Adeno-Associated Virus Vectors as Therapeutic and Investigational Tools in the Cardiovascular System

Serena Zacchigna, Lorena Zentilin, Mauro Giacca

Abstract: The use of vectors based on the small parvovirus adeno-associated virus has gained significant momentum during the past decade. Their high efficiency of transduction of postmitotic tissues in vivo, such as heart, brain, and retina, renders these vectors extremely attractive for several gene therapy applications affecting these organs. Besides functional correction of different monogenic diseases, the possibility to drive efficient and persistent transgene expression in the heart offers the possibility to develop innovative therapies for prevalent conditions, such as ischemic cardiomyopathy and heart failure. Therapeutic genes are not only restricted to protein-coding complementary DNAs but also include short hairpin RNAs and microRNA genes, thus broadening the spectrum of possible applications. In addition, several spontaneous or engineered variants in the virus capsid have recently improved vector efficiency and expanded their tropism. Apart from their therapeutic potential, adeno-associated virus vectors also represent outstanding investigational tools to explore the function of individual genes or gene combinations in vivo, thus providing information that is conceptually similar to that obtained from genetically modified animals. Finally, their single-stranded DNA genome can drive homology-directed gene repair at high efficiency. Here, we review the main molecular characteristics of adeno-associated virus vectors, with a particular view to their applications in the cardiovascular field. (Circ Res. 2014;114:1827-1846.)

Key Words: gene targeting ■ gene therapy ■ viruses

While celebrating the 25th anniversary of its first clinical trial,1 gene therapy is slowly but steadily progressing toward clinical success. The recent achievements in the treatment of several monogenic conditions have awakened interest for a field that has been slow to fulfill its original promises. Yet progress is still to come in relation to the most highly prevalent conditions, including cancer and cardiovascular disorders. For instance, despite the first clinical attempts to induce therapeutic angiogenesis date back to 1994,2 20 years and multiple additional trials in different countries on, we still do not have any reproducible gene therapy medicine for conditions as prevalent as myocardial and peripheral ischemia.

A vast part of the problems so far encountered by most gene therapy applications relate to the intrinsic difficulty of delivering polyanions, such as DNA or RNA, across the cell membranes, consisting of nonpolar, hydrophobic phospholipids. This justifies the use of viral vectors in the majority of gene therapy clinical trials conducted to date. It is in this context that vectors based on the small, defective, and nonpathogenic human parvovirus adeno-associated virus (AAV) have gained increasing popularity in the gene therapy community during the past 2 decades.

AAV is a small, nonpathogenic human virus, belonging to the Parvovirus family, which was originally discovered in the mid-1960s as a contaminant of a cell culture also infected with adenovirus.3,4 After its discovery, it soon became evident that the virus is a ubiquitous commensal of mammals,5 displaying a rather peculiar life cycle: for productive replication to occur, AAV requires a series of still incompletely characterized cellular functions that are triggered by the concomitant infection of the host cell with another virus (typically, adenovirus or herpesvirus) or by cell treatment with chemical or physical agents exerting genotoxic activity.5,7 In the absence of this host cell helper function, the wild-type AAV genome establishes a latent infection by integrating into a specific sequence of human chromosome 19q13.3,8–10 similar to the lambda phage in bacteria. Thus, AAV asserts itself as the only mammalian DNA virus capable of site-specific integration.

Growing interest in AAV molecular biology started in the mid-1980s, fostered by progressive recognition that the virus might be used as a gene delivery vector.11,12 AAV vectors, which are commonly devoid of all viral genes, are unable to integrate into the host genome, neither site-specifically nor in a random manner, but still retain the infectious properties of their wild-type counterpart. Among their attractive characteristics are their genomic simplicity, possibility of generating high-titer vector preparations, lack of inflammatory response once these vectors are injected in vivo, and, most notably, their...
capacity to deliver genes into postmitotic cells and to drive persistent expression of the encoded genes, coinciding virtually for the whole life of the organism. Figure 1A reports a summary of >90 clinical trials that have been performed to date using AAV vectors. A product tested in 3 of these trials—an AAV1 vector coding for the lipoprotein lipase (LPL), to be used by intramuscular injection in patients with familial LPL deficiency—has been the first ever gene medicinal product approved for marketing in the Western world, authorized by the European Commission in 2012 under the commercial name of Glybera (http://ec.europa.eu/health/documents/community-register/html/o194.htm).

Currently, a single clinical trial using an AAV vector, aimed at correcting Leber congenital amaurosis by the intraocular administration of the RPE65 gene, has reached phase III. Another trial, aimed at the treatment of heart failure by overexpressing the sarcoplasmic reticulum Ca2+-ATPase (SERCA2a) pump,13 is now in phase IIb and represents the first and still unique AAV clinical experimentation in the cardiovascular field (Figure 1B).

Besides offering novel therapeutic opportunities, AAV vectors can also be envisaged as valuable tools for investigating the cardiovascular system. In particular, the property of these vectors to transduce the heart efficiently and to drive transgene expression in this organ for long periods of time in the absence of inflammation or immune response permits in vivo phenotypic assessment of gene function. This type of information is conceptually similar to that obtained by using transgenic mice, in the case of vectors carrying protein-coding complementary DNAs (cDNAs) or microRNAs, or knockout mice, when the vector is used to express a short hairpin RNA (shRNA) targeting a specific gene. Compared with genetically modified mice, however, AAV vectors are much easier and quicker to produce and offer the possibility to analyze the combined effect of multiple factors by simply mixing different vector preparations, because transduction usually occurs at a high multiplicity of virions per cell. Importantly, AAV vectors allow for the extension of in vivo disease modeling to animal species different from rodents and for which genetic modification remains impractical, such as pigs, dogs, and non-human primates.

Here, we review the properties of AAV vectors, especially in light of their use in the cardiovascular system, for both therapeutic and investigational purposes.

### Biology of AAV Vectors in the Cardiovascular System

The AAV virion is a simple particle of 18 to 25 nm, consisting of a linear, 4.7-kb single-stranded DNA genome, packed into an icosahedral capsid composed of 60 proteins. The genome contains 3 open reading frames: rep and cap, coding for the replicative proteins and the viral capsid, respectively,14 and AAP, which is nested within the cap gene and codes for a recently discovered protein assisting virion assembly.15 These genes are flanked by two 145-bp inverted terminal repeats, which form a T-shaped hairpin at both ends (Figure 2A). These palindromic sequences are the only cis-acting elements required for the major functions of AAV (viral DNA replication, assembly of the viral particles, integration/excision from the host genome) and the only portion of the original genome maintained in recombinant vectors.16

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<th>Nonstandard Abbreviations and Acronyms</th>
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<td>AAV</td>
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<tr>
<td>cDNA</td>
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<td>scAAV</td>
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<td>SERCA2a</td>
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<td>shRNA</td>
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<td>VEGF</td>
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<td>VLDL</td>
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Figure 1. Adeno-associated virus (AAV) clinical trials. The clinical trials that have used AAV vectors (>90) are presented as a percentage according to the phase of experimentation (A) and of disease treated (B). Data are from http://www.abedia.com/wiley/index.html.
The structure of a recombinant AAV vector backbone is straightforward: it consists of a transgene cassette, <4.5 kb in length, cloned between the viral inverted terminal repeats. This cassette could correspond to a protein-coding cDNA controlled by a polymerase II promoter or to a shRNA, expressed from a polymerase III promoter (Figure 2B and 2C, respectively). Once cloned into a plasmid, such constructs are able to generate infectious particles in the presence of other factors that are provided in trans. These factors comprise the AAV Rep and Cap proteins and additional helper proteins that are usually the products of a few adenovirus or herpes virus genes, which are cotransfected with the AAV vector plasmid into the packaging cells.

The replicating AAV genomes are packaged into preformed empty capsids within the nucleus of the producer cells, and viral particles are then harvested and purified from the cell homogenate by standard biochemical procedures (Figure 2D).

