AAV Vectors for Cardiac Gene Transfer: Experimental Tools and Clinical Opportunities

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Since the first demonstration of *in vivo* gene transfer into myocardium there have been a series of advancements that have driven the evolution of cardiac gene delivery from an experimental tool into a therapy currently at the threshold of becoming a viable clinical option. Innovative methods have been established to address practical challenges related to tissue-type specificity, choice of delivery vehicle, potency of the delivered material, and delivery route. Most importantly for therapeutic purposes, these strategies are being thoroughly tested to ensure safety of the delivery system and the delivered genetic material. This review focuses on the development of recombinant adeno-associated virus (rAAV) as one of the most valuable cardiac gene transfer agents available today. Various forms of rAAV have been used to deliver "pre-event" cardiac protection and to temper the severity of hypertrophy, cardiac ischemia, or infarct size. Adeno-associated virus (AAV) vectors have also been functional delivery tools for cardiac gene expression knockdown studies and successfully improving the cardiac aspects of several metabolic and neuromuscular diseases. Viral capsid manipulations along with the development of tissue-specific and regulated promoters have greatly increased the utility of rAAV-mediated gene transfer. Important clinical studies are currently underway to evaluate AAV-based cardiac gene delivery in humans.

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INTRODUCTION

A number of strategies for cardiac gene transfer have been described since the earliest report of naked DNA delivery to the heart.[1] Each approach has specific attributes and limitations which when understood can be adapted and used for particular applications. Recent clinical trials have demonstrated success using the parvovirus, recombinant adeno-associated virus (rAAV) as a vector for gene transfer to myocardium. This review will cover the advances in rAAV research that have aided its development into a safe, clinically relevant vehicle for cardiac gene therapy applications. Examples of treatments in animal models for a variety of inherited and acquired diseases affecting the heart are provided, current data from rAAV-mediated cardiac gene therapy trials is discussed and future directions for the field are proposed.

WILD-TYPE AND RECOMBINANT AAV BIOLOGY

Wild-type adeno-associated virus (wtAAV) was first discovered as a 20 nm, icosahedral contaminant in adenovirus preparations.[2] It is a nonpathogenic, nonenveloped, DNA virus containing a linear single-stranded genome of 4.6–4.8 kb that requires coinfection with a helper virus for viral replication.[2,3] In a productive infection, the wtAAV DNA becomes uncoated, converted to duplex form and is integrated into host cellular DNA during the latent phase in a site-specific manner.[4] The wtAAV genome consists of two open reading frames flanked by 145 base-pair inverted terminal repeats (ITRs). The viral genes encode alternatively spliced capsid (Cap) proteins and multifunctional replication (Rep) proteins.[5]

To generate recombinant AAV (rAAV) for gene delivery, a plasmid vector is designed containing the control region and complementary DNA of interest flanked by wtAAV ITRs. The size of the promoter elements and transgene are ideally between 4.1 and 4.9 kb for efficient packaging although studies have shown that it is possible to package up to 5.2 kb with decreased efficiency.[3] In a transfection based production scheme, the vector plasmid is co-transfected into mammalian cells with helper plasmids containing the *rep* and *cap* genes and the essential adenoviral gene products which provide helper functions in *trans*.[6] The ITRs supply all *cis* acting sequences necessary for viral packaging. Once the helper genes are expressed, the vector genome is rescued from the plasmid DNA and replication occurs. In the presence of rAAV capsids the single-stranded vector DNA becomes encapsidated to generate mature viral particles. These particles containing the transgene of interest can then be purified from a cell lysate using either anion exchange, affinity chromatography, or density gradient centrifugation techniques.[5] Once a titer is determined the transgene containing rAAV may be frozen or immediately used for experiments. In contrast to the ability of wtAAV to integrate into a host genome, the rAAV used for gene delivery applications has been shown to largely persist as an episome for long periods of time following transduction.[7] This in addition to the lack of *rep* and *cap* genes or helper virus proteins in rAAV stocks further increases the general safety and appeal of rAAV as a vehicle for gene transfer.

