarian cancer | Endostatin + angiostatin | 96, 97 |
| | | Pancreatic cancer | Endostatin | 99 |
| | | Pancreatic cancer | 3TSR | 99 |
| | | Ovarian carcinoma | P125A endostatin | 100, 101 |
| | | Glioblastoma | TFPI-2 | 102 |
| | | Ovarian cancer | Kringle 5 | 103 |
| | AAV2 5YF mutant | Breast cancer | siRNA against IRE1α, XBP-1, or ATF6 | 104 |
| | AAV5 | Lung cancer | Vasostatin | 105 |
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