Abstract: Gene therapy, aimed at the correction of key pathologies being out of reach for conventional drugs, bears the potential to alter the treatment of cardiovascular diseases radically and thereby of heart failure. Heart failure gene therapy refers to a therapeutic system of targeted drug delivery to the heart that uses formulations of DNA and RNA, whose products determine the therapeutic classification through their biological actions. Among resident cardiac cells, cardiomyocytes have been the therapeutic target of numerous attempts to regenerate systolic and diastolic performance, to reverse remodeling and restore electric stability and metabolism. Although the concept to intervene directly within the genetic and molecular foundation of cardiac cells is simple and elegant, the path to clinical reality has been arduous because of the challenge on delivery technologies and vectors, expression regulation, and complex mechanisms of action of therapeutic gene products. Nonetheless, since the first demonstration of in vivo gene transfer into myocardium, there have been a series of advancements that have driven the evolution of heart failure gene therapy from an experimental tool to the threshold of becoming a viable clinical option. The objective of this review is to discuss the current state of the art in the field and point out inevitable innovations on which the future evolution of heart failure gene therapy into an effective and safe clinical treatment relies. (Circ Res. 2013;113:792-809.)

Key Words: cardiac ■ clinical translation ■ gene therapy ■ heart failure
Gene-based therapies are gaining momentum from the incremental number of forthcoming clinical trials in human disease. With the recent approval of Glybera by the European Medical Agency (EMEA) as the first gene-based treatment of defective lipoproteinlipase activity, human gene therapy has become a clinical reality. The treatment introduces a normal lipoproteinlipase gene packaged in a delivery vector derived from adeno-associated virus (AAV), serotype 1 (AAV1), which conveniently has a natural propensity toward muscle cells, the tissue mainly contributing to healthy lipoproteinlipase protein production. Glybera is administered via a 1-time series of small intramuscular injections.

In human end-stage heart failure (HF), the Calcium Up-regulation by Percutaneous administration of gene therapy In cardiac Diseases (CUPID) trial (NCT00454818) pursues therapeutic levels of the calcium (Ca²⁺)-handling enzyme sarcoplasmic reticulum Ca²⁺ ATPase (SERCA2a). The second HF gene therapy phase I/II trial (Ad5.hAC6 Gene Transfer for Congestive Heart Failure; NCT00787059) seeking Food and Drug Administration (FDA)–granted investigational drug status uses human adenylyl cyclase 6 (AC6) as therapeutic target. If positively evaluated in forthcoming phase III studies, this novel therapeutics may open a new chapter in HF therapy. To date, these trials epitomize the quest for novel molecular-targeted HF treatments that could improve conventional clinical regimes, which cannot target underlying molecular defects of failing cardiomyocytes.

HF gene therapy refers to a therapeutic system that uses cardiac-directed delivery technology and viral vectors for biological drug administration to the diseased heart (Figure 1). Formulated DNA or RNA is used to intervene directly within the genetic and molecular foundation of diseased cardiomyocytes with the ultimate aim to correct key molecular defects being out of reach for conventional drugs. Using the cells own transcriptional and translational machinery, the classification (ie, inotropic) is determined by the biology of the DNA/RNA product. Since the first reports of in vivo gene transfer into the myocardium, there has been a series of advancements that have driven the evolution of an experimental tool to the threshold of becoming clinical reality.

Focusing on HF gene therapies that directly restore systolic cardiomyocyte performance, this review discusses state-of-the-art concepts and developments with respect to vectors, delivery technology, expression systems, and translation of targets into effective therapies. With respect to cardioprotective, paracrine, lusitropic, and angiogenic target biology, we refer to previously published excellent reviews. This article further highlights current needs to develop pharmacokinetic (PK) and pharmacodynamic (PD) models so that HF gene therapy can unleash its full potential as an effective and safe clinical treatment. It finally introduces the National Institutes of Health (NIH) Gene Therapy Resource Program (GTRP; http://www.gtrp.org) as catalyst for clinical translation and advocates the integration of systems biology to HF gene therapy.

<table>
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<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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<tr>
<td>AAV adeno-associated virus</td>
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<tr>
<td>AC6 adenyl cyclase 6</td>
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<td>Ad adenovirus</td>
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<td>β-AR β-adrenergic receptor</td>
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<td>βARKct β-adrenergic receptor kinase C terminus</td>
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<td>CMV cytomegalovirus</td>
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<td>CUPID Calcium Up-regulation by Percutaneous administration of gene therapy In cardiac Diseases</td>
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<tr>
<td>EMEA European Medical Agency</td>
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<td>FDA Food and Drug Administration</td>
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<td>GRK2 G-protein–coupled receptor kinase 2</td>
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<tr>
<td>GTRP Gene Therapy Resource Program</td>
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<tr>
<td>LV left ventricular</td>
</tr>
<tr>
<td>MI myocardial infarction</td>
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<tr>
<td>MLC myosin light chain</td>
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<tr>
<td>PD pharmacodynamics</td>
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<tr>
<td>PK pharmacokinetics</td>
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<tr>
<td>rAAV recombinant adeno-associated virus</td>
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<tr>
<td>SERCA2a sarco(endo)plasmic reticulum Ca²⁺ ATPase 2a</td>
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**Figure 1. Heart failure (HF) gene therapy concept.** HF gene therapy uses viral vectors such as adeno-associated vectors (AAV; 1) to deliver therapeutic DNA and RNA to nuclei of dysfunctional cardiomyocytes and (2) to intervene directly within the genetic and molecular foundation of the cells. Myocardial infarction is shown as a common origin for HF development. Ultimate aim is targeted correction of key molecular defects being out of reach for conventional drugs using the cells own transcriptional and translational machinery (3). Adapted with permission from Davis et al (Physiol Rev. 2008;88:1567–651). Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
Safe and Efficient Viral Vector Systems for Clinical Cardiac Gene Delivery