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NATURAL AND MODIFIED AAV CAPSIDS FOR CARDIAC GENE TRANSFER

The efficiency of AAV receptor-mediated cellular entry is controlled by the capsid proteins. The AAV *cap* gene encodes 3 capsid proteins (VP1, VP2, and VP3) and displays considerable sequence diversity between serotypes. These differences alter receptor-binding sites on the surface of the capsid and lead to the observed variations in tissue transduction. One way in which investigators can restrict the location of transgene expression to the heart is to identify a naturally cardiotropic AAV capsid and design a pseudotyped rAAV that is optimized for delivery to the heart. Initial pseudotype experiments in a variety of animal models and using many different injection routes compared various combinations of the AAV capsids 1–5 to establish their respective natural tropisms or affinity for particular tissues. When considered together, the results suggested that rAAV2/1 yielded the highest level of transgene expression in the heart.[8–11] The next general wave of vector comparisons was made between rAAV2/1 and more recently described pseudotypes: rAAV2/6, rAAV2/8, rAAV2/9, and rAAV2/10.[12–16] For a compilation of these studies and the models they were performed in please see (**Table 1**). Taken together, various groups have demonstrated great success with all of these pseudotypes with rAAV2/9 appearing to be the most naturally cardiotropic.[17–22] It is important to note that although extremely high levels of cardiac expression are observed, other organs (including liver, skeletal muscle, and pancreas) also display robust levels of expression.[17] Additional research in nonhuman primates will be required to confirm preliminary capsid comparison studies in rodent, porcine, canine models as well as the initial primate experiments.[18]

In addition to evaluating natural capsids rescued from primate or human tissue, investigators have also manipulated the virus in a variety of ways to enhance the specificity of cardiac transduction. DNA shuffling is a method used to introduce permutations of genetic variations using *in vitro* recombination and has been employed to design cardiotropic rAAV. The technique generates chimeric AAV capsids by shuffling known serotype capsid sequences and then performing direct *in vivo* biopanning to identify novel cardiotropic mutant capsids. This type of screen retrieved a unique capsid (M41) based upon its high transduction frequency in muscle and low frequency in liver in both mice and hamsters.[23] The efficiency of rAAVM41 transduction in the heart was comparable to that of rAAV2/9 but transduction of nonmuscle tissues was greatly reduced. Use of such cardiac-specific chimeras enhances the overall safety of cardiac gene transfer through minimization of off-target effects that could be introduced when performing delivery via the systemic circulation. Another key benefit displayed by the M41 variant is the reduced sequestration of virus in the liver. The decrease in the vector genomes lost in the liver results in a higher effective dose of vector in the heart, essentially providing greater overall efficacy.

Another technique to modify viral tropisms is to integrate peptides for specific cardiovascular targets into the capsid. This has been particularly useful for developing therapies for atherosclerosis, a narrowing of the coronary arteries due to fatty plaque accumulation that is the single leading cause of death in the United States today.[24] Investigators found that vascular transduction was increased by isolating human venous endothelial cell-targeting peptides by phage display and genetically incorporating them into AAV capsids.[25] In another study, two plaque-targeting peptides, CAPGPSKSC (CAP) and CNHRYMQMC (CNH) were inserted into the AAV2 capsid. In mice, this retargeting resulted in substantially higher levels of vector in the brachiocephalic artery (the site of advanced atherosclerotic plaques) and in the aorta.[26]

Table 1 Compilation of the different types of rAAV that have been assessed in their ability to confer cardiac gene expression including the species and delivery routes that have been used as well as the duration of expression (this is the final time point of the experiment and not an indication of termination or significant decrease in expression)

rAAV	Longest published cardiac expression duration (species/route)
rAAV2/1	12 months (mice/I.My.)[9]
rAAV2/2	12 months (mice/I.My.)[9]
rAAV2/3	12 weeks (rats/I.My.)[15]
rAAV2/4	24 weeks (rats/I.My.)[15]
rAAV2/5	27 months (primates/I.T.)[16]
rAAV2/6	24 weeks (rats/I.My.)[15]
rAAV2/7	24 weeks (rats/I.My.)[15]
rAAV2/8	24 weeks (rats/I.My.)[15]
rAAV2/9	27 months (primates/I.T.)[16]
rAAV2/10	27 months (primates/I.T.)[16]
rAAVM41	4 months (hamsters/I.V.)[23]
dsAAV	16 weeks (rats/I.V.)[33]

Abbreviations: dsAAV, double-stranded adeno-associated virus; I.My., intramyocardial; I.T., intrathoracic; I.V., intravenous; rAAV, recombinant AAV.

