Chapter 2
Gene Delivery to Cardiovascular Tissue

Rachel Masson, Stuart A. Nicklin, and Andrew H. Baker

Abbreviations

AAV	Adeno-associated virus
Ad	Adenovirus
CABG	Coronary artery bypass graft
CAR	Coxsackie virus and adenovirus receptor
CVD	Cardiovascular disease
EC	Endothelial cells
eNOS	Endothelial nitric oxide synthase
FGF	Fibroblast growth factor
FGFR1	Fibroblast growth factor receptor-1
FH	Familial hypercholesterolemia
HIV	Human immunodeficiency virus
HO-1	Heme oxygenase-1
HSPG	Heparan sulfate proteoglycans
HSV	Herpes simplex virus
iNOS	Inducible nitric oxide synthase
ITR	Inverted terminal repeats
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
MMPs	Matrix metalloproteinases
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide

R. Masson (✉)
BHF Glasgow Cardiovascular Research Centre, University of Glasgow,
126 University Place, Glasgow, G12 8TA, UK
e-mail: r.masson.1@research.gla.ac.uk

D. Abraham et al. (eds.), *Advances in Vascular Medicine*,
DOI 10.1007/978-1-84882-637-3_2,

NOS	Nitric oxide synthase
NPC	Nuclear pore complex
PDGFR	Platelet-derived growth factor receptor
SERCA2a	Sarco-endoplasmic reticulum calcium ATPase pump
SHR	Spontaneously hypertensive rat
SMC	Smooth muscle cell
TIMPs	Tissue inhibitors of metalloproteinases
VEGF	Vascular endothelial growth factor

2.1 Gene Therapy

Advancement in the understanding of molecular therapeutics has allowed the development of novel treatments to prevent and treat many diseases. Originally conceived for the treatment of inherited monogenic disorders, such as Duchenne's muscular dystrophy and hemophilia, where gene replacement should restore a normal phenotype, gene therapy approaches can now be applied to the treatment of more complex acquired diseases, including cardiovascular diseases (CVDs) and cancers. Before the full potential of gene therapy can be reached, many limitations common to all methods of gene delivery must be overcome. The efficiency of gene transfer will determine how successful the gene therapy application will be. To date, difficulties in achieving sustained gene expression in the target tissue or cell have resulted in limited clinical benefits from gene therapy. The success of gene therapy is restricted by the relative lack of suitable vectors and will depend on the ability of researchers to address a number of still unsolved problems. This can be approached by either the isolation of new viral serotypes that can be developed into vectors or the creation of new vectors by the modification of the existing ones.

2.2 Justification for Gene Therapy for Cardiovascular Disease

CVD remain the leading cause of mortality and morbidity in the western population. An estimated 2.6 million people have CVD in the UK, accounting for over 216,000 deaths in 2004. More than one in three people (37%) die from CVD (www.bhf.org.uk). Despite advances and improvements in treatments, the incidence of CVD continues to increase worldwide. Gene therapy for the treatment of CVD is currently being developed preclinically and tested clinically. Developments in the field of gene therapy have been rapid. Since 1990, over 1,300 clinical trials have been approved worldwide (www.advisorybodies.doh.gov.uk). The majority of clinical trials are for the treatment of cancers (66.5%), with the second biggest field in gene therapy being for the treatment of CVDs (9.1%).

2.3 Therapeutic Genes for Cardiovascular Diseases

With the identification of the genes involved in CVD and the assignment of function to these genes, the potential to translate this information and identify candidate therapeutic genes is enormous. The genes of interest include targets for the treatment of heart failure, such as sarco-endoplasmic reticulum calcium ATPase pump (SERCA2a), targets for treatment of hypertension, including components of the renin–angiotensin system, and targets for the induction of therapeutic angiogenesis, including angiogenic factors, such as the vascular endothelial growth factor (VEGF) and the fibroblast growth factor (FGF).

VEGF production is induced in response to a number of stimuli, such as hypoxia. Its activity can result in a revascularization process, known as therapeutic angiogenesis,[1,2] through the induction of EC growth or proliferation. Being an angiogenic factor, and therefore having the ability to induce the formation of new blood vessels from the existing vascular bed, VEGF is an ideal gene to overexpress in the context of ischemic vascular disease. Direct muscular injection of human VEGF cDNA into patients with ischemic limbs led to an increased blood flow to the limbs and the subsequent healing of ulcers.[3,4] However, some experiments have demonstrated that nonregulated overexpression of pro-inflammatory VEGF can also lead to detrimental effects, including hypotension and arthritis, and so an element of transcriptional control needs to be included. Adeno-associated virus (AAV) vectors expressing the VEGF transgene under the control of hypoxia response elements induced gene expression in ischemic mouse hearts in vivo.[5]

The absence of heme oxygenase (HO)-1 is implicated in the exacerbation of atherosclerosis, demonstrated by the accelerated and more advanced atherosclerotic lesion formation in HO-1-deficient mice.[6] Retroviral-mediated overexpression of HO-1 in the spontaneously hypertensive rat (SHR) resulted in the attenuation of hypertension,[7] while adenoviral-mediated HO-1 gene transfer prevented the development of atherosclerosis in apolipoprotein E deficient mice.[8] Adenoviral-mediated HO-1 overexpression has also resulted in the attenuation of remodeling responses to experimental vascular injury.[9] The many advantageous effects make this gene an important novel target in the treatment of vascular disease.

The potential of gene therapy in the treatment of hypertension has been explored, although this strategy is unlikely to be tested clinically. Nitric oxide (NO) plays an important role in vascular smooth muscle relaxation, and many vascular diseases are influenced by a reduction in NO bioavailability. Gene therapy approaches aim to increase NO bioavailability to improve vascular function. The direct injection of a plasmid expressing human endothelial NO synthase (eNOS) fused to the CMV promoter significantly reduced systemic blood pressure in the SHR, that was prolonged for 5–6 weeks.[10] In vitro, adenoviral-mediated expression of eNOS and inducible nitric oxide synthase (iNOS) had antiproliferative and antiangiogenic effects on endothelial and smooth muscle cells (SMCs).[11-13] Nitric oxide synthase (NOS) is one of the many genes that have beneficial effects on endothelial function and blood pressure. Other vasodilatory promoting genes include atrial natriuretic peptide, human kallikrein, and bradykinin, and are being investigated for their role in the treatment of CVDs.

2.4 Requirements of a Gene Delivery Vector

A multitude of vector systems, viral and nonviral, have been assessed as tools for gene delivery. A generic vector that is suitable for use in all circumstances is unlikely; gene expression is required in different target tissues for varying lengths of time for different conditions. To avoid eliciting host immune responses, a lack of immunogenicity is desirable and would allow for vector readministration. The induction of immune response is a limiting factor, particularly for adenovirus serotype 5 (Ad5) vectors, which target dendritic cells and some monocytes. The removal of virulence genes in viral vectors helps to limit host defenses.[14,15] Vectors capable of sustained transgene expression would avert the problems of vector readministration; however, some gene therapy applications only require transient transgene expression. Vectors must be producible on a large scale resulting in high vector concentrations. To date, no vector possesses all these qualities, although many steps are being made to overcome these hurdles. Each vector system has its own advantages and disadvantages, depending on its intended use.

For cardiovascular gene delivery, vectors with the ability to transduce cells of the vasculature or of the myocardium are being developed. To increase specificity of cardiovascular gene delivery vectors, methods of tropism alteration and incorporation of cell-specific promoters can be applied.[16-18] Vector tropisms need to be modified to allow efficient and selective transgene expression in vascular cells in vivo.

2.5 Ex Vivo and In Vivo Gene Delivery for CVD

Gene delivery approaches are based on two major concepts: ex vivo and in vivo delivery. In ex vivo cell-based gene therapy, autologous cells or tissue are harvested from a patient, incubated with the vector carrying the desired therapeutic gene, and then reintroduced into the patient. Genetically modified cells will express the transgene, usually at high levels. Owing to the lack of effective pharmacological interventions, this method is being developed for gene therapy of vein graft failure during coronary artery bypass graft (CABG) surgery. CABG surgery is performed on patients with significant atherosclerotic narrowing and blockages of the arteries. CABG allows for the incubation of the graft vessel with a gene therapy vector prior to coronary grafting. Late vein graft failure is a common clinical problem[19,20] and occurs due to thrombosis or neointima formation and accelerated atherosclerosis, a process in which a role for matrix-degrading matrix metalloproteinases (MMPs) and neuronal nitric oxide synthase (nNOS), amongst others, has been implicated. Tissue inhibitor of metalloproteinase-3 (TIMP-3) has been shown to inhibit MMP activity and promote apoptosis, thus inhibiting the progression of neointima formation associated with late vein graft failure in human and pig model systems.[21] Adenovirus-mediated overexpression of nNOS-induced beneficial effects on vein graft remodeling and improved endothelial function,[22] demonstrating the potential of this technique.

Transgene expression in nontarget tissue is limited by this ex vivo method by the removal of excess virus prior to engraftment.

Ex vivo gene delivery has also been utilized in the treatment of familial hypercholesterolemia (FH), in which patients have a deficiency of low-density lipoprotein receptors (LDLRs). For this approach, autologous hepatocytes are harvested, transduced with recombinant retroviruses expressing LDLR, and then transplanted back into the patient. This technique has been validated in rabbit models of FH[23] and in patients,[24] both showing persistent and significantly reduced levels of low-density lipoprotein (LDL) cholesterol. However, ex vivo approaches are limited to largely invasive surgical procedures and to tissues and cells that can easily be removed from the body and then reimplanted. Thus, its clinical applications are severely limited. In vivo gene delivery may be able to help overcome this limitation, although faces many challenges of its own.

For in vivo gene delivery, the vector is either administered directly into diseased tissue within a patient, or is systemically delivered and targeted to the site of action by the vector. Local delivery will ensure relatively efficient transduction of target cells unattainable by systemic administration and avoids the need for the delivery vector to cross endothelial barriers, thus resulting in high vector levels in the target tissue.[25] The route of administration has a major influence on the ability of the vector to transduce various cells and tissues. Delivery methods encompass direct injection into the tissue of interest, catheter-mediated gene transfer techniques,[26] or perfusion.[27] Intramyocardial injection of rAAV2 vectors was used to achieve beneficial therapeutic effects in rat ischemia/reperfusion models and demonstrated highly selective transduction of myocardial tissue.[28] Infusion-perfusion catheters have been used in the context of restenosis prevention. In this case, either adenovirus expressing human vascular endothelial growth factor 165 ($hVEGF_{165}$) or plasmid–liposome complexes containing the $hVEGF_{165}$ gene were delivered directly into the artery. However, in both groups there was no significant change in the lumen diameter or clinical restenosis rate when compared with the control group.[29] A surgical technique to improve gene delivery efficiency involves treating the heart with permeability agents in vivo. Simultaneous clamping of all vessels to/from the heart is followed by continuous retrograde perfusion of the heart through a catheter positioned in the aortic root.[30] This technique eliminates excess virus, which ultimately reduces peripheral tissue infection. Local delivery can however result in leakage of transgene expression into nontarget tissues.[31,32]

Systemic delivery is the ultimate goal of gene therapy as it is, in concept, a simple and noninvasive route of delivery. However, the challenge with this approach is that the body has evolved many highly specific systems to remove foreign particles and pathogens from the bloodstream. Many vectors for systemic gene transfer remain ineffective at delivering genes to the vasculature and myocardium, as a result of liver sequestering after vector administration. There is a trend for viral vectors to display tropism for nonvascular tissues. Liver sequestration is a major limitation of Ad vectors, which are mainly based on serotype 5.[33,34] This hepatic tropism limits the use of systemic delivery to gene therapy for liver disorders. Advances in vector technology and development are helping to overcome this major barrier.