The most established method for AAV production, which is widely used in research core facilities, is based on the transient cotransfection of the vector and helper plasmids in adherent HEK293 cells. Production titers within 1×10⁴ and 1×10⁵ AAV particles per cell (as measured by quantifying the number of viral genomes) represent current, consistent, and probably limiting values, still amenable to scaling-up to a certain extent by increasing the number of cultured cells grown on roller bottles or on cell factory stacks. An alternative procedure, which has gained significant momentum during the past few years, is based on a baculovirus expression vector system in nonadherent insect cells grown in bioreactors, with significant methodological simplification. This system seems stable and particularly useful for scaling-up the production of clinical-grade vectors.

**Tissue Tropism and Persistence of AAV Vectors in the Cardiovascular System**

One of the striking characteristics of AAV vectors is their property to be largely ineffective in several cultured, replicating, primary cells and cell lines in vitro while infecting tissues consisting of noncycling cells at high efficiency in vivo. These
include the heart (cardiomyocytes), skeletal muscle (skeletal myofibers), brain (neurons), retina (photoreceptors, retinal pigment epithelium, and ganglionar cells), and, to a lesser extent, liver (hepatocytes) and pancreas (both β-cells and acinar cells). The identification of naturally occurring AAV serotypes, carrying variations in the amino acid sequence of the virion capsid protein, has enabled the development of vectors with improved efficiency in some of these tissues. To date, 13 distinct human or nonhuman primate AAV serotypes have been isolated. The primary receptors for 8 of these serotypes have been identified so far. Whereas the prototype AAV2, as well as AAV3 and AAV13, recognizes heparan sulfate proteoglycans, AAV1, AAV4, AAV5, and AAV6 bind N-linked or O-linked sialic acids and AAV9 cell surface terminal galactose residues. A variety of serotype-specific coreceptors further increases the efficiency of these vectors in specific cell types.

Some serotypes seem to possess the capacity to cross or bypass certain anatomic barriers, including the vascular endothelium. Intraperitoneal or intravenous injections of AAV8 and AAV9 in neonatal and, at higher doses, in adult mice permit widespread transduction throughout the body, especially in the heart and skeletal muscle. Figure 3 shows extensive cardiac transduction after intraperitoneal injection of AAV9 vectors in P7 mice. AAV9 is also able to cross the blood–brain barrier and thus target cells in the central nervous system with relatively high efficiency; some serotypes were also reported to be retrogradely transported from the muscle to spinal cord motor neurons. Together, these observations might pave the way to possible applications of AAV vectors in the therapy of various neurodegenerative conditions.

Several published studies have comparatively analyzed the specific tropism of the known AAV serotypes in different tissues in vivo. As far as cardiac transduction is concerned, AAV9 stands as the most cardiotropic serotype in gene transfer studies in rodents. However, AAV1, AAV6 (which differs from AAV1 by only 6 amino acids in the VP1 capsid protein, 5 of which are also present in VP2 and VP3), and AAV8 were also reported to transduce cardiomyocytes at high efficiency. The performance of a given serotype greatly depends on the route of administration: whereas AAV1 and AAV6 seem more suitable for cardiac gene transfer through intramyocardial, intrapericardial, or intracoronary routes, AAV8 and AAV9 can also achieve efficient cardiac transduction when administered systemically.

Although the availability of these serotype variants has improved the relative efficiency of AAV vectors in specific, susceptible cell types, postmitotic cells still remain the preferred targets for these vectors in vivo. The molecular determinants of this peculiar tropism are still not completely clear. Given the widespread distribution of cell surface receptors, the main factors regulating AAV transduction may reasonably coincide with steps after vector internalization. Among these, one critical determinant restricting cellular permissivity is recognition of the incoming, single-stranded DNA AAV genome as a sort of damaged DNA by the cellular DNA damage response proteins. Work from our laboratory has indeed demonstrated that interaction between AAV genomes and members of the MRN (Mre11, Rad50, Nbs1) complex limits transduction efficiency. Exit from the cell cycle and terminal cell differentiation, as it occurs in skeletal muscle fibers and cardiomyocytes, determines a parallel downregulation of DNA damage response proteins and consequent release from the inhibition they impose.

Table 1 reports critical appraisal of the advantages and disadvantages of the different AAV serotypes for cardiovascular gene transfer. One of the most attractive features of AAV vectors for skeletal and cardiac muscle gene transfer is the ability of these vectors to persist over time and sustain long-term expression of their transgenes. This property has been amply documented in various experimental models for skeletal muscle, and liver, and brain. In humans, analysis of autopic tissues from a patient who was enrolled in a gene therapy trial for hemophilia B and died because of unrelated causes revealed that the AAV2-encoded transgene remained active in the skeletal muscle for 10 years after injection.

Contrary to wild-type AAV, and in the absence of the viral Rep proteins, recombinant AAV vectors do not integrate into the host genome. Rather, in infected cells, the viral genomes persist as extrachromosomal double-stranded DNA (dsDNA) in the form of both linear and supercoiled circular monomers and concatamers, which are most likely formed by random end-joining or homologous recombination (HR). In long-lived, nondividing cells, these molecules, which are organized in a chromatin conformation, are able to sustain expression indefinitely.

**Figure 3. Cardiac transduction after systemic injection of adeno-associated virus (AAV) vectors.** Cardiac expression of Zoanthus green fluorescent protein (ZsGreen) after intraperitoneal injection of AAV9-ZsGreen (5×10¹¹ vector genomes per mouse) in P7 pups (left). Images were taken at 2 magnifications at 3 months. Quantification of the viral DNA in these mice indicated the presence of n=1×10⁹ viral genomes per heart. Scale bars, 100 μm. Green fluorescent protein expression in Z/EG mice (carrying a floxed GFP cassette) at 1 month after intracardiac injection of AAV9-Cre (5×10¹¹ vector genomes per heart) in adult mice (right). Scale bars, 100 μm.
Self-Complementary AAV Vectors

Several studies have established that the conversion of the AAV single-stranded DNA genome into dsDNA, by second strand synthesis or strand annealing, is a rate-limiting step for the onset of AAV-mediated transgene expression. This problem can be circumvented by the self-complementary AAV (scAAV) vector technology. This takes advantage of the property of the intermediate dimeric DNA molecular species formed during AAV DNA replication to fold into a self-complementary dsDNA molecule. Small AAV genomes, less than half the size of wild-type AAV (≈2.5 kb), are efficiently packaged as dimeric inverted repeats and give rise to scAAVs, which can be purified from mixed populations of particles containing monomeric genomes. scAAVs show increased efficiency and faster kinetics of transduction in cell lines in vitro and in several tissues in vivo, including heart and skeletal muscle. However, scAAV vectors seem to show the greatest efficiency compared with ssAAVs in experimental settings in which infection occurs at low multiplicity of vectors per cell. On the contrary, when a large number of vectors enter the same cells, single-stranded DNA to dsDNA conversion of conventional vectors occurs efficiently by strand annealing, and thus the comparative performance of scAAV is significantly lower. Another fact that reduces scAAV performance is that their dsDNA genomes still carry hairpinned inverted terminal repeats and are thus recognized by the same cellular DNA damage response proteins that restrict transduction with conventional AAV vectors. Because of size constrains, scAAVs seem especially useful for the expression of small genes, including shRNAs and microRNAs.

Inflammatory and Immune Response to AAV Vectors

Given their structural simplicity and lack of viral proteins encoded by their genome, recombinant AAV vectors are poorly immunogenic, especially when compared with other viral vectors such as adenoviruses. However, there is evidence that both innate and adaptive immunity, to a different extent, can limit viral transduction. As far as the former is concerned, both the vector DNA itself and the transducing virions can, in principle, be detected by the Toll-like receptor and other pattern recognition receptors. AAV vector genomes depleted from the CpG sequences, which bind Toll-like receptor-9, were in fact shown to avoid innate immune recognition and to improve skeletal muscle transduction.