METHODS TO INCREASE rAAV TRANSGENE EXPRESSION

Although AAV is a single-stranded DNA virus, both *wtAAV* gene and rAAV-mediated transgene expression require the conversion of single-stranded DNA to a double-stranded form before transcription.[27] This process is partially responsible for the delayed onset of transgene expression as compared to other gene delivery vehicles.[28–30] By mutating one of the ITRs it is possible to create vectors that exclusively package hairpin-like double-stranded AAV (dsAAV) DNA genomes which result in greater and more rapid transduction of tissues following intravenous administration in mice.[27,31]

The dsAAV strategy has been employed to develop a gene-based therapy for hypertension. Approximately 74.5 million adults in the United States have been diagnosed with high-blood pressure. If left untreated, hypertension can lead to damage to the heart, coronary arteries, and kidneys as well as increase ones chances of having a stoke, vision loss, or suffering other serious consequences.[32] Investigators have found that dsAAV-mediated gene delivery of adrenomedullin successfully lowered blood pressure in spontaneously hypertensive rats.[33] Of note, one disadvantage to using dsAAV is that the control elements plus transgene are limited to a length of ~2.2 kb. An alternative method to accelerate the onset of expression following rAAV-mediated gene transfer is to inhibit topoisomerase using camptothecine which leads to the rapid conversion of single-stranded rAAV genomes to the

transcriptionally active double-stranded form.[34] Clearly, elegant transgene expression enhancement techniques such as these are powerful tools that could be implemented in many gene transfer applications.

rAAV DELIVERY ROUTES

There are a variety of routes that may be employed to physically introduce rAAV to the heart (**Figure 1**). The optimal choice largely depends upon whether the targeted area is a defined location within the heart (such as an infarct) or the entire heart itself. Options for global cardiac delivery include an assortment of intravenous or intra-arterial routes as well as multiple intramuscular cardiac injections. Injections of rAAV into the coronary artery have been shown to achieve effective and uniform gene transfer throughout the myocardium as demonstrated in rats, pigs, and hamsters.[35–39] Successful rAAV-mediated gene delivery to the whole heart has also been demonstrated following aortic occlusion in mice and pressurized venous infusion into dogs, rats, and pigs.[40–42] In genetic diseases where the heart is only one component of the required treatment, systemic intravenous administration has provided efficient cardiac transduction when the optimal AAV serotype is used.[43,44]

Transgene delivery to a precise location within the heart can be achieved through direct intramyocardial injections from the outside of the heart with a needle or via the endocardial surface using a catheter as demonstrated in hamster and swine models.[39,45]

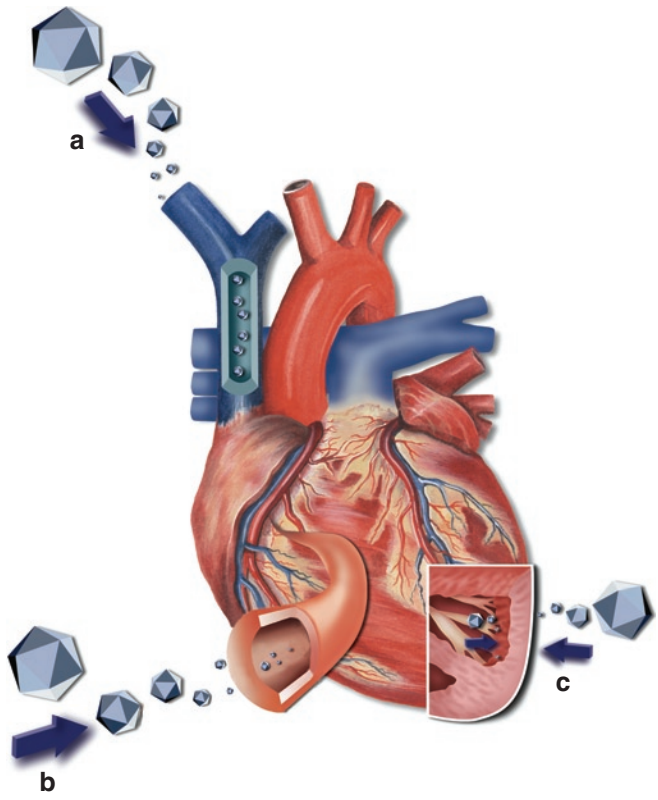

Figure 1 Recombinant adeno-associated virus (rAAV) delivery routes. (**a**) Systemic venous delivery—through the superior vena cava. (**b**) Vascular delivery—through the coronary artery. (**c**) Intramyocardial delivery—showing entry from the inside though endocardium and from the outside through epicardium (both ultimately target the heart muscle directly).

Prenatal administration has been demonstrated in mice using in utero-intraperitoneal injections of the luciferase gene. Whole-body imaging analysis of these animals revealed low level, but positive cardiac expression.[46] In general, each of the outlined routes of delivery can be combined with the most advantageous AAV capsid and incorporated into gene therapy treatments to successfully provide local, regional, or global transduction of the heart.