Some AAV serotypes have been recently shown to efficiently cross the blood vessel barrier and as such can be intravenously injected.[35,36] The major limitation of these vectors is that other noncardiac organs may also be targeted. Transductional and transcriptional targeting strategies can be used to improve transgene expression and cell specificity.

2.6 Nonviral Vectors

Nonviral vectors account for approximately 25% of the clinical trials currently in operation (www.wiley.co.uk/genmed/clinical). The simplest form of the vector is naked plasmid DNA encoding for the gene of interest and can be directly injected into the target tissue. Nonviral vector gene delivery is highly inefficient with levels of transduction being significantly less than those achieved by viral vector gene delivery. Nonviral vectors have no specific mechanism with which to cross cell membranes or traffic the injected DNA into the host cell nucleus.[37,38] Strategies to improve vector delivery can be categorized into two general groups: (1) the association of the DNA with other molecules, and (2) the application of physical energy to aid cell entry through the cell membrane (Table 2.1). The major problems of nonviral vector delivery include the interactions of the vector–DNA complex with blood plasma proteins and nontarget cells, and entrapment within endosomes from which

Table 2.1 Characteristics of nonviral gene delivery techniques

Method of gene transfer		Advantages	Disadvantages
Physical	Hydrodynamic injection	Potent gene transfer to liver	Restricted to the liver
	Bioballistic (gene gun)	High transfection efficiency	Shallow penetration of DNA into the tissue
			Short duration of gene transfer
			Dependent on cell line used
	Ultrasound	Low invasiveness	Relatively short duration of gene expression
		Nontoxic	
Chemical	Liposomes	Large capacity for DNA (>20 kb)	Low transfer efficiency in comparison to viral vectors
		Lack of immunogenicity	Poor efficiency in transduction of nondividing cells
		Broad tropism	
	Polycation DNA complexes	Safe in vivo	Instability
		High transduction efficiency in vitro	Cleared rapidly from blood stream
			Nonspecific interactions with other proteins
	Peptide DNA complexes	Low toxicity	Conjugation reactions may reduce biological activities of the proteins and peptides
		Low immunogenicity	

the vector must escape. Once inside the target cell, the challenge of resisting nonspecific cytoplasmic degradation and passage through the physical barrier of the nuclear envelope must be faced.[38] Additionally, plasmid DNA that reaches the nucleus remains extrachromosomal and is usually lost during breakdown of the nuclear envelope at mitosis.[39] Recent studies have thus focused on the development of specially designed vectors with reduced affinity for intracellular proteins and cellular surfaces[40,41] and on mimicking viral properties that will allow the nonviral vector to be maintained and replicate in the target cells. As plasmids contain no proteins to interact with cellular receptors, physical methods of gene delivery can be applied to bring the vector into closer proximity with the cell membrane or to temporarily disrupt the cell membrane, making it permeable to the DNA. Potentially, the use of nonviral vectors offers several advantages over the use of viral vectors including ease and thrift of mass-production, lessened immunogenicity, and a lower risk of unwanted transgene expression in nontarget tissues. However, clinical applications of nonviral vectors remain impeded by the low efficiency of transfection and transient transgene expression. Producing sustained gene expression and potentiating the efficiency of delivery remains a goal of nonviral gene therapy applications.

2.7 Viral Vectors

Viruses have evolved highly specialized mechanisms to enable them to insert their genomes into target cells, making them an ideal candidate to deliver therapeutic genes. In a direct comparison of gene transfer vectors for myocardial gene transfer, recombinant (E1-/E3-) adenovirus, recombinant AAV, and recombinant (ICP27-) HSV all exhibited robust transgene expression, while uncomplexed and complexed naked DNA displayed very limited expression.[42] The efficiency of viral vectors can be attributed to the viral proteins that interact selectively with cell surface receptors and potentially in the trafficking of the virus to the nucleus.[43,44] However, low-level expression of viral genes often evokes an adaptive immune response, and as such the host would destroy the vector-transduced cell.[45] Ad vectors in particular evoke strong immune responses and on administration, can activate an innate immune response mediated by the viral particle itself.[45] This type of immune response is not specific and is aimed at clearing the body of foreign particles, being the first line of defense. Rapid clearance of the vector by cellular elements of the innate immune response involves Kupffer cells,[46] activation of the classical arm of the complement pathway,[47] and an inflammatory response. Adaptive cellular responses are subsequently induced, which activates cytotoxic T-lymphocytes (CTLs).[48] B-cells are activated during the humoral response, which can result in the production of neutralizing antibodies, thereby eliminating the option of vector readministration. By removing genes necessary for viral replication to provide space in which to insert foreign genes, viruses can be manipulated to express foreign genes in any cells that the virus transduces. This also minimizes host immune responses through removal of the adaptive arm of the immune response. Recombinant vectors are thus replication

Table 2.2 Characteristics of viral vectors for use in gene therapy

Vector	Ability to integrate	Transgene capacity	Tropism	Immune response activation	Reference
Retrovirus	Yes	9 kb	Dividing cells only	Minimal	51
Lentivirus	Yes	7–9 kb	Dividing and nondividing cells. Ideal for endothelial cells	Minimal	57,76
Herpes simplex virus-1	No	152 kb	Dividing and nondividing cells. Natural tropism for neuronal cells	Minimal	83
Adenovirus	No	36 kb	Dividing and nondividing cells	Strong	45
Adeno-associated virus	Yes	4.6 kb	Dividing and nondividing cells	Minimal	145,178

deficient, and to produce them, the replication genes must be provided in *trans*, either integrated into the genome of the packaging cell line or on a plasmid.

In principle, any virus can be used as a vector. There are five main classes of clinically applicable viral vectors being studied for cardiovascular applications; retroviruses, lentiviruses, HSV, adenoviruses (Ad), and AAV, a summary of which can be seen in Table 2.2. These five vector classes can be further subcategorized according to whether the vector genome integrates into the host chromosome or exists extra-chromosomally.[49] Integrating vectors are associated with an increased risk of insertional mutagenesis,[50] although careful engineering may be applied to minimize these risks. For example, the engineering of vectors that integrate into predetermined sites could allow long-term transgene expression while preventing the detrimental effects through inappropriate integration.[51] Since each vector system has its own unique set of properties, one vector may be preferential above another in a particular setting and will determine its range of uses in gene therapy.

2.7.1 Retrovirus

Retroviruses were the first viral vectors to be used in human gene therapy[52] and approximately 25% of the world's gene therapy clinical trials use retroviruses as their platform vector (www.wiley.co.uk/genmed/clinical). Retroviruses can be further subdivided into oncoretroviruses, lentiviruses, and spumaviruses, all of which are being developed for gene therapy applications to varying extents. Retroviruses are small enveloped RNA viruses, which replicate via an integrated DNA intermediate by the actions of the enzyme reverse transcriptase. The viral genome is approximately 10 kb, comprising at least three genes: *gag* (group-specific antigens), *pol* (reverse transcriptase), and *env* (the viral envelope protein). These viral genes are flanked by

long terminal repeats (LTRs), which are required for integration into the host genome and control viral gene expression. The genome also contains a packaging sequence that allows it to be distinguished from the host-cell RNA.[53]

Retroviral vectors have all their viral genes removed and replaced with the transgene of interest, thus rendering them replication-incompetent.[54] Despite their wide use as gene delivery vectors, the small genome of retroviruses allows for only 9 kb of foreign sequence to be inserted. Production of high-titer preparations required for gene therapy applications is problematic. Retroviruses are associated with low-efficiency gene transfer owing to their inability to deliver genes to nondividing cells.[55] Thus, their utility as gene delivery vectors for vascular applications is severely limited as they are not able to infect nondividing vascular cells. These inefficiencies have led to the development of lentiviral vectors, which are capable of infecting both dividing and nondividing quiescent cells.[56-58]

The genome of retroviruses integrates into the host's genome leading to the potential for long-term transgene expression. However, integration is not site-specific and subsequently this vector has many safety concerns associated with it. Random insertion of an LTR sequence adjacent to a cellular proto-oncogene can lead to inappropriate expression of a protein involved in cellular regulation. Random insertional mutagenesis could also disrupt tumor suppressor genes, potentially leading to dysregulation and a malignancy. In 2000, a clinical trial carried out in France to treat children with severe combined immunodeficiency-X1, illustrated the oncogenic potential of retroviral vectors.[59] This study was based on ex vivo transfer of the γc gene into CD34+ cells using a defective gamma Moloney retrovirus-derived vector. After 10 months, the therapy was found to provide sustained full correction of disease phenotype demonstrating the unique potential of gene therapy. However, by 2003, two patients had developed a serious adverse complication consisting of uncontrolled leukemia-like clonal lymphocyte proliferation,[50] with a third case of leukemia-like illness being reported in 2005.[60] Two of the three patients were found to have retrovirus integration within or in close proximity to the LM02 proto-oncogene promoter, which is associated with childhood leukemia. This integration resulted in the inappropriate upregulation of the proto-oncogene and proved fatal in one of the patients.[61] However, the beneficial outcomes in the remaining patients are not to be overlooked. To date, 17 out of 20 patients in both the Paris and London clinical trials have had their immune system restored and has remained functional for over 7 years.[62] One adverse effect has recently been reported in the UK-based clinical trial (www.news.bbc.co.uk/1/hi/health).

2.7.2 Lentivirus

Lentiviruses are a subclass of retroviruses that are increasing being used in gene therapy. In particular, they are being developed for the treatment of neurodegenerative disorders, because of their ability to efficiently transduce cells of the nervous system.[63,64] The lentiviruses used are usually derived from human immunodeficiency

virus-1 (HIV-1) and so raise many potential clinical safety concerns. The vector integrated into the genome randomly. To improve the biosafety of these vectors, significant modification to the HIV-1 genome can be made.[65] Deletion of accessory genes *tat, vif, vpr, vpu,* and *nef* produces minimal vectors that contain only genes necessary for replication and packaging, thus minimizing deleterious effects.[65] Development of nonhuman lentiviral-based systems, including simian,[66] feline,[67,68] and bovine immunodeficiency viruses,[69,70] has also been given attention to increase the safety profile of these vectors.