The role of adaptive responses in determining the efficiency and duration of AAV transduction in patients seems far more relevant. Although preclinical animal studies had not indicated a significant immune response against these vectors, experience from clinical trials in humans showed clearly that AAV virions cannot completely hide the immune system and that both pre-existing antibodies and cytotoxic T-cell effectors may impair long-term efficacy of gene therapy. Neutralizing antibodies recognizing AAV2 and, to a lesser extent, other serotypes are found naturally in a large proportion of individuals (≈70%). A cytotoxic, CD8+ T-cell response, possibly elicited by degraded and myosin heavy chain-I–exposed capsid antigens, was also reported in a clinical study for hemophilia B. In this study, which entailed liver transduction with an AAV2 vector, the immune response contributed to the clearance of

Table 1. Critical Appraisal of the Advantages and Disadvantages of Different AAV Vector Serotypes for Cardiac Gene Transfer

<table>
<thead>
<tr>
<th>AAV serotypes used for cardiac gene transfer</th>
<th>Pros</th>
<th>Cons</th>
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<tr>
<td>All AAV vectors</td>
<td>Broad receptor/coreceptor expression</td>
<td>Limited cloning capacity</td>
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<td></td>
<td>Long-term persistence in muscle cells as episomes</td>
<td>High multiplicity of infection requirement for efficient transduction</td>
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<td></td>
<td>Low risk of mutagenesis/carcinogenesis</td>
<td>Limited efficiency in poorly differentiated cells</td>
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<td></td>
<td>Relatively weak innate and acquired immune response</td>
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<tr>
<td>AAV1</td>
<td>High transduction efficiency by intramuscular injection</td>
<td>Poor diffusion</td>
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<tr>
<td></td>
<td>Transduction only in the vicinity of the injection site</td>
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<tr>
<td></td>
<td>Not suitable for systemic injection</td>
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<tr>
<td></td>
<td>Tropism variability among different species</td>
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<tr>
<td>AAV2</td>
<td>Moderate efficiency</td>
<td>Poor diffusion</td>
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<tr>
<td>AAV6</td>
<td>High transduction efficiency by intramuscular injection</td>
<td>High prevalence of neutralizing antibodies (≈70%)</td>
</tr>
<tr>
<td></td>
<td>Poor diffusion</td>
<td>Not suitable for systemic injection</td>
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<tr>
<td></td>
<td>Tropism variability among different species</td>
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<tr>
<td>AAV8</td>
<td>High transduction efficiency by systemic delivery</td>
<td>Broad biodistribution in off-target organs after systemic administration</td>
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<td></td>
<td>Lowest incidence of pre-existing immunity (22–38% of neutralizing antibodies)</td>
<td></td>
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<tr>
<td>AAV9</td>
<td>High transduction efficiency by systemic delivery</td>
<td>Tropism variability among different species</td>
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AAV indicates adeno-associated virus.
transduced cells and consequent short-lived transgene expression. A subsequent clinical effort using an AAV8 vector with a self-complementary genome and expressing factor IX from a hepatocyte-specific promoter also elicited T-cell responses against the capsid and, in some patients, determined an elevation of aminotransferases accompanied by a partial loss of transgene expression; this was rapidly resolved by a short course of glucocorticoid therapy, without loss of transgene expression. The largely unsuccessful attempts to establish an animal model that duplicates these human findings still leave the debate open about the significance of this anticapsid effect.

A transient cellular response and a stable humoral immune response against the AAV1 capsid were also detected in patients injected intramuscularly with an AAV1-expressing LPL, despite preventive pharmacological immune suppression; in this case, however, the transgene was nonetheless successfully expressed for a long time without adverse clinical events. Several cells infiltrating the transduced muscles displayed phenotypic markers of regulatory T cells, which might have suppressed antisuicide cytotoxic T lymphocyte activity and contributed to long-term maintenance of transgene expression.

To avoid pre-existing immune recognition, in more recent clinical studies with AAV vectors, preventive strategies have been adopted by excluding patients with detectable neutralizing antibody titers or including mild immunosuppressive pharmacological regimens. In the Calcium Upregulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID) trial for gene therapy of heart failure, only subjects with a neutralizing antibody titer against AAV1 capsid <1:2 were enrolled in the study.

Apart from preventive immunosuppression, other strategies can be conceived to avoid immune reactivity against AAV vectors. A widely investigated approach involves the modification of capsid immunogenic epitopes by either random mutagenesis or rational design, also by including epitopes specifically activating suppressive T regulatory cells. Another possibility involves the use of empty capsids as decoys to adsorb anti-AAV antibodies, although increasing the viral particle load may increase the cytotoxic T lymphocyte response.

Despite these caveats, it must be emphasized that, to date, no evidence of acute clinical response or appearance of unwanted events that might be attributed to immune system activation by AAV vectors has been recorded in any of the several hundred subjects enrolled in the clinical trials conducted with these vectors.

**In Vivo Cardiac Expression of Small RNAs Using AAV Vectors**

The prolonged persistence of AAV vectors in vivo and their relative genetic simplicity render these vectors interesting tools for the expression of short regulatory RNAs. Specific target gene silencing is usually achieved by polymerase III–directed transcription of shRNAs of 60 to 70 bp, directed against the gene of interest, which then enter the endogenous microRNA pathway to be processed by Dicer to form mature, 21- to 23-bp small interfering RNAs. The AAV vector backbone provides sufficient space to accommodate even multiple shRNA expression cassettes, for instance, 3 to 4 copies of the H1 or U6 promoter. shRNAs can also be expressed using polymerase II promoters. Albeit less efficient, these promoters offer the possibility to obtain tissue-specific or inducible expression and to avoid the risk of saturating the RNA interference pathway, with possible side effects.

In the cardiovascular field, particular efforts have been made in the development of AAV carrying shRNAs targeting genes that regulate the cardiac Ca2⁺ cycle. In a rat model of heart failure, a scAAV9 encoding an shRNA against phospholamban was able to restore cardiac function and reduce pathological hypertrophy and dilation after systemic administration. Beneficial effects were also observed by targeting the phospholamban-associated protein phosphatase 1β in a mouse model of dilated cardiomyopathy; in this study, the shRNA was used to silence the inducible brain natriuretic peptide promoter, and its expression, therefore, increased along with disease progression. Another interesting study used an AAV9 vector carrying an shRNA against the coxsackievirus RNA polymerase to protect the heart against virus-induced myocarditis. Finally, AAV vectors expressing shRNAs are also serving as precious tools to dissect disease mechanisms. For instance, it is well established that estrogens protect women from hypertension although the specific receptor mediating this effect remained undetermined for years. Using selective estrogen receptor agonists and recombinant AAV vectors carrying shRNAs to silence either estrogen receptor α or β, a recent study rather elegantly showed that estrogen receptor β in the paraventricular nucleus and rostroventrolateral medulla is essential to prevent aldosterone/salt-induced hypertension in female rats. Besides opening new avenues for the treatment of systemic hypertension in general, these results also hold important implications for the design of effective and safe estrogen replacement therapies.

AAV vectors can also be used to overexpress microRNAs. In this case, the microRNA gene sequence is usually expressed under the control of constitutive or tissue-specific promoters. Previous work from our laboratory has shown that AAV9 vectors expressing the precursor DNAs for human microRNA-590-3p and microRNA-199-3p, once injected into the heart, trigger cardiomyocyte proliferation and cardiac regeneration after myocardial infarction. Other reports indicate that AAV9–microRNA378 attenuates cardiac hypertrophy after pressure overload because of thoracic aortic constriction and that AAV9–microRNA-669a alleviates chronic dilated cardiomyopathy in dystrophic mice.

**AAV Vectors With Cardiac-Specific or Inducible Promoters**

Efficient in vivo transduction and long-term transgene expression in susceptible tissues are hallmarks of AAV-mediated gene transfer. Although this is a highly desirable property in several circumstances, it becomes disadvantageous when transgene expression is exclusively sought in an organ (eg, in the heart but not in the liver) or when its prolonged expression becomes deleterious (eg, in the case of angiogenic factors).