TISSUE RESTRICTED AND INDUCIBLE PROMOTERS

When using an intravenous delivery route even the most cardiotropic capsid choices may result in low-level transduction of noncardiac tissue. Many investigators have demonstrated the utility of cardiac promoters and their ability to augment specificity for either all striated muscle (useful for diseases that affect both the heart and skeletal muscle) or cardiac tissue alone in mice and rats.[47–49] Promoters that have shown the most cardiac-specific expression include: cardiac myosin light chain and cardiac myosin heavy chain.[47,50,51]

Another cardiac selective promoter was designed by ligation of a 316 bp fragment of the mouse α-cardiac actin gene enhancer containing 2 myocyte enhancing factor-2 sequences and 2 MyoD (a myogenic regulatory factor) enhancer sequences attached to the elongation factor 1α promoter. The promoter was used to drive expression of a therapeutic transgene (the calcium ($Ca2^{+}$)-sensing *S100A1*) in a rat model of heart failure (HF) and resulted in improved contractile function and left ventricular (LV) remodeling. Importantly, no S100A1 expression was observed in the other tissues analyzed.[52]

Promoters have also been developed which function as switches that are controlled under specific conditions. One such example is a hypoxia response element concatemer combined with either a minimal simian virus 40 promoter or a cardiac-specific promoter that becomes induced under ischemic conditions.[53–55] Other useful promoters are those that manipulate transgene expression pharmacologically such as the tet-on or tet-off systems which are controlled by the antibiotic tetracycline or analogs such as doxycycline.[56,57] In general, the use of tissue-specific or regulatable promoters adds another layer of control over the location, timing and amount of transgene expression and can further increase the safety of virtually any gene delivery system.

GENE THERAPY FOR HEART FAILURE (HF) AND HYPERTROPHY

HF is a chronic, progressive condition in which the heart is unable to pump enough blood to meet the body's demand. In the United States, ~5 million people suffer from HF and it contributes to ~300,000 deaths each year.[58] Several approaches have been developed to ameliorate HF using rAAV-mediated gene transfer. One strategy is through manipulation of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) pump. SERCA2a controls the transport of Ca^{2+} to the sarcoplasmic reticulum during relaxation in the cardiac cycle. Intracoronary rAAV2/1-mediated gene transfer of *SERCA2a* into a swine model of volume-overload HF resulted in positive LV inotropic effects and LV reverse-remodeling through increased of calcium availability.[59] This SERCA overexpression strategy is the basis of the "Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease" or CUPID,

the SERCA2a Gene Therapy in LAVD Patients and the "AAV6-CMV-Serca2a GENe Therapy Trial in HF" or AGENT-HF clinical trials which are discussed in greater detail later in this review.[60]

Another approach to augment Ca^{2+} release is through reduction of phospholamban—an inhibitor of the SERCA2a pump. Competitive inhibition by delivery of a pseudophosphorylated form of phospholamban improved LV systolic function and contractility through enhanced calcium release in cardiomyopathic hamsters and in a rat infarction model.[36,61] Additionally, rAAV-mediated delivery of RNA interference and small hairpin RNA have also shown signs of enhanced contractility and calcium handling *in vivtro* in cardiomyocytes as well as improved cardiac function in a rat model of HF and through direct knockdown of phospholamban expression.[62–64]

Other groups have reversed cardiac dysfunction in models of HF through the inhibition of protein phosphatase 1, a phosphatase that becomes activated in HF and plays a key role in depressed cardiac function. Studies designed to investigate whether protein phosphatase 1 could be repressed by inhibitor-2 (INH-2), an endogenous protein phosphatase 1 inhibitor, showed that rAAV2-mediated *INH-2* delivery through the coronary arterial route increased survival in the cardiomyopathic hamster model of HF by 3 months.[65]

Sick euthyroid syndrome can accompany cardiac HF and hypertrophy and lead to lower serum triiodothyronine (T3) levels, decreased expression of the thyroid hormone receptor isoforms TRα1 and TRβ1 and ultimately lower SERCA expression.[66,67] SERCA expression increases when T3 occupied receptors bind to the thyroid response elements located in the SERCA promoter. A study in mice took advantage of this association by showing rAAV2-mediated overexpression of the thyroid receptor isoforms α1 and β1 improved contractile function in pressure overload-induced cardiac hypertrophy through increased SERCA expression.[66]