Lentiviruses have a relatively large packaging capacity of up to 8 kb and an ability to infect a wide range of cells. They are also minimally immunogenic having been shown to sustain gene expression for several months[71] without detectable pathology.[72-74] Gene transfer through lentiviruses is relatively stable, as the transgene integrates into the host genome and is copied along with the host genome every time the cell divides. One of the most appealing features of these vectors is that unlike other retroviruses, lentiviruses can infect nondividing cells, being able to enter the nucleus without mitosis.[75,76] This ability makes these vectors ideal for targeting the endothelium, which is largely composed of nondividing cells. Lentivirus transduction of both primary human saphenous vein endothelial cells (EC) and SMC was shown to be efficient and without toxicity,[57] but there are relatively few studies to date. Lentivirus-based vectors have been also shown to be successful at transducing adult cardiomyocytes of a transplanted heart,[77] and the hearts of SHR in a study of cardiac physiology.[78]

Recently, a new generation of lentiviral vectors has been produced with enormous potential. These are in the form of nonintegrating lentiviral vectors. By introducing mutations into highly conserved acidic residues in the viral integrase gene, catalytic site or chromosome binding site, vectors can be rendered integration defective without interrupting viral DNA synthesis or accumulation in the nucleus.[79-81] Efficient sustained transgene expression in vivo is attainable with nonintegrating lentiviral vectors as has been demonstrated in muscle[81] and in rat ocular and brain tissue at levels high enough to improve retinal degeneration in an appropriate disease model.[82]

2.7.3 Herpes Simplex Virus (HSV)

HSV type 1 is an enveloped double-stranded DNA virus containing an icosahedral-shaped capsid surrounded by a layer of proteins referred to as tegument. It has a relatively large genome of 150 kb, which facilitates large foreign DNA inserts of up to 30–40 kb.[83] HSV is able to infect a broad range of cell types including nondividing cells. Natural viral infection can take the form of a cycle of lytic replication or can enter a latent state in which the viral genome persists without the expression of any viral proteins, possibly for the life of the host. Latently infected neurons function normally and do not illicit an immune response.[84] HSV-1 has many key features making it a highly desirable vector for gene delivery. First, it has a large transgene capacity, which is provided by the deletion of genes superfluous for viral replication.

However, because its genome does not integrate, HSV vectors are unlikely to be suitable for the treatment of conditions requiring long-term gene expression. Because of its natural tropism for neuronal cells, it has become a promising vector for the treatment of neurological disorders such as Parkinson's disease.[85] HSV vectors have also emerged as promising vectors in cancer therapies in the form of replication-selective oncolytic vectors.[86-88] These vectors fail to replicate efficiently in healthy cells and will replicate in cancer cells only, destroying them through oncolysis.

2.7.4 Adenovirus

Adenoviruses are nonenveloped dsDNA viruses with an icosahedral capsid consisting of three main structural proteins, hexon, fiber, and penton base, and several minor capsid proteins. Their genomes range in size from 26 to 45 kb. Adenoviruses were first isolated from tonsils and adenoid tissue[89] and are infectious human viruses, which often cause mild infection of the gastrointestinal, upper respiratory tract and eye. Most adenoviral infections are self-limiting being efficiently counteracted by the host's immune system. Deletion of the virulent genes during vector production may help in reducing the pathogenesis of these viruses.

Adenoviral vectors, most commonly Ad5 and adenovirus serotype 2 (Ad2), are a popular choice in gene therapy and such a status has led to much information about them becoming widely available. As such, adenovirus is well characterized and can be easily genetically altered and grown to high titers. They have a high capacity for the insertion of foreign DNA allowing up to 36 kb (helper-dependent Ads) to be accommodated. They were initially deemed promising vectors for cardiovascular gene therapy applications as they were shown to transduce human vascular cells in vitro[90] and in vivo.[91,92] Adenoviral vectors exhibit a tropism for many human cells and can infect quiescent as well as dividing cells,[93] an important characteristic for the transduction of vascular EC and SMC, which have low mitotic rates, even in diseased states.[94] Adenovirus replicates episomally, thus reducing the risk of random integration into the host genome. However, because Ad vectors are nonintegrating, it means that their genomes are lost in proliferating cells, and so transgene expression will be transient, although this may be advantageous in certain clinical applications. Transient gene expression coupled with hepatic tropism is a major limiting factor for adenoviral vectors and has led to their use in niche areas such as vein grafting, where gene transfer can be carried out ex vivo.[21,95]

The major inadequacy of adenoviral vectors is their high immunogenicity. Many individuals produce neutralizing antibodies and memory T cells directed at Ad proteins after exposure to the vectors. This is a result of the expression of viral genes, which trigger a cascade of humoral and innate immune responses.[48] This is a significant problem, as gene expression is consequently short-lived[96] and vector readministration is less effective.[97] In view of this, current studies focus on strategies to eliminate host immune responses,[14,98] and also on engineering vectors with increased transduction of cardiovascular cells. This can be achieved in several

ways, one of which involves the abolition of the natural tropism of the virus and subsequently endowing it with a new tropism for the target cell type.[99-101]

2.7.4.1 Ad Vector Development

To reduce the immunogenicity of Ad vectors and create additional space for the insertion of new genetic material, Ad has been altered in several ways to remove unnecessary parts of the genome (Fig. 2.1). Expression of adenovirus proteins occurs in phases – early and late. The adenovirus genome contains five early transcription units (E1A, E1B, E2, E3, E4), two early delayed (intermediate) transcription units, and five late units (L1–L5), and encodes over 70 gene products.[102] The genome is flanked by inverted terminal repeats (ITRs) of 100–140 bp in size that serve as replication origins. Early genes (E1A and E1B) are involved in gene expression regulation and their activation leads to the expression of viral late genes (involved in the expression of structural proteins) and the production of infectious viral particles. The foreign gene can be inserted into the region occupied by either E1 or E3 genes with one or both being deleted in the vector construct. In the first generation Ad vector, the E1 (E1A and E1B) gene is replaced by the gene of interest and the resultant defective virus is propagated in cell lines, such as 293 cells,[103] which provide the early gene products in *trans*. The progeny virus cannot replicate in normal cells and on introduction into the host, it will infect cells and express the foreign gene, but no progeny virus will be produced. As the E3 region of the genome is dispensable in viral replication, many first-generation vectors will also have all or part of the E3 region deleted. Despite these deletions, first-generation vectors still express wild-type late viral genes at low levels and trigger a CTL immune response,[104] resulting in a short duration of transgene expression.

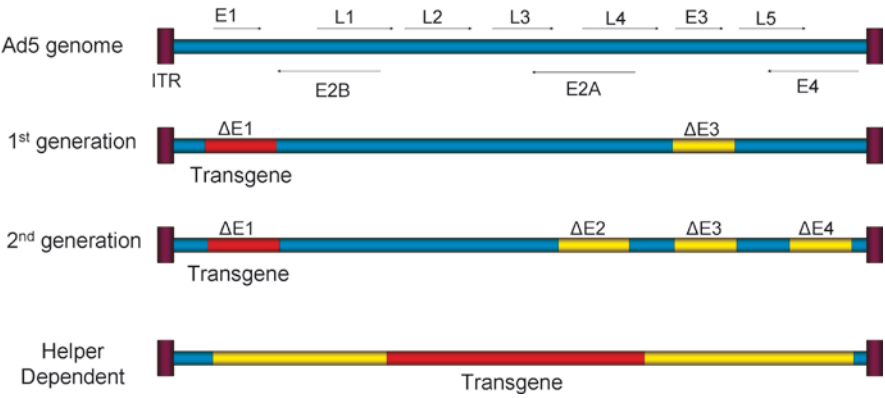

Fig. 2.1 Adenovirus serotype 5 (Ad5) vector development. Adenovirus 5 genome and maps of first-, second-, and third-generation adenoviral vectors showing regions of the genome deleted to facilitate transgene insertion. Adapted from ref.[186]

Second-generation Ad vectors have the E2 and/or E4 regions deleted from their genomes in addition to the E1±E3 deletion. However, second-generation vectors were not found to reduce inflammation of humoral immune response to adenovirus in rabbit models in comparison with first-generation vectors, and most disappointingly did not increase longevity of transgene expression.[96]

Helper virus-dependent or gutless vectors have essential regions of the viral genome (L1, L2, VA, and TP) deleted and rely on the provision of essential viral functions from a helper virus. The gutless adenovirus only keeps the two ITRs and the packaging signal from the wild-type adenovirus required for DNA replication and packaging.[105-108] By deleting most of the viral genomes, it is possible to accommodate up to 37 kb of insert DNA into defective vectors. In vivo studies have shown substantially longer transgene expression with helper-dependent vectors[14,98] sustained up to a year in baboons. However, an innate immune response is still activated against the vectors.[48] Recently, however, Barcia et al demonstrated that helper-dependent Ad vectors mediated sustained transgene expression for up to 1 year in the brains of mice preimmunized against adenovirus.[15] This highlights the potential of these vectors in the treatment of chronic diseases, as the immune system was unable to inhibit transgene expression.

2.7.4.2 Vector Capsid Engineering

There are more than 50 different serotypes of adenovirus, classified into six groups (A–F) based on biochemical and immunological properties. These viruses infect different cell types through the utilization of different primary cellular receptors and thus have a wide tissue tropism range. Most adenoviruses, except subgroup B and the short fiber of subgroup F, use the coxsackie virus and adenovirus receptor (CAR).[109,110] The two-step mechanism of Ad5 infection is well characterized, making it possible to reengineer it to alter its tropism. Ad5 virus interacts in vitro with CAR by means of the knob domain of the capsid fiber, bringing the capsid into close proximity with integrins. After attachment, the RGD motif in the penton base at the N-terminus of the fiber interacts with coreceptors $\alpha_v\beta_3/\alpha_v\beta_5$ integrins.[111] Adenovirus is then internalized by receptor-mediated endocytosis and released by endosomal acidification in fiber-free form to the cytosol, before trafficking to the nucleus. CAR is relatively ubiquitous, resulting in the infection of undesirable tissues as well as target tissues. Ad5 can transduce EC,[90] coronary arteries,[92] the heart[112] and at lower efficiency vascular SMC.[113] This is reflective of the distribution of CAR expression, with high CAR expression leading to high transduction efficiency. Indeed, after systemic injection in the rat and mouse models, Ad5 virions preferentially accumulated in the liver and spleen.[33,114] This highlights the need to substantially alter Ad5 tropism to retarget it to alternative sites, for example the brain, kidney, and heart vasculature.