A feasible strategy to confine the expression of a transferred gene to the tissue of choice is by transcriptional targeting by...
the inclusion of tissue-specific promoters. Unfortunately, heart-specific promoters often comprise genetic elements spread over a genomic distance that exceeds the packaging capability of AAV vectors. Therefore, promoter choice is often a compromise between cardiac specificity and transcription efficiency. The promoters considered so far to achieve restricted transgene expression in cardiomyocytes include those of the α-myosin heavy chain, the ventricular form of myosin light chain 2, and the cardiac troponin C genes. Heterologous fusion of myosin light chain 2 promoter sequences, which is reported to be highly specific for the heart during postnatal life, with the cytomegalovirus enhancer was also shown to drive predominant cardiac expression in adult mice and in a pig model of heart failure. Other regulatory cassettes drive prevalent expression in both skeletal and cardiac muscles and seem useful for gene transfer in diseases such as Duchenne muscular dystrophy, which affects both tissues.

When the diffusible AAV9 and AAV8 vectors are used systemically, they not only transduce skeletal muscle and heart, but also the liver. Of interest, one of the most commonly used constitutive promoters, the cytomegalovirus immediate early promoter, is rapidly silenced in the liver by DNA methylation, thus limiting expression to muscle tissues. A more specific strategy to achieve selective transgene silencing in the liver is the incorporation of microRNA-122 target sites into the transgene transcript 3′ untranslated region, because this microRNA is specifically expressed in hepatocytes. Such a tissue-specific microRNA-based detargeting strategy can be used to silence transgene expression in the skeletal muscle in addition to the liver, to achieve relatively pure cardiac specificity.

Different types of inducible transcription systems, in which transgene expression is modulated by small molecules, have been engineered into the AAV backbone, essentially by adapting the systems broadly used in other vector platforms. Currently, the 2 most considered methods to achieve pharmacologically inducible transcription are the tetracycline-inducible (TetOn) or tetracycline-repressible (TetOff) systems and the method based on rapamycin-induced dimerization of FKBP12 (FK506 binding protein 12)- and mammalian target of rapamycin–derived proteins. In the former case, regulation is based on 2 transcriptional units, one coding for the tetracycline regulator under the control of a constitutive promoter and the other containing the transgene downstream of a tetracycline-binding promoter (Figure 4A and 4B). In the case of rapamycin-inducible promoters, the system is composed of 3 transcriptional units, 2 coding for the 2 factors undergoing dimerization in the presence of the antibiotic and another containing the transgene with an upstream promoter controlled by the dimer (Figure 4C). In both cases, therefore, multiple transcriptional cassettes are needed to achieve regulation, which poses a problem of design when dealing with the relatively small AAV vector genome. This problem can be solved either by engineering 2 transcriptional units within the same vector or, more frequently, by the codelivery of 2 vectors, one containing the regulated transgene and the other coding for the regulator(s). Cardiac tissue specificity can then be achieved by expressing the regulators under the control of a cardiac-specific promoter.

Common experience indicates that, often in contrast with the results obtained with plasmids or lentiviral vectors, the abovedescribed pharmacologically inducible system works satisfactorily when in the context of AAV vectors, probably because the viral genomes remain episomal inside the nucleus, thus escaping the epigenetic influence imparted by host cell chromatin at the integration locus. However, the need for multiple expression cassettes or viruses renders these inducible systems rather cumbersome for clinical application. In addition, in contrast to rodents, the tetracycline regulator has been proven immunogenic in nonhuman primates. For these reasons, the use of these regulatable systems has so far been confined to preclinical applications.

Application of AAV Vectors for Cardiovascular Disorders

We next consider a series of in vivo gene transfer applications using AAV vectors that were aimed at either treating a pathological condition (gene therapy in preclinical models of human disease) or exploiting the properties of AAVs for biological investigation in the cardiovascular system. The examples reported are paradigmatic, albeit, inevitably, not exhaustive. The more advanced applications are summarized in Table 2.

Cardiac Delivery Routes

A reliable and clinically applicable delivery system is an obvious prerequisite for effective cardiac gene therapy. Some AAV serotypes have been shown to possess high cardiac tropism after intravenous administration in mice; however, these results are more difficult to reproduce in larger animals. Furthermore, the dose of vector required to transduce the human heart by systemic intravenous injection would likely be prohibitive, or certainly impractical.

The simplest and most obvious delivery method is that of direct injection into the cardiac muscle. Direct, transepicardial injection of vector after left thoracotomy allows delivery throughout the left ventricular free wall but is highly invasive and cannot target the interventricular septum. Percutaneous myocardial gene transfer into normal and ischemic myocardium has been achieved through endocardial delivery using electromechanical mapping for 3-dimensional image reconstruction of the ventricles. Promising preclinical and phase I/II results were obtained using this system to guide transendocardial injections of either plasmid DNA or adenoviral vectors via a percutaneous injection catheter. The same method has recently allowed efficient scAAV6 delivery to the canine heart under fluoroscopic guidance. This approach, however, still holds several limitations, including patchy transduction of a limited number of myocytes and vector delivery to organs other than the heart, because of significant spillage of vectors into the systemic circulation. Because of these overall pitfalls, the most substantial value of intramyocardial injection continues to lie in animal research.

Infusion of AAV vectors into the coronary arteries represents a highly attractive delivery route, because it would allow...
gene delivery to the whole myocardium during percutaneous coronary intervention in humans. However, the efficiency of AAV-mediated gene delivery by this method is shown in different studies as being highly variable. In addition, optimal gene transfer often requires highly invasive procedures, such as cardiopulmonary bypass, with the use of potentially dangerous pharmacological vasodilators. Before the implementation of the first AAV-based gene therapy trial for heart failure, various catheter-based delivery systems and devices were considered and compared, including anterograde coronary artery infusion, catheter-based anterograde intracoronary viral gene delivery with coronary venous blockade, and V-Focus Cardiac Delivery System (an investigational device that isolates the coronary circulation from the remainder of the systemic circulation while maintaining perfusion of the myocardium). The trial eventually adopted a femoral approach to obtain a percutaneous access to the coronary arteries, and a programmable pump to achieve slow infusion rate.

Different from anterograde intracoronary delivery, pressure-regulated retroinfusion of the cardiac veins should prolong the adhesion time of the vector with the cardiac endothelium and increase endothelial permeability. Indeed, selective retroinfusion of AAV vectors into the coronary vein allowed for efficient, long-term, myocardial gene expression and an absence of transgene expression in other organs, although vector genomes could also be detected in lung and liver.

In conclusion, an optimal, clinically translatable technique for global cardiac myocyte delivery is still to be developed and
should ideally incorporate retrograde or anterograde coronary infusion, extended vector residence time in the coronary circulation, increased endothelial permeability, isolation of cardiac circulation to minimize collateral gene expression, and minimization of technique-associated morbidity.97

Induction of Therapeutic Angiogenesis
During the past 20 years, a copious number of studies have led to the identification of a panel of cytokines and growth factors involved in blood vessel formation both during development and in adulthood. From these studies, it soon became clear that a few of these molecules, such as the members of vascular endothelial growth factor (VEGF) and fibroblast growth factor families, were extremely potent in initiating the angiogenic response. Around the mid-1990s, therefore, these molecules became the center of intense investigation for potential use in patients with ischemic cardiomyopathy or critical limb ischemia.