In HF, the upregulation of G protein–coupled receptor kinase 2 contributes to dysfunctional β-adrenergic receptor (β-AR) signaling and ultimately impaired cardiac function.[68] The β-AR kinase inhibitor (β-ARKct) can inhibit the activation of G protein–coupled receptor kinase 2 and improve β-AR signaling. The current findings are that rAAV2/6-mediated delivery of β-*ARKct* results in sustained improvement of global cardiac function and a reversal of negative remodeling in a rat model of HF. Interestingly, β-ARKct overexpression improved outcomes to a greater degree than did administration of a β-blocker alone.[69] In sum, a theme that emerges from all of these investigations is that multiple pathways can be approached to manage the many varied aspects of HF. Careful selection of a therapeutic approach that is best suited to address the specific pathobiologies of HF patients will enable future treatments to be tailored to individual needs.

CARDIO-PROTECTIVE GENE TRANSFER

Ischemic heart disease is the lack of blood flow and oxygen to parts of the heart that is caused by a narrowing of the coronary arteries and can ultimately lead to heart muscle damage.[70] Many investigators have worked to develop rAAV gene therapy as a preventative treatment for ischemic heart disease. One strategy involving rAAV2-mediated delivery of transforming growth factor β1 suggested that the hearts of treated rats were protected from ischemia-reperfusion injury via an antioxidant mechanism through reduced activation of nicotinamide adenine dinucleotide phosphate oxidase and nuclear factor κ-light-chain-enhancer of activated B-cells (NF-κB).[71] Another approach promoted neovascularization in ischemic mouse hearts following rAAV2-mediated transfer of the stress inducible enzyme heme oxygenase-1 (*HO-1*) through coinduction of vascular endothelial growth factor (*VEGF*) and stromal-cell derived factor 1 (*SDF-1*).[72]

HO-1 gene transfer has also protected tissue from ischemia-reperfusion injury. rAAV2-mediated transfer of the human *HO-1* gene (*hHO-1*) to rats 8 weeks before acute coronary artery occlusion led to a dramatic reduction (>75%) in LV myocardial infarction size.[73] This was accompanied by decreases in myocardial lipid peroxidation, proapoptotic Bcl-2-associated X protein (Bax) and proinflammatory interleukin-1β protein abundance as well as an increase in the level of antiapoptotic Bcl-2 protein. The investigators concluded that the *hHO-1* transgene exerts its cardioprotective effects by reducing oxidative stress, inflammation, and apoptotic cell death.[73] Similarly, other groups have shown that rAAV-mediated gene delivery of the nuclear factor-κB (*NF-κB*) inhibitor—*iκBα*, the extracellular superoxide dismutase (*Ec-SOD*), and the inducible heat-shock protein 70 (*HSP70i*) are each able to limit infarct size when predelivered to rodent models of ischemia-reperfusion injury.[74–78]

An early *in vitro* study showed that rAAV mediated transfer of the human *VEGF* gene into rat cardiomyocytes increased the concentration of VEGF protein expression both in cells as well as in the culture medium of those myocytes in a dose-dependent manner.[79] Subsequent *in vivo* studies have demonstrated improvement in rodent and dog models of ischemia and infarction following rAAV-mediated *VEGF*, angiogenin, and human growth hormone delivery through increased angiogenesis.[80–83] Future studies will be necessary to evaluate the long-term effects and ensure the safety of each of these approaches to temper the severity and improve outcomes for patients with ischemic heart disease.

Gene transfer can also be a useful method to provide immunoprotection to a heart that is to be transplanted or to a particular area of the heart that is susceptible to injury. Investigators have found that rAAV2-mediated delivery of a dominant-negative suppressor of cytokine signaling (*SOCS1*) transgene in mice increased resistance to acute cardiac injury caused by enteroviral infection. These results imply that inhibition of SOCS in the heart augments the host-cell antiviral system, thus preventing viral-mediated end-organ damage during the early stages of infection.[84] Additional rodent studies that demonstrate protective gene transfer include the prevention of heart allograft rejection and prolonged allograft survival in cardiac transplantation models through rAAV-mediated delivery of *HO-1* or the immunosuppressive *CTLA4Ig* gene.[85,86] Given the existing deficiencies in current management options, a host of additional gene transfer opportunities exist in the realm of cardio-protective gene transfer.