Genetic strategies to alter adenovirus tropism can either focus on pseudotyping the Ad5 fiber with that of another serotype, or on modification of the

existing receptor binding mechanisms. Native hepatic tropism can be altered by mutating the virus in areas integral to cellular receptor binding. The identification and mutation of the residues in the knob involved in CAR binding[115] has allowed the production of modified Ad5.[116]

2.7.4.3 Adenoviral Retargeting by Pseudotyping

The adenovirus fiber protein mediates primary binding of adenovirus to its receptor. Vector retargeting may be achieved through the use of chimeric vectors that incorporate the entire fiber or part of the fiber from a different serotype in place of its own. This could potentially ablate the virus's natural tropism by removal of both the CAR- and heparan sulfate proteoglycan (HSPG)-binding sites and bestow a new tropism upon the vector. Several adenovirus serotypes have shown increased transduction of specific tissues. Proof of the concept of chimeric vectors was first shown in 1996 with the production of functional adenoviral vectors in which the fiber was composed of the tail and shaft domains of Ad5 and the knob domain of serotype 3.[117] Alterations in adenoviral tropism were achieved through primary binding via the Ad3 receptor with subsequent internalization steps achieved via domains of the penton base of Ad5.

Ad5 vectors pseudotyped with serotype 37 and 19p fibers have demonstrated a lack of native tropism for mouse, rat, and human hepatocytes in vitro and demonstrated greatly reduced transduction of liver after systemic injection into rats.[118] Further genetic modifications can allow the development of targeted and thus more efficient vectors. Isolated targeting peptides can be genetically incorporated into the HI loop of the fiber of Ad19p between amino acids 331 and 332. Kidney targeting peptides HTTHREP and HITSLLS, which were identified through in vivo phage display, were incorporated into Ad19p-pseudotyped vectors. These peptide-modified vectors were shown in vitro and in vivo, after systemic administration, to display a significant increase in selective renal targeting with higher levels of transduction than the unmodified Ad19p vectors.[119]

2.7.4.4 Nongenetic Targeting

A simple way of altering vector tropism without genetic modification is the coating of the viral particle with a bispecific antibody. One domain of the bispecific molecule binds to the virus capsid, while the other domain binds to a novel receptor thus acting as a molecular bridge. This concept has been used in vitro to enhance Ad-mediated transduction of human umbilical vascular EC,[120] and in vivo to redirect Ad vectors to a new cellular receptor after systemic delivery.[121,122] Although the addition of a protein adapter enhances the affinity of Ad vectors for their targets, it also increases the difficulty of crossing the barrier from laboratory to clinic as there are more components to be considered and reproduced without batch variation.

2.7.4.5 Retargeting Detargeted Vectors by Ligand Insertion

The insertion of targeting peptides into the fiber gene of Ad5 can provide new tropism to detargeted vectors. The exposed HI loop has been identified as a preferred insertion site for peptides[123,124] without detriment to virion assembly or fiber trimerization. As the fiber is present at a frequency of 36 copies per virion, the vector can display the targeting peptide a maximum of 36 times. Foreign peptides have also been successfully incorporated in the hypervariable region 5 surface loop of the hexon of Ad vectors.[125] In this region, peptides can be displayed at a copy number of 720. However, in a direct comparison of peptide-modified fiber and hexon vectors, hexon-mediated targeting failed to change the tropism of the vectors.[126]

Recent work has shown the application of the phage display technology to identify sequences with desired biological properties, and subsequently introduced these sequences in the retargeting site of the vector.[127-129] One disadvantage of these small targeting peptides is their often weak binding affinity for their targets. The concept of phage display of exogenous peptides was first conceived in 1985, and is simply the display of peptides or proteins on the surface of bacteriophage.[130] The technology of phage display has since been developed and is now used in a wide range of applications, including the rapid isolation of novel peptides with the ability to bind to defined target molecules in vitro or in vivo.[131] For use in cardiovascular applications, phage display could potentially identify ligands, which are specific for the vasculature. Highly efficient and selective peptides can be isolated through the process known as biopanning, which can be carried out in vitro and in vivo. Successive rounds of biopanning enrich the pool of phage with clones that specifically bind the target.

The distinct disadvantage of using in vitro biopanning is that the question remains as to whether the ligands isolated in vitro will display the same specificity in vivo. Phage libraries can be directly introduced into live animals, to select for targeting peptide sequences. However, targeting peptides identified in animal models may not always be applicable and achieve the same level of targeting in humans. In 2002, the first in vivo screening of a peptide library in a patient was carried out.[132] Isolated motifs from tissue biopsies showed high similarity to ligands for cell-surface proteins of the human vasculature. This method has since been used in stage IV cancer patients to identify tumor-targeting ligands.[133] This study displays how this method can be directly applicable in a clinical setting.

2.7.5 Adeno-Associated Virus (AAV)

AAV vectors have developed rapidly over the past decade and have become promising vectors for several genres of gene therapy. RAAV2 vectors have been extensively researched and are the most characterized and predominantly used of the AAV vectors. The potential of these vectors in cardiovascular gene delivery was first shown through rAAV-mediated expression of the cytoprotective gene HO-1 in rat myocardium.[28] The safety and efficiency of these vectors was further proven through

rAAV-mediated myocardial gene transfer in mice. Transgene expression was observed 1 year postinfusion with no significant inflammatory response or adverse affects on LV systolic function.[134] In a study by Xiao et al,[135] the introduction of rAAV vectors expressing the *lacZ* gene into the muscles of immunocompetent mice resulted in persistent gene expression for more than 1.5 years.[135] AAV vectors are thus minimally pathogenic and possess the ability to mediate long-term transgene expression, and so could prove useful in clinical situations where prolonged transgene expression is desirable. Stable transgene expression is a prerequisite for vectors to treat inherited disorders and would be desirable in the treatment of many acquired CVDs, which progressively worsen over time. However, the progress of AAV vectors has been hampered by their poor transduction of many target tissues.

RAAV vectors evoke little innate immune response, with only transient infiltration of neutrophils and chemokines.[136] Immune response against the virus appears to be restricted to the generation of antibodies specific for the viral capsid protein.[137] AAV vectors are inefficient transducers of antigen presenting cells such as macrophages and dendritic cells, which are believed to be necessary in the production of cellular immune responses.[138] However, recently the duration of transgene expression in the liver mediated by rAAV2 vectors was found to be limited to 8 weeks.[139] Upon further investigation, it was suggested that transduced hepatocytes were destroyed by the activation of T-cells against the capsid of rAAV2.[139,140] Direct comparison of T-cell responses activated against the capsids of rAAV serotypes 2, 7, and 8 revealed little evidence of T-cell activation against rAAV7 and 8 and postulated a potential role for heparin binding in directing immune response against the capsid proteins.[140] Thus, utilization of alternative serotypes that do not use HSPG as their receptor for cell entry may help to avoid this limitation.

In AAV vectors, the viral DNA, except the ITRs, has been eliminated to allow for foreign DNA insertion. This adds a safety feature that will reduce host immune responses directed at viral gene expression and eliminate the possibility of the generation of replication competent pseudo-wild-type AAV. One important safety concern with AAV vectors is the potential for germ-line transmission. Intramyocardial injection of AAV vectors expressing *lacZ* into Sprague–Dawley rats resulted in the detection of *lacZ* expression and β-galactosidase activity in the testes at 6 months postinfusion.[141] In a similar study, Arruda et al found that while vector DNA could be detected in the gonad of rat, mouse, rabbit, and dog, no AAV vector sequences could be detected in the semen.[142] Another major safety concern lies among reports of high incidences of hepatic carcinomas after rAAV vector infusion into mice.[143] Carcinomas that developed in these mice were subsequently found to contain AAV vector proviruses at a specific chromosomal locus,[144] implicating insertional mutagenesis by AAV vectors as a causative factor. These findings raise questions of rAAV vector safety.

2.7.5.1 AAV Biology

AAVs are small 4.7-kb linear single-stranded DNA nonenveloped viruses. Their genomes are organized in similar ways, being extremely simple in composition and

containing only two large open reading frames (ORFs) flanked by ITRs of approximately 145 bp, which are required for viral genome replication and packaging (Fig. 2.2). The two ORFs encode two genes, rep (replication) and cap (capsid), which are, respectively, involved in gene expression regulation and structure. Four multifunctional rep isoforms with molecular masses of 78, 68, 52, and 40 kDa are encoded by the 5′ ORF and are transcribed from two different promoters. The rep proteins are involved in specific DNA-binding, helicase, and site-specific endonuclease and modulation of transcription of viral genome promoters. The 3′ ORF encodes three capsid proteins (VP1, VP2, and VP3) through alternate splicing of the cap gene. All three proteins use the same stop codon, and so VP2 and VP3 are successive amino-terminal truncated forms of VP1. The three proteins interact together to form a capsid with icosahedral symmetry. When used as gene delivery vectors, the rep & cap genes, which make up 96% of the genome, are replaced by the transgene. Recombinant vectors are produced by supplying these deleted genes in *trans*. The resultant vectors are less likely to evoke a host immune response. The small size of the AAV virion is responsible for the limited DNA packaging capacity and is a major disadvantage of AAV vectors. Transgenes can be packaged as long as they are not significantly larger (119% maximum capacity) or smaller than the wild-type genome.[145] Without these limits, the resultant vectors are severely defective with regard to producing infectious virions. One method to overcome this limitation is the trans-splicing of larger genes between two independent AAV vectors that will be coadministered.[146] This technique utilizes the ability of AAV genomes to combine, although results in lower transgene expression as a result of the complexity of the system. However, further development may increase the utility of AAV vectors allowing them to appeal to a wide range of applications.