Initial attempts to revascularize ischemic tissues were made by delivering VEGF-A or fibroblast growth factor-2 as recombinant proteins; a few years later, gene therapy approaches also moved to the clinic, pioneered by Isner and collaborators and sustained by extensive experimentation performed in both small and large animal models. Treatment essentially consisted in the delivery of angiogenic genes by either naked plasmid DNA or adenoviral vectors.98 Collectively, these approaches have shown a common trend: initial enthusiasm for early studies, often based on an open-labeled design and a short-term follow-up, was soon tempered by the less successful outcome of subsequent, double-blinded, randomized, placebo-controlled and long-term trials.99 The overall failure of these studies was not because of the poor efficiency of the chosen genes, which are in fact extremely potent in triggering angiogenesis, but was more related to the lack of an appropriate delivery system. Recombinant proteins would require continuous administration because of their rapid degradation; plasmid delivery is largely inefficient even when high quantities of DNA are injected; adenoviral vectors are powerful gene transfer systems, but are fraught with the induction of strong inflammatory and immune responses, which severely limit the dose that can be used and the persistence of vectors in the injected tissues.100

In this scenario, preclinical research has started taking advantage of the appealing features of AAV vectors. So far, animal studies using AAV vectors expressing different VEGF-A isoforms have provided at least 3 important pieces of information, which can also explain the poor outcome of clinical trials conducted so far. First, the comparison of different VEGF-A family members and splicing variants has indicated that these are not equivalent in driving the angiogenic process.101 In particular, vessels formed on stimulation with the 121-amino acid isoform of VEGF-A (VEGF-A121), which had been used in some of the original experiments, mainly consist in unstructured capillaries devoid of a smooth muscle layer. Structured arterioles require the action of the 165-amino acid form of the factor (VEGF-A165).102,103 Second, proper timing is essential to drive persistent and functional angiogenesis. The use of an AAV vector expressing VEGF-A165 under the control of an inducible promoter has indicated that the expression of this factor >15 days is required for the newly formed vessels to become stable and persist over time. One of the reasons explaining the failure of approaches based on recombinant proteins or adenoviral vectors is possibly related to the too short duration of the angiogenic stimulus. Third, to achieve functional angiogenesis, the expression of VEGF-A165 should be controlled. Whereas the vasculature formed by constitutive VEGF-A165 expression is immature, cessation of the stimulus leads to maturation and acquisition of functional competence.89,104,105

Considering the complexity of the angiogenic process, in which an initial phase of endothelial sprouting has to be followed by a maturation phase when pericytes and smooth muscle cells are recruited to cover the new endothelial tubes, the promotion of functional neovascularization is a highly ambitious objective, requiring the coordinated action of multiple molecular players. In this respect, a reasonable approach to promote the formation of mature vessels might consist in the combined delivery of different angiogenic genes, acting on both early and late phases of the process. This can be achieved either by inserting multiple transgenes into the same vector or by mixing multiple vector preparations, each one encoding for a different gene. Animal experimentation has already shown that the combined delivery of AAV vectors expressing VEGF-A as the prototypical angiogenic factor, with agents stabilizing the newly formed blood vessels, such as angiopoietin-1,106-109 platelet-derived growth factor-B,110 or fibroblast growth factor-4,111 allows for the formation of a properly structured and functional neovascular. Using AAV vectors to express angiogenic factors that provide synergistic activities to produce stable and functional blood vessels seems to be a promising, as yet underexploited, option in cardiovascular gene therapy.

Gene Therapy of Heart Failure
With aging of the human population, the incidence and economic burden of managing heart failure is increasing considerably, as is the need to develop curative strategies. Overwhelming preclinical evidence from small and large animal models has shown the importance of SERCA2a in heart failure and the power of using AAV vectors to treat this condition by increasing SERCA2a expression.112,113 In 2007, the Celladon Corporation sponsored an open-label, phase I/II clinical trial for AVV1-mediated SERCA2a gene transfer (CUPID),113 which was followed by a small, 3-dose, randomized, double-blinded, placebo-controlled phase II trial. Overall, these studies have extolled the safety of the strategy and have reported clinical improvement in terms of cardiac remodeling and symptoms, accompanied by reduced cardiovascular hospitalization and major clinical events for >3 years after high-dose treatment.114 Based on these promising results, a larger, randomized, double-blinded, placebo-controlled, multinational, and multicenter phase IIb clinical trial is currently underway.

Apart from these clinically relevant results, AAV vectors have contributed to the definition of additional molecules that are potential therapeutic targets in heart failure. For instance, the critical role of phospholamban in the negative regulation of SERCA2a activity was clearly confirmed by AAV-based in vivo gene transfer experiments. In particular, AAV-mediated expression of a dominant-negative phospholamban mutant suppressed heart failure progression in a small animal model.
Accordingly, the AAV-mediated knockdown of phospholamban by 3 different strategies, namely RNA interference, antisense oligonucleotides, and engineered zinc-finger protein transcription factor, was effective in restoring cardiac function and geometry in rat models of heart failure. The enthusiasm for these promising results has been recently lessened by a study in which an shRNA against phospholamban delivered by an scAAV6 vector in canines resulted in serum troponin elevation and depressed cardiac function, possibly attributed to the dysregulation of endogenous microRNA pathways.

AAV-based experiments have proven the important role of additional proteins in controlling the levels of functional SERCA2a. Among these, SUMO1 is abundantly expressed by guest on May 20, 2017 http://circres.ahajournals.org/ Downloaded from of nonischemic cardiomyopathy. Accordingly, the AAV-mediated knockdown of phospholamban by 3 different strategies, namely RNA interference, antisense oligonucleotides, and engineered zinc-finger protein transcription factor, was effective in restoring cardiac function and geometry in rat models of heart failure. The enthusiasm for these promising results has been recently lessened by a study in which an shRNA against phospholamban delivered by an scAAV6 vector in canines resulted in serum troponin elevation and depressed cardiac function, possibly attributed to the dysregulation of endogenous microRNA pathways.

### Table 2. Overview of Some of the Currently More Advanced AAV-Based Applications for Cardiovascular Disorders

<table>
<thead>
<tr>
<th>AAV Serotype</th>
<th>Target Cells</th>
<th>Route of Administration</th>
<th>Promoter</th>
<th>Transgene</th>
<th>Comments</th>
<th>Progress of Research</th>
<th>Citation</th>
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<tr>
<td>AAV2, AAV1</td>
<td>Muscle fibers, cardiomyocytes</td>
<td>Intramuscular, intramyocardial</td>
<td>CMV, cardiac-specific or inducible</td>
<td>VEGF165, VEGF165 plus angiopoietin 1</td>
<td>Functional vessel maturation requires controlled VEGF expression, which can be achieved by using inducible promoters or gene combinations</td>
<td>Preclinical</td>
<td>89,106–109</td>
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<tr>
<td>Heart failure</td>
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<tr>
<td>AAV1</td>
<td>Cardiomyocytes</td>
<td>Intracoronary</td>
<td>CMV</td>
<td>SERCA2a</td>
<td>Long-term expression required for heart failure therapy</td>
<td>Clinical phase IIb</td>
<td>13,113</td>
</tr>
<tr>
<td>AAV6, AAV9</td>
<td>Cardiomyocytes</td>
<td>Intracoronary, retrograde venous delivery</td>
<td>CMV</td>
<td>S1001A</td>
<td>Expected to achieve results similar to SERCA2a overexpression</td>
<td>Preclinical</td>
<td>78,79</td>
</tr>
<tr>
<td>AAV6</td>
<td>Cardiomyocytes</td>
<td>Recirculating delivery (MCARD)</td>
<td>CMV</td>
<td>jARKct</td>
<td>Alternative to β-blocker therapy: heart specificity and long-term effects by single treatment</td>
<td>Preclinical</td>
<td>120,193</td>
</tr>
<tr>
<td>AAV9</td>
<td>Cardiomyocytes</td>
<td>Intracardiac</td>
<td>CMV</td>
<td>VEGF-B</td>
<td>Effective in protecting the heart against heart failure in mice, rats, and dogs</td>
<td>Preclinical</td>
<td>124–126</td>
</tr>
<tr>
<td>Cardiac regeneration</td>
<td>AAV9</td>
<td>Cardiomyocytes</td>
<td>Intracardiac</td>
<td>CMV</td>
<td>hsa-miR-590-3p, hsa-miR-199a-3p</td>
<td>Effective in driving proliferation of already differentiated cardiomyocytes</td>
<td>Preclinical</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>AAV8</td>
<td>Hepatocytes</td>
<td>Intravenous</td>
<td>Thyroxin-binding globulin</td>
<td>LDLR</td>
<td>No therapy available, persistent expression</td>
<td>Clinical trial proposed</td>
</tr>
<tr>
<td>AAV8</td>
<td>Hepatocytes</td>
<td>Intravenous</td>
<td>H1</td>
<td>shApoB</td>
<td>Decreases LDL cholesterol with no effect on HDL cholesterol</td>
<td>Preclinical</td>
<td>138</td>
</tr>
<tr>
<td>AAV8</td>
<td>Muscle fibers</td>
<td>Intramuscular</td>
<td>CMV</td>
<td>LPLSer(447)X</td>
<td>Very good safety and tolerability data</td>
<td>On the market</td>
<td>142,143</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>AAV6, AAV9</td>
<td>Cardiomyocytes</td>
<td>Intravenous</td>
<td>CMV</td>
<td>Microdystrophin</td>
<td>Provides cardiac protection in mdx mice</td>
<td>Preclinical</td>
</tr>
<tr>
<td>AAV6, AAV9</td>
<td>Cardiomyocytes</td>
<td>Intravenous</td>
<td>U7</td>
<td>Modified U7 snRNA</td>
<td>Induces effective dystrophin exon skipping in mdx mice and GRMD dogs</td>
<td>Preclinical</td>
<td>149–151</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>AAV9</td>
<td>Cardiomyocytes</td>
<td>Intraperitoneal</td>
<td>CMV</td>
<td>CASQ2</td>
<td>Prevention of life-threatening arrhythmias in mice</td>
<td>Preclinical</td>
</tr>
</tbody>
</table>