TREATMENT OF METABOLIC OR NEUROMUSCULAR DISEASES THAT HAVE CARDIAC INVOLVEMENT

Extensive studies have been conducted to investigate the use of rAAV-mediated gene delivery to treat rare genetic disorders

resulting in metabolic alterations, such as Pompe disease, Fabry disease, and mucopolysaccharidosis type VII as well as others. Pompe disease is caused by a deficiency in the acid α-glucosidase enzyme and has an estimated incidence of 1 in 40,000.[87] The build-up of glycogen in the lysosomes of severely affected patients leads to an enlarged heart, abnormal electrocardiogram readings and if left untreated it can result in cardiorespiratory failure within the first year of life. Both intramuscular and systemic delivery of acid α-glucosidase using rAAV2 or rAAV2/1 (respectively) have demonstrated clearance of glycogen from the lysosomes of cardiomyocytes and improved cardiac function in a model of Pompe disease as measured by enzyme detection assays and periodic acid Schiff staining as well as magnetic resonance imaging and electrocardiogram analysis.[43,88,89]

A deficiency in the lysosomal enzyme, α-galactosidase A (GLA) results in Fabry disease. The estimated prevalence of Fabry disease in the general population is ~1 in 117,000.[90] With age their vital organs become increasingly affected and Fabry patients can experience complications such as heart disease and stroke. A single injection of rAAV2-*GLA* into the quadriceps muscle of a mouse model of the disease provided structural improvement of cardiac hypertrophy by echocardiographic examination 25 weeks after administration.[91] Similarly, other groups have demonstrated restoration of α-galactosidase A to wild-type levels in the hearts of Fabry mice following a single intravenous injection of rAAV2-*GLA* to either the hepatic portal vein or tail vein.[92,93]

Mucopolysaccharidosis type VII is a lysosomal storage disease caused by deficiency of the acid hydrolase β-glucuronidase. It is estimated to occur in 1 in 250,000 newborns and can cause valvular heart disease and aortic regurgitation.[94,95] Investigators working to find a treatment for mucopolysaccharidosis type VII found that one intrahepatic administration of rAAV2-β-glucuronidase was sufficient to reduce the lysosomal storage deficiency of this disease within the heart via the therapeutic effect of the secreted protein, much like enzyme replacement therapy.[96]

Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is a condition that blocks the conversion of very long-chain fatty acids to energy, particularly during periods of fasting. It occurs in ~1 in 30,000 individuals in the United States and the most rare but severe, early onset form typically presents in the first months of life with hypertrophic or dilated cardiomyopathy, pericardial effusion, and arrhythmias, as well as hypotonia, hepatomegaly, and intermittent hypoglycemia.[97] Studies have shown that a single IV administration of rAAV2/8-*VLCAD* provided long-term cardiac expression and corrected the biochemical phenotype in *VLCAD*-deficient mice.[98]

Other examples of genetic diseases affecting the heart that may be treatable using a gene therapy approach are those which are primarily neuromuscular disorders. Several groups have demonstrated successful treatment of the cardiac phenotype in animal models such as the dystrophic hamster (Bio 14.6, Bio TO-2) which has a defect in the δ-sarcoglycan (*SGCD*) gene. This was achieved by delivery of rAAV2 or dsAAV2/8-*SGCD* through either intramural, intraperitoneal, intravenous administration, direct infusion of the vector into the coronary artery *ex vivo* in a heterotopically transplanted heart and by transcoronary rAAV2-mediated transfer of a dominant-negative form of apoptosis signal-regulating kinase 1.[35,45,99–101]

The *mdx* mouse model of Duchene muscular dystrophy has also been utilized as a tool to test cardiac gene delivery methods. Investigations have shown that rAAV2 delivery of the microdystrophin gene to the newborn *mdx* mouse cardiac cavity yields efficient expression throughout the myocardium, restores the critical dystrophin-glycoprotein complex and improves sarcolemmal integrity in the heart for up to at least 10 months postadministration.[102] Additional studies showed that systemic administration of rAAV2/6 carrying microdystrophin provided restoration of cardiac geometry and prevented dobutamine-induced cardiac failure.[103] Once rAAV-mediated treatments are confirmed to be safe, diseases such as these that result from single gene defects will likely be among the first to be primarily treated using gene therapy approaches.