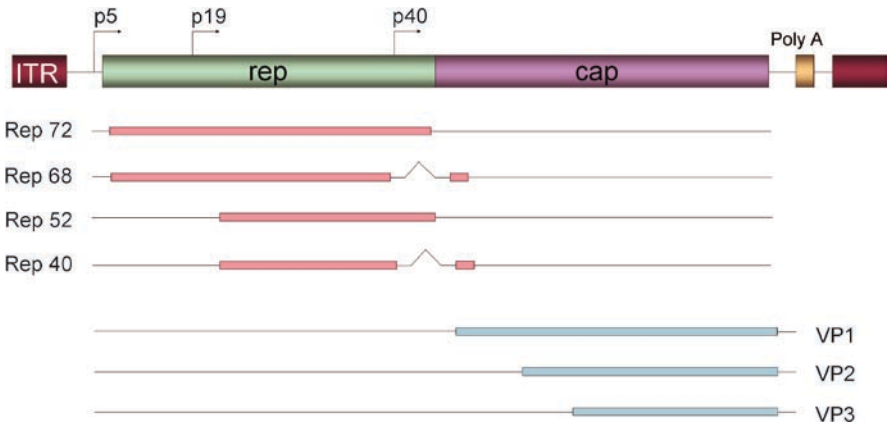

Fig. 2.2 Genome organization of adeno-associated virus (AAV). The AAV genome is a 4.7 kb single-stranded linear DNA genome and is made up of 2 genes, rep and cap, with two flanking inverted terminal repeats (ITRs). Three different promoters drive transcription: P5, P19, and P40. Four transcripts encode nonstructural genes (Rep72, 68, 52, and 40) and three transcripts encode structural proteins (VP1, 2, and 3)

2.7.5.2 AAV Replication

AAVs are helper-dependent viruses with a bi-phasic life cycle. They cannot replicate autonomously, instead requiring coinfection with an unrelated virus, such as Ad or HSV, to complete its life cycle. In the absence of coinfection, AAV can undergo latent infection as an episome or may integrate its viral DNA into the host genome[147] in human chromosome 19 by site-specific recombination directed by the viral *rep* function.[148] AAV genomes can be excised from the host genome in the presence of helper factors and can lead to a productive infection cycle.[149] It is important to note that rAAV vectors lack the integration function as their viral genes have been removed. Advances in AAV vector production have eliminated the need for helper adenovirus infection.[150] Instead, to be packaged into functional vectors, genomes must be provided with all rep, cap, and helper functions *in trans* on exogenous plasmids.[150,151]

There are several common stages for replication of all AAV vectors that must be carried out for successful transgene expression. The first step in infection is the attachment of the vector to the cell surface receptor, and in the case of AAV vectors will require the use of coreceptors to assist in internalization. The virus must then be internalized into the cell by the process of receptor-mediated endocytosis. The vector is subsequently trafficked from early endosomes to late endosomal compartments.[152] It must then escape the endosome to be released into the cytosol and undergo nuclear translocation. Endosomal processing is thought to be an essential step for AAVs, exemplified by the fact that AAV2 directly injected into the cytosol fails to reach the nucleus.[44] After endosomal release, which may occur through weak acidification of the vesicle, AAV rapidly traffics to the nucleus and accumulates in the perinuclear region.[153] Nuclear translocation was initially thought to occur through the virus slowly penetrating the nuclear pore complex (NPC) into the nucleus, with the majority of the virus remaining in perinuclear compartments.[153] However, entry into the nucleus has since been shown to occur independently of the NPC through the use of agents that block NPC function.[154] It is unknown whether viral uncoating to release the genome occurs within or outside the nucleus. However, capsid proteins[155] and the necessary machinery for virion uncoating[154] have been identified within the nucleus, suggesting that nuclear virion uncoating may be a reality, although direct evidence is lacking. The single-stranded DNA genome is converted to double-stranded DNA within the nucleus and is then the transcription template. After entry into the host cell nucleus, the virus can either establish a lytic or lysogenic life cycle, which is determined by the presence or absence of helper virus. The efficiency of all these replication steps will determine the overall efficiency of the vector.

2.7.5.3 AAV Serotypes and Receptors

To date, over 100 AAV genetic variants have been isolated.[156] Twelve known serotypes of AAV have been identified, all displaying a variety of tissue tropisms and receptor-binding characteristics (Table 2.3). The sequence identities among the

Table 2.3 Adeno-associated virus (AAV) serotypes and their varying tropisms and receptors

Serotype	Tropism	Receptor
AAV1	Skeletal muscle[179] cardiac tissue[112]	α2–3 linked or α2–6 linked sialic acid
AAV2	Broad tropism – muscle, brain, retina, liver, lung.	HSPG, αVβ5 integrin, fibroblast or hepatocyte growth factor receptors, 37/67-kDa laminin receptor
AAV3	Cochlear inner hair cells[180]	heparin, heparan sulfate, and FGFR-1, 37/67-kDa laminin receptor
AAV4	Ependymal cells[181]	α2–3 O-linked sialic acid
AAV5	Neurons[182], dendritic cells[183]	PDGFR, α2–3 N-linked sialic acid
AAV6	Skeletal muscle, cardiac tissue[35]	α2–3 linked or α2–6 linked sialic acid
AAV7	Skeletal muscle[184]	Unknown
AAV8	Liver[184]	37/67-kDa laminin receptor
AAV9	Liver, skeletal muscle, cardiac tissue[36]	37/67-kDa laminin receptor
AAV10	Liver, heart, skeletal muscle, lung, kidney, uterus[185]	Unknown
AAV11	Muscle, kidney, spleen, lung, heart, stomach[185]	Unknown

different serotypes are high with a general homology in nucleotide sequence of approximately 80%. The greatest divergence in sequence can be observed in the capsid proteins, especially in regions thought to lie on the utmost exterior of the virion.[157] This may account for the differing natural tropisms of these viruses. The pattern of transgene expression has been demonstrated to be affected by the serotype of AAV,[158] which may be due, in part, to viral receptor distribution, as receptor binding is the primary step in viral infection. The discrepancies in tissue tropisms between serotypes are likely as a result of different mechanisms of uptake into a target cell. To comprehend the differences in transduction efficiencies of the different serotypes, it is important to understand the full mechanism of the initial AAV binding and internalization steps.

AAV2 has a wide host range and utilizes HSPG as an attachment receptor,[159] and at least three different coreceptors including αVβ5 integrin,[160] and the fibroblast or hepatocyte growth factor receptors.[161] AAV3 has been shown to bind to heparin, heparan sulfate, and fibroblast growth factor receptor-1 (FGFR-1), making its array of receptors similar to those of AAV2.[162] Competition assays identified that closely related serotypes AAV1 and AAV6 use either α2–3 linked or α2–6 linked sialic acid as primary receptors when transducing numerous cell types.[163] Platelet-derived growth factor receptor (PDGFR) has been identified as a coreceptor for AAV5, with the in vivo tropism of AAV5 correlating with the distribution of PDGFR.[164] AAV5 also requires α2–3 sialic acid for binding and transduction.[165] AAV4 shares the requirement of AAV5 for sialic acid; however, the difference between these two vectors lies in linkage specificity; AAV4 requires O-linked sialic acid, whereas AAV5 requires N-linked sialic acid, offering an explanation for tropism differences.[166] A 2-yeast hybrid screen with subsequent functional studies revealed the 37/67-kDa laminin receptor (LamR) as important in binding and transduction of AAV8.[167]

It was also shown to be important in the binding of AAV2, -3, and -9. AAV10 and -11 have not yet been fully characterized.

AAV2 vectors have been quite disappointing in the area of cardiovascular gene therapy due to inefficiencies in transduction of both myocardial cells and EC. Direct comparison of Ad5 and AAV2 for transduction of vascular cells has revealed the poor tropism of AAV2 for EC.[57] Transduction of vascular EC has been shown to be inefficient with AAV2 vectors resulting in virion degradation by the proteasome during the trafficking process.[168] Although no AAV serotype appears substantially more efficient than AAV2 in transduction of the vascular endothelium, other EC have been transduced by alternate serotypes. AAV6-based vectors demonstrate a higher transduction efficiency of airway epithelia than AAV2,[169] illustrating the potential of exploiting naturally occurring serotypes. Thus, alternate serotypes with naturally occurring tropism differences can be exploited as potential gene therapy vectors to see if they offer an enhanced tropism for cardiovascular tissues. AAV serotypes 1 and 6 have shown preferential transduction of the musculature.

2.7.5.4 AAV Transcapsidation

RAAV vectors are based on the AAV2 genome and onto which the capsid proteins from a different serotype have been pseudotyped. Capsid proteins from most serotypes have been successfully cross-packaged with ITRs from AAV2. Several studies have been carried out to compare the transduction efficiencies of the ever increasing array of alternate serotype AAV vectors. In a study by Du et al,[170] the capacity of AAV serotypes 1–5 for in vitro myocardial transduction was tested.[170] This study demonstrated the differing capacities of the alternative serotypes, and identified AAV1 as having the highest enhanced ability to transduce adult human cardiomyocytes. In another study that compared the efficiency of recombinant vectors of eight different serotypes in transducing rat myocardium in vivo, AAV1, 6, and 8 demonstrated the highest efficiency in transducing rat hearts in vivo.[112] It is difficult to compare between AAV serotype studies as no standard for titering AAV has been set up, and different routes of administration and different aged animals have been used. However, general trends can be observed, demonstrating that AAV serotypes 1, 6, 8, and 9 show high levels of cardiac transduction.

2.7.5.5 Retargeting AAV Vectors

Although several serotypes of AAV have been identified, several cell types remain nonpermissive to AAV infection. Retargeting vectors may encompass these nonpermissive cells into AAVs vast repertoire, and may improve the efficiency of transduction of cells already permissive to infection. Retargeting of AAV vectors has mainly been applied to AAV2 vectors, and has been achieved in vitro through two main strategies. These are (1) the use of bi-functional antibodies[171] and (2) the genetic modification of the capsid through the insertion of targeting peptides.[172] Vector binding is enhanced

by the use of bi-specific antibodies. During this process, one arm of the antibody binds to the surface of the cell of interest, and the other arm to the AAV capsid structure. Bartlett et al[171] achieved AAV2-mediated transduction of nonpermissive human megakaryocytic cells through the interaction of a bispecific F $(ab)_2$ antibody with both the cell surface receptor $\alpha_{IIb}\beta 3$, and the viral capsid. This facilitated the binding and internalization of the vector via an alternative receptor and represents the potential to improve the binding and transduction profile of AAV2. This technique has been used to redirect AAV binding by insertion of an immunoglobulin binding domain to couple it to various antibodies to mediate altered receptor binding.[173] However, this relies on a very stable interaction between the antibody and the vector.

The AAV capsid protein is important in the initial stages of viral infection and primarily interacts with the cell surface receptor. The capsid protein determines the tissue tropisms of the virus through its selective interactions. Short peptide sequences can be cloned into the capsid gene to change or expand the vector tropism and can even be used to disrupt the native tropism. Targeting peptides may be derived from phage-display techniques previously described. To be successful, the peptide insertion should have minimal effects on subsequent vector assembly, packaging, and infectivity. Several suitable sites for insertion of targeting peptides into the AAV2 capsid have been identified and evaluated for tolerance to insertions and mutations; peptides may be inserted at the optimal position of 587 in the AAV2 capsid to be displayed on the surface of the virion.[172,174] Genetic incorporation of peptides into the AAV capsid has been used to enhance transduction of human EC[129] and to alter tropism toward cells expressing the CD13 receptor[128] and human luteinizing receptor (LH-R).[175]

A variant of this technique is the use of AAV libraries, which are similar in concept to phage libraries. A random peptide is inserted into the AAV2 capsid sequence in a position that allows it to be displayed on the surface of the virion, while at the same time ablating HSPG binding. Chimeric capsid AAV libraries are screened to identify vectors that exclusively transduce a particular target cell or tissue type. This technique was first developed by Müller et al,[176] who used the AAV library to identify vectors that could transduce human coronary artery EC more readily than nonendothelial control cells. Others have used this approach to identify AAV vectors that efficiently transduce acute myeloid leukemia cell lines,[177] a cell type that no other vectors have been found to efficiently transduce. AAV libraries allow the selection of vectors with targeting peptides that have been identified while already in the AAV2 capsid. This eliminates the possibility of the targeting peptide losing its specificity when incorporated into the vector.