jARKct indicates β-adrenergic receptor kinase carboxyl-terminus; AAV, adeno-associated virus; CMV, cytomegalovirus; GMRD, Golden Retriever muscular dystrophy; HDL, high-density lipoprotein; LDLR, low-density lipoprotein receptor; MCARD, molecular cardiac surgery with recirculating delivery; SERCA2a, sarcoplasmic reticulum Ca2+-ATPase; shApoB, shRNA for apolipoproteinB; snRNA, small nuclear RNA; and VEGF, vascular endothelial growth factor.
by healthy hearts, where it modifies SERCA2a on 2 specific lysine residues, thereby increasing both activity and stability of the protein. Consistent with reduced SUMO1 expression in failing hearts, its downregulation by AAV-mediated shRNA delivery accelerated pressure overload–induced deterioration of cardiac function.116 Although these results provide a platform for the design of novel therapeutic strategies for heart failure, the broad action of SUMO1 in cardiomyocytes will possibly represent a major limitation to rapid clinical translation. The experiments based on the overexpression of S100A1, a chaperone protein that enhances the activity of other proteins including SERCA2a, are more advanced; both AAV6 and AAV9 vectors have been exploited to deliver this protein in small and large animal models of heart failure.78,79

In addition to calcium cycling, the β-adrenergic control of cardiac contractility also stands as an important molecular target for heart failure gene therapy. It is well recognized that the chronic activation of sympathetic activity, in particular through the increased activity of G-protein–coupled receptor kinase 2, has adverse biological effects and can initiate or accelerate heart failure.119 An AAV6 encoding for the inhibitory peptide β-adrenergic receptor kinase carboxyl-terminus, which blocks the interaction between G-protein–coupled receptor kinase 2 and β-adrenergic receptors, was tested for its therapeutic activity in rodent, swine, ovine, and bovine models of chronic cardiomyopathy; overall, the vector improved left ventricular dysfunction.120–122 Of interest, the persistent β-adrenergic receptor kinase carboxyl-terminus cardiac expression allowed by AAV vectors could offer major advantages versus β-blocker therapy, namely the achievement of long-term therapeutic benefits after a single treatment and the lack of unspecific blocking of β-adrenergic receptor signaling pathway in other organs.123

An alternative promising strategy stems from the use of AAV vectors to overexpress diffusible growth factors, which can extend their effect beyond the individually transduced cells. Multiple intramyocardial injections of an AAV9 vector expressing VEGF-B, a member of the VEGF family that selectively binds VEGFR-1 expressed on cardiomyocytes, remarkably improved cardiac function in rats after myocardial infarction,124 in mice after aortic constriction,125 and in dogs chronically instrumented to develop tachypacing-induced dilated cardiomyopathy.126 Diffusible factors such as insulin-like growth factor 1 were also shown to exert protective effect against cardiac dysfunction after secretion from a remote site, in particular on AAV5-mediated transduction of the skeletal muscle.127

AAV Gene Transfer for Hyperlipidemia

Most cases of diet-induced and heterozygous familial hypercholesterolemia because of mutations in the low-density lipoprotein receptor (LDLR) can be successfully managed using conventional pharmacological treatment, essentially based on statins combined with additional lipid-lowering drugs when necessary. However, homozygous familial hypercholesterolemia still represents an important target for permanent correction by gene therapy, because this form of the disease leads to premature cardiovascular death in young adulthood and because current therapies, namely LDL apheresis and liver transplantation, are laborious. Functional and persistent LDLR expression in the liver, which can be achieved by AAV-mediated gene transfer, should be able to correct the phenotype. In fact, the reconstitution of LDLR expression using AAV7 and AAV8 injected into the portal vein of LDLR-deficient mice on a high-fat diet resulted in nearly complete normalization of serum lipids lasting ≤21 weeks.128 The same strategy also worked efficiently in novel models of homozygous familial hypercholesterolemia such as a double-knockout mouse lacking both LDLR and APOBEC1 genes129 and a humanized mouse that, besides double gene depletion, also expressed a human apolipoprotein B100 transgene.130 To advance this approach to the clinic, a recent study analyzed vector distribution after intravenous administration and revealed that, despite minimal presence of AAV genomes in all organs for ≈180 days, vector DNA was sequestered mainly in the liver, which contained levels that were 3 orders of magnitude higher than those found in other organs.131

Numerous alternative strategies are currently being pursued with success at the preclinical phase to deliver genes exerting beneficial effects on lipid metabolism using AAV vectors, in most instances AAV8. These include vectors coding for apolipoprotein E,132,133 apolipoprotein A1,134 apolipoprotein F,135 a soluble macrophage scavenger receptor,136 and the lecithin–cholesterol acyltransferase enzyme.137

In addition, several proteins are being recognized as proatherogenic and can be therapeutically silenced by small regulatory RNAs. For instance, apolipoprotein B knockdown by a scAAV-delivered shRNA in murine liver resulted in a specific reduction of LDL cholesterol in diet-induced dyslipidemic mice, whereas high-density lipoprotein (HDL) cholesterol remained unaffected.138 An additional option for treatment stems from retrospective profiling studies, which link aberrant microRNA expression to disease development and progression. A recent study showed that microRNA-122 inhibition by scAAV-delivered Tough Decoy RNA (a recently developed, compact, RNA polymerase III–driven microRNA decoy)139 lowers both HDL and LDL in healthy mice.140 However, because the current view that HDL protects against heart attack argues that therapy for dyslipidemia should lower LDL but raise HDL levels, simultaneous inhibition of microRNA-122 and other HDL biogenesis suppressors such as microRNA-33141 could offer advantages for the therapy for hypercholesterolemia. In this respect, AAV is foreseen as the vector of choice, as mixed vector stocks; each one coding for a single microRNA inhibitor, or a single vector carrying pairs of microRNA inhibitors, could achieve a more balanced and healthy cholesterol profile and provide long-lasting therapy.