GENE TRANSFER TO INFLUENCE CARDIAC ELECTROPHYSIOLOGY

Several investigations have utilized rAAV as a delivery tool to manipulate cardiac electrophysiology. One study showed that rAAV2-mediated delivery of the voltage dependent, 4-aminopyridine-sensitive outward potassium current (*Kv1.5*) gene provided long-term normalization of the action potential duration in a mouse model for long-QT syndrome.[104] Another study corrected the characteristically shortened PR interval in a mouse model of Pompe disease following rAAV2/9-mediated delivery of the acid α glucosidase (acid α-glucosidase) gene necessary for glycogen hydrolysis in lysosomes.[18] Others have shown that rAAV2/9 transfer of the microdystrophin gene is able to ameliorate the electrocardiographic abnormalities in the *mdx* mouse model of Duchenne muscular dystrophy.[105] In principle, a number of other channel genes could be accommodated into rAAV vectors for gain of function studies. Additionally, arrhythmias could be alleviated through rAAV-mediated gene-specific knockdown of dominant mutations. Finally, there is the potential to establish ectopic automatic pacemaker activity, which would be especially useful for the treatment of complete congenital heart block or other situations which require long-term restoration of intrinsic pacemaker activity.

rAAV AS A TOOL TO UNDERSTAND *IN VIVO* PROCESSES

In addition to being used as a therapeutic delivery system, rAAV can also be a valuable tool for increasing our knowledge of *in vivo* biological processes. The protective anti-inflammatory and antiapoptotic effects of smooth muscle were revealed following rAAV2-mediated intracoronary infusion of angiopoietin 1 and 2 into rat-cardiac allografts.[106] Further investigations also uncovered the diverse effects of platelet-derived growth factor (PDGF) ligands in cardiac allograft vasculopathy and fibrosis following administration of rAAV2 carrying *PDGF-A, B, C* and *D*. This work led to the suggestion that a targeted therapy using monoclonal antibodies to block the active sites of PDGF-A, -C, and -D may be beneficial to heart transplant survival.[107]

A novel target for regenerative therapy was discovered in a study that showed proliferation of immature rat cardiomyocytes could be stimulated through the NOTCH1 signaling pathway following rAAV2/8 delivery of the activated form of *NOTCH1*.[108] Other investigations revealed the mechanisms by which hypoxia/

reperfusion induces signals for remodeling through blockade of hypoxia-reoxygenation-mediated collagen type I expression and matrix metalloproteinase activity following rAAV2-mediated overexpression of transforming growth factor β1 in mouse cardiomyocytes.[109]

To gain a better understanding of the role of angiotensin-converting enzyme type 2 (ACE2) in the regulation of cardiac structure and function, a study was conducted which showed induction of fibrosis in the myocardium of stroke-prone spontaneously hypertensive rats following administration of rAAV2/6-*ACE*.[110] Finally, the ability of hypoxia-induced mitogenic factor (HIMF) to provoke the vascular and hemodynamic changes observed in pulmonary hypertension was established following *HIMF* gene transfer using rAAV2 to transduce rat lungs.[111] Beyond gene transfer studies, increased use of rAAV vectors to introduce stable, long-term expression of gene-specific silencing sequences could yield vast amounts of information regarding cardiac development and cardiovascular disease progression.

TRANSDUCTION OF CELLS PRIOR TO CELL THERAPY

Overall, rAAV has not been the most efficient vehicle option for stem cell transduction. One key investigation showed that lentiviral vectors achieved significantly higher transduction efficiencies in cardiosphere-derived resident cardiac stem cells than any of nine rAAV serotypes tested.[112] Despite this, other researchers have found that mesenchymal stem cells can be genetically manipulated through rAAV-mediated delivery of tumor necrosis factor receptor (*TNFR*). Transfer of these modified mesenchymal stem cells improved LV function in a rat model of myocardial infarction through antiapoptotic and anti-inflammatory mechanisms.[113] Other studies established that the modification of tyrosine on the AAV2 capsid increases viral transduction of stem cells.[114,115] Novel approaches to direct the molecular evolution of AAV capsids will allow for the selection of additional unique structures with affinities for specific stem cell types.[23,116]

CLINICAL TRIALS

A careful assessment of data from preclinical biodistribution and toxicity studies to evaluate the safety profile of a gene transfer treatment is absolutely necessary prior to introduction of that approach to the clinic. One of the foremost aims of biodistribution studies is to assess the potential spread of vector sequences beyond the intended site of delivery. The chosen route of delivery greatly impacts this behavior. A study evaluating the biodistribution of rAAV2 vector DNA following myocardial delivery in rats showed that vector expression in extra cardiac tissues such as liver, kidney, and testes was present 6 months postadministration.[117] Other detailed studies in mice and rabbits were designed to track the biodistribution of rAAV2/1 vectors delivered to striated muscle. Those results indicated transient distribution to blood at high doses; however, no toxicities were observed.[118] It is of the utmost importance that studies such as these be performed with a well-characterized vector stock. Additionally, they must be conducted using a good laboratory practice design under an established protocol with well-defined endpoints.