References

1. Josko J, Gwozdz B, Jedrzejowska-Szypulka H, Hendryk S. Vascular endothelial growth factor (VEGF) and its effect on angiogenesis. *Med Sci Monit*. 2000;6(5):1047–1052.
2. Lee M, Rentz J, Bikram M, Han S, Bull DA, Kim SW. Hypoxia-inducible VEGF gene delivery to ischemic myocardium using water-soluble lipopolymer. *Gene Ther*. 2003;10(18):1535–1542.

3. Baumgartner I, Pieczek A, Manor O, et al. Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation*. 1998;97(12):1114–1123.
4. Shyu KG, Chang H, Wang BW, Kuan P. Intramuscular vascular endothelial growth factor gene therapy in patients with chronic critical leg ischemia. *Am J Med*. 2003;114(2):85–92.
5. Su H, Arakawa-Hoyt J, Kan YW. Adeno-associated viral vector-mediated hypoxia response element-regulated gene expression in mouse ischemic heart model. *Proc Natl Acad Sci U S A*. 2002;99(14):9480–9485.
6. Yet SF, Layne MD, Liu X, et al. Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. *Faseb J*. 2003;17(12):1759–1761.
7. Sabaawy HE, Zhang F, Nguyen X, et al. Human heme oxygenase-1 gene transfer lowers blood pressure and promotes growth in spontaneously hypertensive rats. *Hypertension*. 2001;38(2):210–215.
8. Juan SH, Lee TS, Tseng KW, et al. Adenovirus-mediated heme oxygenase-1 gene transfer inhibits the development of atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2001;104(13):1519–1525.
9. Tulis DA, Durante W, Liu X, Evans AJ, Peyton KJ, Schafer AI. Adenovirus-mediated heme oxygenase-1 gene delivery inhibits injury-induced vascular neointima formation. *Circulation*. 2001;104(22):2710–2715.
10. Lin KF, Chao L, Chao J. Prolonged reduction of high blood pressure with human nitric oxide synthase gene delivery. *Hypertension*. 1997;30(3 pt 1):307–313.
11. Sato J, Nair K, Hiddinga J, et al. eNOS gene transfer to vascular smooth muscle cells inhibits cell proliferation via upregulation of p27 and p21 and not apoptosis. *Cardiovasc Res*. 2000;47(4):697–706.
12. Kibbe MR, Li J, Nie S, et al. Inducible nitric oxide synthase (iNOS) expression upregulates p21 and inhibits vascular smooth muscle cell proliferation through p42/44 mitogen-activated protein kinase activation and independent of p53 and cyclic guanosine monophosphate. *J Vasc Surg*. 2000;31(6):1214–1228.
13. Cooney R, Hynes SO, Duffy AM, Sharif F, O'Brien T. Adenoviral-mediated gene transfer of nitric oxide synthase isoforms and vascular cell proliferation. *J Vasc Res*. 2006;43(5):462–472.
14. Morral N, O'Neal W, Rice K, et al. Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc Natl Acad Sci U S A*. 1999;96(22):12816–12821.
15. Barcia C, Jimenez-Dalmaroni M, Kroeger KM, et al. One-year expression from high-capacity adenoviral vectors in the brains of animals with pre-existing anti-adenoviral immunity: clinical implications. *Mol Ther*. 2007;12:2154–2163.
16. Wickham TJ. Targeting adenovirus. *Gene Ther*. 2000;7(2):110–114.
17. Reynolds PN, Nicklin SA, Kaliberova L, et al. Combined transductional and transcriptional targeting improves the specificity of transgene expression in vivo. *Nat Biotechnol*. 2001;19(9):838–842.
18. Barnett BG, Tillman BW, Curiel DT, Douglas JT. Dual targeting of adenoviral vectors at the levels of transduction and transcription enhances the specificity of gene expression in cancer cells. *Mol Ther*. 2002;6(3):377–385.
19. Campeau L, Enjalbert M, Lesperance J, Vaislic C, Grondin CM, Bourassa MG. Atherosclerosis and late closure of aortocoronary saphenous vein grafts: sequential angiographic studies at 2 weeks, 1 year, 5 to 7 years, and 10 to 12 years after surgery. *Circulation*. 1983;68(3 pt 2):II1–II7.
20. Davies MG, Hagen PO. Pathobiology of intimal hyperplasia. *Br J Surg*. 1994;81(9):1254–1269.
21. George SJ, Lloyd CT, Angelini GD, Newby AC, Baker AH. Inhibition of late vein graft neointima formation in human and porcine models by adenovirus-mediated overexpression of tissue inhibitor of metalloproteinase-3. *Circulation*. 2000;101(3):296–304.
22. West NE, Qian H, Guzik TJ, et al. Nitric oxide synthase (nNOS) gene transfer modifies venous bypass graft remodeling: effects on vascular smooth muscle cell differentiation and superoxide production. *Circulation*. 2001;104(13):1526–1532.

23. Chowdhury JR, Grossman M, Gupta S, Chowdhury NR, Baker JR Jr, Wilson JM. Long-term improvement of hypercholesterolemia after ex vivo gene therapy in LDLR-deficient rabbits. *Science*. 1991;254(5039):1802–1805.
24. Grossman M, Rader DJ, Muller DW, et al. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. *Nat Med*. 1995;1(11):1148–1154.
25. Schwarz ER, Speakman MT, Patterson M, et al. Evaluation of the effects of intramyocardial injection of DNA expressing vascular endothelial growth factor (VEGF) in a myocardial infarction model in the rat – angiogenesis and angioma formation. *J Am Coll Cardiol*. 2000;35(5):1323–1330.
26. Sasano T, Kikuchi K, McDonald AD, Lai S, Donahue JK. Targeted high-efficiency, homogeneous myocardial gene transfer. *J Mol Cell Cardiol*. 2007;42(5):954–961.
27. Bridges CR, Burkman JM, Malekan R, et al. Global cardiac-specific transgene expression using cardiopulmonary bypass with cardiac isolation. *Ann Thorac Surg*. 2002;73(6):1939–1946.
28. Melo LG, Agrawal R, Zhang L, et al. Gene therapy strategy for long-term myocardial protection using adeno-associated virus-mediated delivery of heme oxygenase gene. *Circulation*. 2002;105(5):602–607.
29. Hedman M, Hartikainen J, Syvanne M, et al. Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT). *Circulation*. 2003;107(21):2677–2683.
30. O'Donnell JM, Lewandowski ED. Efficient, cardiac-specific adenoviral gene transfer in rat heart by isolated retrograde perfusion in vivo. *Gene Ther*. 2005;12(12):958–964.
31. Champion HC, Georgakopoulos D, Haldar S, Wang L, Wang Y, Kass DA. Robust adenoviral and adeno-associated viral gene transfer to the in vivo murine heart: application to study of phospholamban physiology. *Circulation*. 2003;108(22):2790–2797.
32. Ikeda Y, Gu Y, Iwanaga Y, et al. Restoration of deficient membrane proteins in the cardiomyopathic hamster by in vivo cardiac gene transfer. *Circulation*. 2002;105(4):502–508.
33. Huard J, Lochmuller H, Acsadi G, Jani A, Massie B, Karpati G. The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Ther*. 1995;2(2):107–115.
34. Mizuguchi H, Koizumi N, Hosono T, et al. CAR- or alphav integrin-binding ablated adenovirus vectors, but not fiber-modified vectors containing RGD peptide, do not change the systemic gene transfer properties in mice. *Gene Ther*. 2002;9(12):769–776.
35. Blankinship MJ, Gregorevic P, Allen JM, et al. Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. *Mol Ther*. 2004;10(4):671–678.
36. Pacak CA, Mah CS, Thattaliyath BD, et al. Recombinant adeno-associated virus serotype 9 leads to preferential cardiac transduction in vivo. *Circ Res*. 2006;99(4):e3–e9.
37. Lechardeur D, Sohn KJ, Haardt M, et al. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Ther*. 1999;6(4):482–497.
38. Johnson-Saliba M, Jans DA. Gene therapy: optimising DNA delivery to the nucleus. *Curr Drug Targets*. 2001;2(4):371–399.
39. Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther*. 2002;9(24):1647–1652.
40. Ogris M, Brunner S, Schuller S, Kircheis R, Wagner E. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Ther*. 1999;6(4):595–605.
41. Kursa M, Walker GF, Roessler V, et al. Novel shielded transferrin-polyethylene glycol-polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer. *Bioconjug Chem*. 2003;14(1):222–231.
42. Wright MJ, Wightman LM, Lilley C, et al. In vivo myocardial gene transfer: optimization, evaluation and direct comparison of gene transfer vectors. *Basic Res Cardiol*. 2001;96(3):227–236.
43. Roelvink PW, Mi Lee G, Einfeld DA, Kovesdi I, Wickham TJ. Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science*. 1999;286(5444):1568–1571.

44. Ding W, Zhang L, Yan Z, Engelhardt JF. Intracellular trafficking of adeno-associated viral vectors. *Gene Ther*. 2005;12(11):873–880.
45. McConnell MJ, Imperiale MJ. Biology of adenovirus and its use as a vector for gene therapy. *Hum Gene Ther*. 2004;15(11):1022–1033.
46. Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum Gene Ther*. 1997;8(1):37–44.
47. Cichon G, Boeckh-Herwig S, Schmidt HH, et al. Complement activation by recombinant adenoviruses. *Gene Ther*. 2001;8(23):1794–1800.
48. Muruve DA. The innate immune response to adenovirus vectors. *Hum Gene Ther*. 2004;15(12) :1157–1166.
49. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. *Nat Rev Genet*. 2003;4(5):346–358.
50. Hacein-Bey-Abina S, von Kalle C, Schmidt M, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med*. 2003;348(3):255–256.
51. Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. *Nat Med*. 2001;7(1):33–40.
52. Nabel EG, Plautz G, Nabel GJ. Site-specific gene expression in vivo by direct gene transfer into the arterial wall. *Science*. 1990;249(4974):1285–1288.
53. Verma IM, Somia N. Gene therapy – promises, problems and prospects. *Nature*. 1997;389(6648):239–242.
54. Young LS, Searle PF, Onion D, Mautner V. Viral gene therapy strategies: from basic science to clinical application. *J Pathol*. 2006;208(2):299–318.
55. Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol*. 1990;10(8):4239–4242.
56. Lewis P, Hensel M, Emerman M. Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J*. 1992;11(8):3053–3058.
57. Dishart KL, Denby L, George SJ, et al. Third-generation lentivirus vectors efficiently transduce and phenotypically modify vascular cells: implications for gene therapy. *J Mol Cell Cardiol*. 2003;35(7):739–748.
58. Tsui LV, Kelly M, Zayek N, et al. Production of human clotting Factor IX without toxicity in mice after vascular delivery of a lentiviral vector. *Nat Biotechnol*. 2002;20(1):53–57.
59. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science*. 2000;288(5466):669–672.
60. Couzin J, Kaiser J. Gene therapy. As Gelsinger case ends, gene therapy suffers another blow. *Science*. 2005;307(5712):1028.
61. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, et al. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science*. 2003;302(5644):415–419.
62. Cavazzana-Calvo M, Fischer A. Gene therapy for severe combined immunodeficiency: are we there yet? *J Clin Invest*. 2007;117(6):1456–1465.
63. Mitrophanous K, Yoon S, Rohll J, et al. Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther*. 1999;6(11):1808–1818.
64. Wong LF, Azzouz M, Walmsley LE, et al. Transduction patterns of pseudotyped lentiviral vectors in the nervous system. *Mol Ther*. 2004;9(1):101–111.
65. Kim VN, Mitrophanous K, Kingsman SM, Kingsman AJ. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J Virol*. 1998;72(1):811–816.
66. Fischer-Lougheed JY, Tarantal AF, Shulkin I, et al. Gene therapy to inhibit xenoantibody production using lentiviral vectors in non-human primates. *Gene Ther*. 2007;14(1):49–57.
67. Browning MT, Schmidt RD, Lew KA, Rizvi TA. Primate and feline lentivirus vector RNA packaging and propagation by heterologous lentivirus virions. *J Virol*. 2001;75(11):5129–5140.
68. Lin YL, Noel D, Mettling C, et al. Feline immunodeficiency virus vectors for efficient transduction of primary human synoviocytes: application to an original model of rheumatoid arthritis. *Hum Gene Ther*. 2004;15(6):588–596.