An additional paradigmatic example of AAV-based gene therapy is that of LPL deficiency, an autosomal recessive disorder of lipid metabolism that leads to various clinical consequences, including acute pancreatitis. Eight years after the first proof of concept in LPL-deficient mice,142 in November 2012, an LPL-expressing AAV vector, AAV1-LPLS447X, was approved by the European Medicines Agency as the first gene therapy product in the Western world. The same vector has recently provided the opportunity to address systematically
one of the safety concerns in human subjects related to the use of AAV. The analysis of DNA from muscle biopsies of 5 patients injected intramuscularly with AAV1-LPLS447X indeed confirmed that AAV DNA inserts itself into the genome infrequently and that integration, whenever it happens, is safe and occurs randomly in both the cellular and, unexpectedly, the mitochondrial genomes.\textsuperscript{143}

Another common form of dyslipidemia is hypertriglyceridemia, a metabolic disorder characterized by abnormally elevated concentrations of triglyceride levels that represent a major risk factor for coronary artery disease. Based on the evidence in human populations that single nucleotide polymorphisms and mutations in apolipoprotein A5 positively correlate with increased triglycerides, gene therapy can offer a chance to overexpress the wild-type protein. Indeed, AAV8-mediated gene transfer of apolipoprotein A5 improved the severe hypertriglyceridemia phenotype of apolipoprotein A5-deficient mice.\textsuperscript{144} An alternative successful approach consisted in the intramuscular injection of an AAV2 encoding the human very low-density lipoprotein (VLDL) receptor gene, which reduced triglycerides and normalized glycemia in type 2 diabetic rats.\textsuperscript{145}

Also in the case of lipid metabolisms, AAV is being used as a precious investigational tool to dissect pathophysiological mechanisms and explore novel therapeutic targets. An elegant example is a recent study aimed at understanding the reason why human apolipoprotein E4, which differs from apolipoprotein E3 uniquely for a single amino acid substitution (C112R), causes a more proatherogenic plasma lipoprotein cholesterol distribution.\textsuperscript{146} AAV8 vectors were used to express human apolipoprotein E3, apolipoprotein E4, and several C-terminal truncation and internal deletion variants in apolipoprotein E-\textsuperscript{null} mice that exhibit marked dysbetalipoproteinemia. This strategy revealed that apolipoprotein E4 possesses enhanced lipid- and VLDL-binding ability relative to apolipoprotein E3, which gives rise to impaired lipolytic processing of VLDL and reduced VLDL remnant clearance from plasma.\textsuperscript{148} Increased VLDL cholesterol can be considered a major determinant of the more proatherogenic lipoprotein profile induced by apolipoprotein E4.

**Genetic and Phenotypic Correction of Cardiomyopathies by AAV-Mediated Gene Transfer**

Several genetically determined cardiomyopathies, either specific for the heart or affecting also the skeletal muscle, offer excellent opportunities for treatment by gene therapy. Because patients with these diseases would require transgene expression for their entire life, AAV vectors seem attractive in this respect. A comprehensive review of AAV-based approaches for the specific gene therapy of dystrophic heart disease is provided elsewhere.\textsuperscript{147}

As discussed before, a hurdle that AAV vectors pose is the relatively short size of the inserts they can accommodate. In the case of Duchenne muscular dystrophy, truncated versions of dystrophin can be included into the vector genome to be delivered systemically to the whole muscle mass, including the heart. Indeed, AAV-mediated delivery of such a microdystrophin has yielded amazing cardiac protection in young mdx mice, also improving the outcome of electrocardiographic and dobutamine stress assays at the most severe, terminal stage of dystrophic cardiomyopathy.\textsuperscript{147,148}

In the case of genetic cardiomyopathies, the potential of gene therapy extends beyond the traditional concept of providing a wild-type copy of the mutated gene. In particular, RNA-based therapeutics and splice-site switching approaches have been developed during the past decade to directly correct the genetic defect at the level of mRNA. Besides overcoming the difficulties of cloning large cDNAs for replacement gene therapy, these strategies also guarantee expression of the correct gene at the appropriate dosage and with preserved spatial and temporal regulation. In contrast to the use of modified oligonucleotides administered as naked nucleic acids, the use of vectors to induce exon skipping also overcomes the need for repeated injections.

A few recent studies underline the potential of AAV-based approaches to achieve exon skipping in Duchenne muscular dystrophy and other myopathies. Vector-mediated delivery of modified U7 small nuclear RNA carrying antisense sequences to target essential splicing regulatory signals turned out to correct the disrupted reading frame of dystrophin in skeletal muscle from both human Duchenne muscular dystrophy mice\textsuperscript{149} and Golden Retriever muscular dystrophy dogs\textsuperscript{150} and also to be effective on transendochardial delivery in the dog heart.\textsuperscript{151} A similar U7 small nuclear RNA--based exon-skipping approach was also adopted to rescue hypertrophic cardiomyopathy because of mutations in the cardiac myosin-binding protein C3 in a knockin mouse model of the disease.\textsuperscript{152} The same mice, an AAV vector was also effective in correcting cardiomyopathy by inducing trans-splicing between the endogenously transcribed myosin-binding protein C3 RNA and the correct transcript expressed from the vector DNA.\textsuperscript{153} Allele-specific silencing of mutant transcripts by RNA interference is an alternative therapeutic strategy for dominant cardiomyopathies, which are often determined by missense mutations in sarcomere proteins. The expression of myosin heavy chain 6 R403Q mutation in mice could selectively be silenced by an RNA interference cassette delivered by an AAV9 vector.\textsuperscript{154} In this study, although only a 25% reduction in the levels of mutant transcripts could be achieved, the main pathological manifestations of the diseases, namely hypertrophy and myocardial fibrosis, were prevented for several months.

When considered collectively, the abovedescribed experimental results provide convincing proof of principle that disease mRNA--targeted interventions can be pursued as a therapeutic strategy. However, the therapeutic effects are still transient in most cases, and further improvements in the overall efficiency of transduction and mutation targeting seem necessary in the vast majority of cases before moving forward to the clinic.

**Corrections of Arrhythmias by AAV Gene Transfer**

Abnormalities in heart electric function often severely impair cardiac performance with potentially fatal consequences. Gene therapy has started targeting a few classes of cardiac arrhythmias, including ventricular arrhythmias, atrial fibrillation, and bradyarrhythmias. Although short-term proof of concept has been obtained by various vehicles, in particular adenoviral vectors, AAV is emerging as the preferred choice
 Emerging Concepts in AAV Biology: Toward an Ideal Vector for Cardiovascular Applications

Although the number of preclinical and clinical applications that have taken advantage of the properties of AAV have steadily increased during the past few years, these vectors, as any other at this moment, are still far from being a magic bullet that can be injected to drive efficient and controlled therapeutic gene expression in a desired tissue. In particular, the use of AAV in the cardiovascular field has been massively exploited for research purposes during recent years, but has resulted in a single clinical trial. This seems to be a paradox for a vector that exhibits a peculiar tropism for heart and muscle cells. What are the reasons for such a delayed transition of AAV-based approaches into real therapies for cardiovascular disorders? On one hand, the prolonged transgene expression driven by AAV vectors and their diffusible properties might raise important safety concerns, which need to be carefully, experimentally addressed before clinical transition. On the other hand, the complexity of most cardiovascular disorders significantly lowers the possibility to achieve clinical benefit through the delivery of a single gene. Clinical experience in the use of conventional drugs and small molecules has clearly indicated that in the heart, more than in any other organ, the same compound can have distinct and even opposite effects in the short versus the long term, or at low versus high dose. The as-yet poor control of the kinetics and levels of transgene expression achievable by AAV-mediated gene transfer thus represents an additional relevant limitation to successful clinical application.

Despite these negative considerations, there seems to be ample room for improvement. We now review emerging AAV vector technologies that we think will foster clinical use of these vectors during the next few years.

 Capsid Engineering to Achieve Improved and Selective Tropism

Since the discovery of AAV, a plethora of naturally occurring AAV serotypes and well >100 variants have been described. All these viruses share a similar structure, genome size, and genetic organization, but differ significantly in the amino acid composition of the capsid proteins. By transencapsidation, it is possible to obtain chimeric viral particles packaging an identical genome, usually bearing the first and prototypic AAV2 inverted terminal repeats, in any of the different capsid serotypes.

The properties and relative efficiency of the different serotypes have been compared in several studies. The scenario that has emerged is complex, and the choice of the best vector for specific applications is far from evident. An additional level of complexity comes from a few in vivo experiments, which have revealed an unanticipated variability in terms of transduction efficiency and transgene expression in different animal species. Examples of discordant results have recently been published. For instance, different from mice, AAV9 was less efficient in mediating dog and rhesus monkey cardiac gene transfer compared with AAV6 and AAV8 after transendocardial administration. Similarly, AAV8, which is effective in transducing the liver in many animal models, transduced human hepatocytes in a relatively poor manner. On the contrary, AAV3 efficiently transduced human but not murine hepatocytes. Together, these data suggest that AAV interacts with cellular receptors or blood proteins important for transduction in a species-specific manner, or that poorly controlled variables influence transduction in different species. As a practical consequence, the results obtained in 1 animal model cannot automatically be translated to another animal species or, most importantly, to humans.