A cellular immune response to transgenes encoding foreign proteins is another essential consideration for rAAV-based cardiac gene therapy. One investigation demonstrated this significance through a comparison between the delivery of empty rAAV2 capsids and rAAV2-mediated delivery of the human TNF receptor II immunoglobulin G-Fc fusion protein (rAAV2-*TNFRII-Fc*) following direct injections into baboon hearts.[119] The results showed that the baboons which had received rAAV2-*TNFRII-Fc* developed myocardial infiltrates including $CD8^+$ cells whereas those receiving empty rAAV2 capsids did not. This study highlights the importance of investigations into transgene as well as delivery vehicle safety in a large animal model where the response to transgene expression may be more relevant to human clinical studies.

A clinical trial to evaluate intracoronary administration of rAAV2/1-*SERCA2a* in patients with HF was recently performed.[120] The initial study evaluated the potential for calcium upregulation by percutaneous administration of gene therapy in cardiac disease (CUPID).[121] As a phase I study, the focus was on safety and this was demonstrated in the open label portion of the trial. No significant changes were observed in exams of major organs, blood pressure, heart rate, or body temperature following rAAV2/1-*SERCA2a* administration. Of those adverse events that were observed only two events of fatigue, one of a fever, and one of muscle spasms were determined to have been possibly related to rAAV2/1-*SERCA2a* delivery. Significantly, the two patients in the trial with pre-existing neutralizing antibodies (Nab) to the AAV1 capsid failed to improve while several of those without pre-existing Nab did demonstrate improvement as determined by several endpoints over a 6-month period. Recent evaluation of patients enrolled in the phase II double-blind, placebo controlled portion of the trial has showed a favorable safety signal and demonstrated early indications of efficacy. These included a decreased frequency of cardiovascular events per patient from each of the three dose cohorts.[122] Importantly, no increases in adverse events, disease-related events, laboratory abnormalities, or arrhythmias were observed in any of the treated patients compared to those receiving placebo.[60]

Two other cardiac gene therapy trials are being conducted which employ rAAV6 as the gene delivery vehicle for SERCA2a. The SERCA2a Gene Therapy in LAVD Patients is a currently enrolling phase I/II trial in the United Kingdom that is targeting patients with advanced HF that have received an LV assist device.[60] The AGENT-HF trial is a currently enrolling phase II trial in France that is focused on determining the effects of *SERCA2a* delivery on cardiac remodeling.[60] Although larger studies will be required to truly establish rAAV-*SERCA2a* as a proven treatment modality for advanced HF, these highly promising initial results substantiate the utility of rAAV as a clinically relevant vehicle for cardiac gene delivery.

CONCLUSIONS

Years of investigation using a number of gene delivery systems have provided a firm foundation of data recognizing rAAV as a preferred vehicle to achieve stable cardiac gene transfer. The high efficiency of transduction by newly characterized AAV capsids, both natural and those derived from directed evolution is an exciting advance in the field that opens the door to additional clinical opportunities.[23,123] The field is slowly but steadily designing an ideal cardiac gene delivery

system which will combine the utilization of a safe and highly cardiotropic capsid with an optimized delivery route and a tissue-specific and/or regulatable promoter. The most attractive transgene for each application would be those in which the expressed protein remains safe even after many years of sustained expression. While great advances have been made in all of these areas, the incorporation of optimized capsid, promoter, route and transgene into one gene delivery system is still under construction.

An important consideration for advancing rAAV gene delivery in the clinical arena will continue to be the presence of pre-existing antibodies or memory B-cells to AAV capsids which may diminish efficacy.[121] Solutions to the challenges presented by pre-existing immunity are currently being evaluated. One simple option to minimize this effect is to choose vectors in which the presence of neutralizing antibodies to those capsids in the sera of healthy humans is rare and/or minimal.[87]

Another remaining challenge to moving rAAV-mediated therapies to the forefront of biological medicine is large scale clinical-grade production of the virus. While there have been several advances in the area of saleable rAAV vector manufacturing, these methods have yet to be widely implemented.[124–126] Although the manufacturing of biologics will always be more costly than that of pharmaceuticals, the durable therapy provided by such treatments could ultimately result in a more commercially viable product. These factors are certainly a strong driving force behind the clinical development of therapeutics that could benefit large patient populations.[124,126]

The ability to provide highly specific treatment for the spectrum of inherited and acquired heart disease makes cardiac gene therapy an important clinical approach that fulfills the principles of molecular medicine. During the next phase of rAAV vector development for cardiac gene transfer, we should continue to see useful experimental tools develop into viable clinical opportunities.

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