69. Takahashi K, Luo T, Saishin Y, et al. Sustained transduction of ocular cells with a bovine immunodeficiency viral vector. *Hum Gene Ther*. 2002;13(11):1305–1316.
70. Molina RP, Ye HQ, Brady J, et al. A synthetic Rev-independent bovine immunodeficiency virus-based packaging construct. *Hum Gene Ther*. 2004;15(9):865–877.
71. Zhang XY, La Russa VF, Bao L, Kolls J, Schwarzenberger P, Reiser J. Lentiviral vectors for sustained transgene expression in human bone marrow-derived stromal cells. *Mol Therapy*. 2002;5(5 pt 1):555–565.
72. Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*. 1996;93(21):11382–11388.
73. Abordo-Adesida E, Follenzi A, Barcia C, et al. Stability of lentiviral vector-mediated transgene expression in the brain in the presence of systemic antivector immune responses. *Human Gene Therapy*. 2005;16(6):741–751.
74. Azzouz M, Ralph GS, Storkebaum E, et al. VEGF delivery with retrogradely transported lentivector prolongs survival in a mouse ALS model. *Nature*. 2004;429(6990):413–417.
75. Uchida N, Sutton RE, Friera AM, et al. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 1998;95(20):11939–11944.
76. Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell*. 2000;101(2):173–185.
77. Zhao J, Pettigrew GJ, Thomas J, et al. Lentiviral vectors for delivery of genes into neonatal and adult ventricular cardiac myocytes in vitro and in vivo. *Basic Res Cardiol*. 2002;97(5): 348–358.
78. Diez-Freire C, Vazquez J, de Adjounian MF Correa, et al. ACE2 gene transfer attenuates hypertension-linked pathophysiological changes in the SHR. *Physiol Genomics*. 2006;27(1): 12–19.
79. Leavitt AD, Robles G, Alesandro N, Varmus HE. Human immunodeficiency virus type 1 integrase mutants retain in vitro integrase activity yet fail to integrate viral DNA efficiently during infection. *J Virol*. 1996;70(2):721–728.
80. Engelman A. In vivo analysis of retroviral integrase structure and function. *Adv Virus Res*. 1999;52:411–426.
81. Apolonia L, Waddington SN, Fernandes C, et al. Stable gene transfer to muscle using non-integrating lentiviral vectors. *Mol Ther*. 2007;15(11):1947–1954.
82. Yanez-Munoz RJ, Balaggan KS, MacNeil A, et al. Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med*. 2006;12(3):348–353.
83. Latchman DS. Gene delivery and gene therapy with herpes simplex virus-based vectors. *Gene*. 2001;264(1):1–9.
84. Jacobs A, Breakefield XO, Fraefel C. HSV-1-based vectors for gene therapy of neurological diseases and brain tumors: part I. HSV-1 structure, replication and pathogenesis. *Neoplasia*. 1999;1(5):387–401.
85. Burton EA, Glorioso JC, Fink DJ. Gene therapy progress and prospects: Parkinson's disease. *Gene Ther*. 2003;10(20):1721–1727.
86. Kirn D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: biological principles, risk management and future directions. *Nat Med*. 2001;7(7):781–787.
87. Han ZQ, Assenberg M, Liu BL, et al. Development of a second-generation oncolytic Herpes simplex virus expressing TNFalpha for cancer therapy. *J Gene Med*. 2007;9(2):99–106.
88. Liu BL, Robinson M, Han ZQ, et al. ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. *Gene Ther*. 2003;10(4):292–303.
89. Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med*. 1953;84(3):570–573.
90. Lemarchand P, Jaffe HA, Danel C, et al. Adenovirus-mediated transfer of a recombinant human alpha 1-antitrypsin cDNA to human endothelial cells. *Proc Natl Acad Sci U S A*. 1992;89(14):6482–6486.

91. Lemarchand P, Jones M, Yamada I, Crystal RG. In vivo gene transfer and expression in normal uninjured blood vessels using replication-deficient recombinant adenovirus vectors. *Circ Res*. 1993;72(5):1132–1138.
92. French BA, Mazur W, Geske RS, Bolli R. Direct in vivo gene transfer into porcine myocardium using replication-deficient adenoviral vectors. *Circulation*. 1994;90(5):2414–2424.
93. Berkner KL. Development of adenovirus vectors for the expression of heterologous genes. *Biotechniques*. 1988;6(7):616–629.
94. Gordon D, Reidy MA, Benditt EP, Schwartz SM. Cell proliferation in human coronary arteries. *Proc Natl Acad Sci U S A*. 1990;87(12):4600–4604.
95. Turunen P, Puhakka HL, Heikura T, et al. Extracellular superoxide dismutase with vaccinia virus anti-inflammatory protein 35K or tissue inhibitor of metalloproteinase-1: combination gene therapy in the treatment of vein graft stenosis in rabbits. *Hum Gene Ther*. 2006;17(4): 405–414.
96. Wen S, Schneider DB, Driscoll RM, Vassalli G, Sassani AB, Dichek DA. Second-generation adenoviral vectors do not prevent rapid loss of transgene expression and vector DNA from the arterial wall. *Arterioscler Thromb Vasc Biol*. 2000;20(6):1452–1458.
97. Yang Y, Li Q, Ertl HC, Wilson JM. Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol*. 1995;69(4):2004–2015.
98. Schiedner G, Morral N, Parks RJ, et al. Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat Genet*. 1998;18(2):180–183.
99. Biermann V, Volpers C, Hussmann S, et al. Targeting of high-capacity adenoviral vectors. *Hum Gene Ther*. 2001;12(14):1757–1769.
100. Dmitriev I, Krasnykh V, Miller CR, et al. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol*. 1998;72(12):9706–9713.
101. Haviv YS, Blackwell JL, Kanerva A, et al. Adenoviral gene therapy for renal cancer requires retargeting to alternative cellular receptors. *Cancer Res*. 2002;62(15):4273–4281.
102. Mizuguchi H, Kay MA, Hayakawa T. Approaches for generating recombinant adenovirus vectors. *Adv Drug Deliv Rev*. 2001;52(3):165–176.
103. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*. 1977;36(1):59–74.
104. Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A*. 1994;91(10):4407–4411.
105. Mitani K, Graham FL, Caskey CT, Kochanek S. Rescue, propagation, and partial purification of a helper virus-dependent adenovirus vector. *Proc Natl Acad Sci U S A*. 1995;92(9):3854–3858.
106. Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT. A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc Natl Acad Sci U S A*. 1996;93(12):5731–5736.
107. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A*. 1996;93(24):13565–13570.
108. Ng P, Beauchamp C, Evelegh C, Parks R, Graham FL. Development of a FLP/frt system for generating helper-dependent adenoviral vectors. *Mol Ther*. 2001;3(5 pt 1):809–815.
109. Bergelson JM, Cunningham JA, Droguett G, et al. Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*. 1997;275(5304):1320–1323.
110. Tomko RP, Xu R, Philipson L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci U S A*. 1997;94(7):3352–3356.
111. Wickham TJ, Mathias P, Cheresh DA, Nemerow GR. Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*. 1993;73(2):309–319.
112. Palomeque J, Chemaly ER, Colosi P, et al. Efficiency of eight different AAV serotypes in transducing rat myocardium in vivo. *Gene Ther*. 2007;14(13):989–997.