The characterization of AAV capsid structure and biology has favored the development of several synthetic capsid variants that expand the repertoire of available vectors. Capsid re-engineering approaches have contributed to optimize receptor-binding and transduction efficiency, biodistribution and tissue target selectivity after systemic administration, as well as to escape neutralizing antibodies in vivo. Several techniques have been proposed to engineer new AAV variants, including rationale or random capsid mutagenesis, DNA shuffling, in vitro and in vivo molecular evolution, and direct selection. Thanks to rational receptor footprint engineering, new AAV2 chimera, such as AAV218, AAV218G9, and AAV-SASTG, are now available, which transduce cardiac and skeletal muscle with high efficiency but are detargeted from liver tissue. Similarly, new AAV9 variants have been generated by random mutagenesis and selected for strong cardiac tropism and significantly less transgene expression in off-target applications.
organisms (liver, kidney, pancreas).\textsuperscript{36,170} Even better, AAV variants have been obtained by both AAV-based peptide display libraries\textsuperscript{721} and DNA-shuffled libraries and direct evolution in mice (AAV2-PSVSPRP, AAV2-VNSTRLP, AAVM41).\textsuperscript{172} Other AAV2-derived mutants, AAV2.15 and AAV2.4, contain mutations at critical antigenic sites and are thereby capable of evading neutralizing antibodies in human serum.\textsuperscript{165}

Notably, AAV capsid modification not only modifies receptor interaction and virus adsorption but also influences other steps in the viral life cycle, including endosomal release, trafficking, decapsidation, and even gene expression,\textsuperscript{173} all of which contribute to increased transduction efficiency. However, extensive capsid engineering might also increase the complexity of the regulatory pathways necessary to obtain permission for the clinical use of modified AAV vectors.

**Expression of Large Genes in Heart and Skeletal Muscle**

The cDNAs of several genes expressed by cardiac and skeletal muscle cells (eg, those coding for dystrophin, other cytoskeletal proteins, or different ion channels) cannot be efficiently packaged into AAV vectors because of their length, which exceeds $\approx 4.7$ kb size of the wild-type AAV genome. Different approaches have been proposed to circumvent this problem, based essentially on the property of AAV vectors to transduce the cells at high multiplicity and to form intermolecular concatamers through recombination (Figure 5A). One possibility is to deliver 2 independent vectors simultaneously into the same cell, one carrying the 5’ and the other the 3’ ends of the same transcription cassette, with appropriate splice signals to promote trans-splicing inside the target cells.\textsuperscript{174,175} Alternatively, a dual hybrid vector system can contain overlapping sequence elements, either from the transgene itself\textsuperscript{176} or from exogenous bridging sequences,\textsuperscript{177} to drive HR. Such a dual vector transduction system seems to work efficiently in skeletal muscle and heart for the expression of mini-dystrophins.\textsuperscript{175,176} Recently, a triple AAV vector coinfection was tested with success in mice to reconstitute the functional, full-length dystrophin-coding sequence.\textsuperscript{178}

Although attractive, these strategies are still weakened by several limitations. To increase the probability of transgenic recombination, cells need to be infected at a high multiplicity of infection, which greatly depends on total vector dose, capsid serotype, route of administration, and, ultimately, on target organ permisiosity. Technical improvement in this area will likely depend on a better understanding of the mechanisms that drive the molecular processes that underlie HR or trans-splicing in the infected cells, including the definition of the relevant cellular factors involved, which are currently not completely understood.

**AAV-Based Strategies for Gene Editing**

Understandably, an attractive property of AAV vectors is the ability to intercept the cellular HR machinery and to mediate precise gene targeting at chromosomal loci. A few years ago, indeed, it was noted that when the AAV genomes carry sequences that are homologous to the cellular genome, these recombine at relative high frequency.\textsuperscript{179} This property, which is likely linked to the single-stranded nature of the AAV genome or to the capacity of its hairpins to associate with cellular proteins of the HR machinery,\textsuperscript{37} has paved the way for the use of AAV vectors to achieve gene correction (Figure 5B).

Several proof-of-concept experiments in cultured cells have demonstrated the feasibility of AAV-mediated introduction of point mutations or small insertions and deletions into the host cell genome at a frequency of $\approx 1\%$ of transduced cells, which is orders of magnitude higher than that achieved with a plasmid vector.\textsuperscript{180} In situ gene correction, as opposed to current gene addition, would represent the ideal therapy for monogenic diseases, in particular for those with dominant inheritance.

The possibility of translating these findings to the clinic, however, still seems to be in its infancy. AAV-directed gene targeting depends on the activity of the cellular HR machinery, which is downregulated in postmitotic tissues.\textsuperscript{28} The genome editing applications, therefore, are so far restricted to ex vivo cultured cells, which also offer the possibility to select cells in which genome targeting has occurred. Proposed ex vivo applications of therapeutic interest include the modification of various types of adult stem cells or induced pluripotent stem cells.\textsuperscript{181,182} Recently, proof of concept of the feasibility of this approach has been provided by the efficient correction of a recessive form of junctional epidermolysis bullosa through editing the mutated gene in primary keratinocytes.\textsuperscript{183} In principle, such an approach can also be conceived for cells of other organs, including the heart, on differentiation and expansion of cardiomyocytes from embryonic stem cells or induced pluripotent stem cells.

Multiple evidence indicates that the process of HR-mediated gene targeting can significantly be increased by introducing a dsDNA break in the targeted region.\textsuperscript{184,185} In the presence of an AAV vector carrying a sequence homologous to the one being repaired, gene correction is stimulated. Targeting a dsDNA break to a specific genomic sequence can be achieved by sequence-specific designer nucleases, in particular those based on zinc-fingers\textsuperscript{186,187} or the highly specific DNA-binding domains of transcription activator-like effectors.\textsuperscript{188}

Both the nuclease-encoding sequence and the HR donor template can be engineered into vectors to achieve efficient gene transfer. Ideally, the nuclease, as well as the donor DNA, should be present in the target cell only transiently and at a high concentration. AAV vectors seem to be valuable tools for this purpose, because they enter into the nucleus en masse and then persist as episomes, which are subsequently diluted on cell division.\textsuperscript{189} A recent demonstration in support of the applicability of AAV vectors for in vivo nuclease-mediated gene targeting was successfully achieved in the neonatal liver of a mouse model of Factor IX deficiency.\textsuperscript{190}

These approaches, in conjunction with AAV vectors, seem truly exciting for their capacity to markedly boost site-specific gene correction. However, deeper investigation is still required to improve restricted delivery and expression of the nucleases in vivo and to solve concerns on their off-target genomic effects.\textsuperscript{191} In addition, the applicability of this therapeutic approach to postmitotic tissues, such as muscle and heart, seems to require further technological implementation. On a final note, it
remains to be seen whether the recently developed CRISPR/ Cas (Cas9) system, once adapted to AAV vectors, will lead to more satisfactory results in terms of editing specificity.

Conclusions

Thirty years after AAV was originally conceived as a gene delivery vector, it can now safely be concluded that AAV technology is mature and rapidly progressing toward the clinics in various applications. These include cardiovascular disorders, after the ice-breaking SERCA2a clinical trial for the treatment of heart failure. In addition, an increasing number of investigators are taking advantage of AAV vectors for phenotypic assessment in animal models of cardiomyopathy, arrhythmia, atherosclerosis, and hypertension. Genetic
manipulation of mice has provided invaluable information on gene function during the past 2 decades; gene transfer with AAV vectors can contribute to this information in a more straightforward manner and, more importantly, in animal species in which genetic modification is not so amenable.

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