113. Ohno T, Gordon D, San H, et al. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. *Science*. 1994;265(5173):781–784.
114. Koeberl DD, Alexander IE, Halbert CL, Russell DW, Miller AD. Persistent expression of human clotting factor IX from mouse liver after intravenous injection of adeno-associated virus vectors. *Proc Natl Acad Sci U S A*. 1997;94(4):1426–1431.
115. Kirby I, Davison E, Beavil AJ, et al. Mutations in the DG loop of adenovirus type 5 fiber knob protein abolish high-affinity binding to its cellular receptor CAR. *J Virol*. 1999;73(11):9508–9514.
116. Jakubczak JL, Rollence ML, Stewart DA, et al. Adenovirus type 5 viral particles pseudotyped with mutagenized fiber proteins show diminished infectivity of coxsackie B-adenovirus receptor-bearing cells. *J Virol*. 2001;75(6):2972–2981.
117. Krasnykh VN, Mikheeva GV, Douglas JT, Curiel DT. Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J Virol*. 1996;70(10):6839–6846.
118. Denby L, Work LM, Graham D, et al. Adenoviral serotype 5 vectors pseudotyped with fibers from subgroup D show modified tropism in vitro and in vivo. *Hum Gene Ther*. 2004;15(11):1054–1064.
119. Denby L, Work LM, Seggern DJ, et al. Development of renal-targeted vectors through combined in vivo phage display and capsid engineering of adenoviral fibers from serotype 19p. *Mol Ther*. 2007;15(9):1647–1654.
120. Nettelbeck DM, Miller DW, Jerome V, et al. Targeting of adenovirus to endothelial cells by a bispecific single–chain diabody directed against the adenovirus fiber knob domain and human endoglin (CD105). *Mol Ther*. 2001;3(6):882–891.
121. Printz MA, Gonzalez AM, Cunningham M, et al. Fibroblast growth factor 2-retargeted adenoviral vectors exhibit a modified biolocalization pattern and display reduced toxicity relative to native adenoviral vectors. *Hum Gene Ther*. 2000;11(1):191–204.
122. Reynolds PN, Zinn KR, Gavrilyuk VD, et al. A targetable, injectable adenoviral vector for selective gene delivery to pulmonary endothelium in vivo. *Mol Ther*. 2000;2(6):562–578.
123. Krasnykh V, Dmitriev I, Mikheeva G, Miller R, Belousova N, Curiel DT. Characterisation of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J Virol*. 1998;72(3):1844–1852.
124. Dmitriev I, Krasnykh V, Miller CR, et al. An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilisation of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol*. 1998;72:9706–9713.
125. Vigne E, Mahfouz I, Dedieu JF, Brie A, Perricaudet M, Yeh P. RGD inclusion in the hexon monomer provides adenovirus type 5-based vectors with a fiber knob-independent pathway for infection. *J Virol*. 1999;73(6):5156–5161.
126. Campos SK, Barry MA. Comparison of adenovirus fiber, protein IX, and hexon capsomeres as scaffolds for vector purification and cell targeting. *Virology*. 2006;349(2):453–462.
127. Engelstadter M, Bobkova M, Baier M, et al. Targeting human T cells by retroviral vectors displaying antibody domains selected from a phage display library. *Hum Gene Ther*. 2000;11(2):293–303.
128. Grifman M, Trepel M, Speece P, et al. Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. *Mol Ther*. 2001;3(6):964–975.
129. Nicklin SA, Buening H, Dishart KL, et al. Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells. *Mol Ther*. 2001;4(3):174–181.
130. Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*. 1985;228(4705):1315–1317.
131. Johnsson K, Ge L. Phage display of combinatorial peptide and protein libraries and their applications in biology and chemistry. *Curr Top Microbiol Immunol*. 1999;243:87–105.
132. Arap W, Kolonin MG, Trepel M, et al. Steps toward mapping the human vasculature by phage display. *Nat Med*. 2002;8(2):121–127.
133. Krag DN, Shukla GS, Shen GP, et al. Selection of tumor-binding ligands in cancer patients with phage display libraries. *Cancer Res*. 2006;66(15):7724–7733.
134. Woo YJ, Zhang JC, Taylor MD, Cohen JE, Hsu VM, Sweeney HL. One year transgene expression with adeno-associated virus cardiac gene transfer. *Int J Cardiol*. 2005;100(3):421–426.

135. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol*. 1996;70(11):8098–8108.
136. Zaiss AK, Liu Q, Bowen GP, Wong NC, Bartlett JS, Muruve DA. Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. *J Virol*. 2002;76(9): 4580–4590.
137. Bessis N, GarciaCozar FJ, Boissier MC. Immune responses to gene therapy vectors: influence on vector function and effector mechanisms. *Gene Ther*. 2004;11(suppl 1):S10–S17.
138. Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol*. 1998;72(5):4212–4223.
139. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med*. 2006;12(3):342–347.
140. Vandenberghe LH, Wang L, Somanathan S, et al. Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. *Nat Med*. 2006;12(8):967–971.
141. Pachori AS, Melo LG, Zhang L, Loda M, Pratt RE, Dzau VJ. Potential for germ line transmission after intramyocardial gene delivery by adeno-associated virus. *Biochem Biophys Res Commun*. 2004;313(3):528–533.
142. Arruda VR, Fields PA, Milner R, et al. Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. *Mol Ther*. 2001;4(6):586–592.
143. Donsante A, Vogler C, Muzyczka N, et al. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Ther*. 2001;8(17):1343–1346.
144. Donsante A, Miller DG, Li Y, et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science*. 2007;317(5837):477.
145. Hermonat PL, Quirk JG, Bishop BM, Han L. The packaging capacity of adeno-associated virus (AAV) and the potential for wild-type-plus AAV gene therapy vectors. *FEBS Lett*. 1997;407(1):78–84.
146. Yan Z, Zhang Y, Duan D, Engelhardt JF. Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. *Proc Natl Acad Sci U S A*. 2000;97(12):6716–6721.
147. Cheung AK, Hoggan MD, Hauswirth WW, Berns KI. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. *J Virol*. 1980;33(2):739–748.
148. Kotin RM, Menninger JC, Ward DC, Berns KI. Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter. *Genomics*. 1991;10(3):831–834.
149. Berns KI, Pinkerton TC, Thomas GF, Hoggan MD. Detection of adeno-associated virus (AAV)-specific nucleotide sequences in DNA isolated from latently infected Detroit 6 cells. *Virology*. 1975;68(2):556–560.
150. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol*. 1998;72(3):2224–2232.
151. Grimm D, Kern A, Rittner K, Kleinschmidt JA. Novel tools for production and purification of recombinant adenoassociated virus vectors. *Hum Gene Ther*. 1998;9(18):2745–2760.
152. Douar AM, Poulard K, Stockholm D, Danos O. Intracellular trafficking of adeno-associated virus vectors: routing to the late endosomal compartment and proteasome degradation. *J Virol*. 2001;75(4):1824–1833.
153. Bartlett JS, Wilcher R, Samulski RJ. Infectious entry pathway of adeno-associated virus and adeno-associated virus vectors. *J Virol*. 2000;74(6):2777–2785.
154. Hansen J, Qing K, Srivastava A. Infection of purified nuclei by adeno-associated virus 2. *Mol Ther*. 2001;4(4):289–296.
155. Sanlioglu S, Benson PK, Yang J, Atkinson EM, Reynolds T, Engelhardt JF. Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. *J Virol*. 2000;74(19):9184–9196.
156. Gao G, Vandenberghe LH, Alvira MR, et al. Clades of adeno-associated viruses are widely disseminated in human tissues. *J Virol*. 2004;78(12):6381–6388.

157. Gao G, Alvira MR, Somanathan S, et al. Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc Natl Acad Sci U S A*. 2003;100(10):6081–6086.
158. Rabinowitz JE, Rolling F, Li C, et al. Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol*. 2002;76(2):791–801.
159. Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol*. 1998;72(2):1438–1445.
160. Summerford C, Bartlett JS, Samulski RJ. AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med*. 1999;5(1):78–82.
161. Qing K, Mah C, Hansen J, Zhou S, Dwarki V, Srivastava A. Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat Med.* 1999;5(1):71–77.
162. Blackburn SD, Steadman RA, Johnson FB. Attachment of adeno-associated virus type 3H to fibroblast growth factor receptor 1. *Arch Virol*. 2006;151(3):617–623.
163. Wu Z, Miller E, Agbandje-McKenna M, Samulski RJ. Alpha2, 3 and alpha2, 6 N-linked sialic acids facilitate efficient binding and transduction by adeno-associated virus types 1 and 6. *J Virol*. 2006;80(18):9093–9103.
164. Di Pasquale G, Davidson BL, Stein CS, et al. Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med*. 2003;9(10):1306–1312.
165. Walters RW, Yi SM, Keshavjee S, et al. Binding of adeno-associated virus type 5 to 2, 3-linked sialic acid is required for gene transfer. *J Biol Chem*. 2001;276(23):20610–20616.
166. Kaludov N, Brown KE, Walters RW, Zabner J, Chiorini JA. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J Virol*. 2001;75(15):6884–6893.
167. Akache B, Grimm D, Pandey K, Yant SR, Xu H, Kay MA. The 37/67-kilodalton laminin receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *J Virol*. 2006;80(19):9831–9836.
168. Nicklin SA, Von Seggern DJ, Work LM, et al. Ablating adenovirus type 5 fiber-CAR binding and HI loop insertion of the SIGYPLP peptide generate an endothelial cell-selective adenovirus. *Mol Ther*. 2001;4(6):534–542.
169. Halbert CL, Allen JM, Miller AD. Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *J Virol*. 2001;75(14):6615–6624.
170. Du L, Kido M, Lee DV, et al. Differential myocardial gene delivery by recombinant serotype-specific adeno-associated viral vectors. *Mol Ther*. 2004;10(3):604–608.
171. Bartlett JS, Kleinschmidt J, Boucher RC, Samulski RJ. Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab′gamma)2 antibody. *Nat Biotechnol*. 1999;17(2):181–186.
172. Wu P, Xiao W, Conlon T, et al. Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol*. 2000;74(18):8635–8647.
173. Ried MU, Girod A, Leike K, Buning H, Hallek M. Adeno-associated virus capsids displaying immunoglobulin-binding domains permit antibody-mediated vector retargeting to specific cell surface receptors. *J Virol*. 2002;76(9):4559–4566.
174. Girod A, Ried M, Wobus C, et al. Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med*. 1999;5(9):1052–1056.
175. Shi W, Arnold GS, Bartlett JS. Insertional mutagenesis of the adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors targeted to alternative cell-surface receptors. *Hum Gene Ther*. 2001;12(14):1697–1711.
176. Muller OJ, Kaul F, Weitzman MD, et al. Random peptide libraries displayed on adeno-associated virus to select for targeted gene therapy vectors. *Nat Biotechnol*. 2003;21(9):1040–1046.
177. Perabo L, Buning H, Kofler DM, et al. In vitro selection of viral vectors with modified tropism: the adeno-associated virus display. *Mol Ther*. 2003;8(1):151–157.
178. Xiao W, Chirmule N, Berta SC, McCullough B, Gao G, Wilson JM. Gene therapy vectors based on adeno-associated virus type 1. *J Virol*. 1999;73(5):3994–4003.

179. Hauck B, Xiao W. Characterization of tissue tropism determinants of adeno-associated virus type 1. *J Virol*. 2003;77(4):2768–2774.
180. Liu Y, Okada T, Sheykholeslami K, et al. Specific and efficient transduction of Cochlear inner hair cells with recombinant adeno-associated virus type 3 vector. *Mol Ther*. 2005;12(4): 725–733.
181. Davidson BL, Stein CS, Heth JA, et al. Recombinant adeno-associated virus type 2, 4, and 5 vectors: transduction of variant cell types and regions in the mammalian central nervous system. *Proc Natl Acad Sci U S A*. 2000;97(7):3428–3432.
182. Alisky JM, Hughes SM, Sauter SL, et al. Transduction of murine cerebellar neurons with recombinant FIV and AAV5 vectors. *Neuroreport*. 2000;11(12):2669–2673.
183. Xin KQ, Mizukami H, Urabe M, et al. Induction of robust immune responses against human immunodeficiency virus is supported by the inherent tropism of adeno-associated virus type 5 for dendritic cells. *J Virol*. 2006;80(24):11899–11910.
184. Gao GP, Alvira MR, Wang L, Calcedo R, Johnston J, Wilson JM. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci U S A*. 2002;99(18):11854–11859.
185. Mori S, Wang L, Takeuchi T, Kanda T. Two novel adeno-associated viruses from cynomolgus monkey: pseudotyping characterization of capsid protein. *Virology*. 2004;330(2):375–383.
186. Alba R, Bosch A, Chillon M. Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Ther*. 2005;12(suppl 1):S18–S27.