Among different nonviral and viral vector systems developed for tissue and organ gene delivery, recombinant AAVs (rAAV) have emerged as the most valuable gene transfer agents available today (Figure 2). Because of their efficiency and safety in transducing both dividing and nondividing cells, rAAV vectors are used in >99 clinical trials worldwide (http://www.abedia.com/wiley/vectors.php).12,13 Promising results have been obtained from phase I/II trials, including Leber’s congenital amaurosis, hemophilia B, Parkinson disease, cystic fibrosis, and, most recently, chronic HF. AAV1 is the viral vector used by the first EMEA-approved gene therapy targeting a rare human disease caused by defective lipoproteinlipase.1

Wild-type AAV was first discovered as a 20-nm, icosahedral contaminant in adenovirus (Ad) preparations and belongs to the family of Parvoviridae.12,14 It is a nonpathogenic, nonenveloped DNA virus containing a linear single-stranded genome of 4.6 to 4.9 kb that requires coinfection with a helper virus for viral replication. Its genome consists of 2 open reading frames flanked by 145 base pair inverted terminal repeats. The genes encode alternatively spliced capsid (cap) proteins and multifunctional replication (rep) proteins (Figure 3A). To generate rAAV for gene delivery, a plasmid devoid of cap and rep genes is designed containing the control region and complementary DNA of interest flanked by wild-type AAV inverted terminal repeats.12,13 The only cis-elements apparently required for rAAV vectors are the inverted terminal repeats that flank the recombinant viral genome and aid in episomal concatamer formation in the nucleus after the single-stranded vector DNA is converted by host DNA polymerase into double-stranded DNA.

The size of promoter elements and transgene is ideally between 4.1 and 4.9 kb for efficient packaging. The involved lack of expressing viral genes most likely contributes to the favorable low immunogenicity of rAAV when compared with other vectors.15,16 In contrast to the ability of wild-type AAV to integrate specifically into the host chromosome 19q13.4, rAAV serotypes used for cardiac gene delivery applications have been shown to persist largely as an episome for long periods of time after transduction. This accounts for >24 months of reported long-term cardiac expression duration, which renders AAV highly suitable for chronic HF therapy.17,18 Figure 3B illustrates key steps of cellular entry of AAV and its nuclear import in target cells.

Inherent cardiotropic, that is, supposedly preferential cardiomyocyte transduction of distinct rAAV serotypes such as rAAV9 in rodent models, adds to the attractive profile of the vector as current viral vehicle of choice irrespective of the in vivo delivery mode. Systemic application of rAAV9, for example, in rodents transduced both the heart and the extracardiac tissue (see the following section for details), but usage of a cardiac-directed promoter sequence restricted long-term expression of the encoded protein to the heart.19,20 Thus, the term cardiotropic for these vectors might somehow be misleading because it suggests cardiac restriction, which does not exist so far. Nonetheless, different options are currently being explored to develop rAAV mutants with optimal cardiotropic, cardiac specificity being the ultimate goal. One such possibility is transductional targeting, meaning directed capsid engineering and subsequent creation of mutant rAAV libraries.13,21 This can be achieved by random ligation of DNA fragments derived from different rAAV serotypes each encoding a different domain of the capsid protein; a technique also referred to as capsid DNA shuffling. Another method, directed

![Figure 2. Vectors commonly used in cardiovascular gene transfer and specific characteristics.](http://circres.ahajournals.org/)

To date, naked plasmid DNA, adeno-associated viruses (AAV), and adenovirus are used in 18.2%, 5.2%, and 23% of 1.902 registered gene therapy clinical trials (see www.abedia.com/wiley/vectors.php for continuous update).
capсид evolution, starts with the insertion of engineered peptide sequences into the AAV capsid thereby focusing on the major capsid protein VP3, which mostly accounts for receptor binding of AAV serotype to their target cells.16,21

Both methods select the most promising cardiotope rAAV variants by their either in vivo or in vitro transduction efficiency, and the corresponding capsid DNA is identified and sequenced by polymerase chain reaction amplification. In light of well-known differences of AAV serotypes with regard to species, optimization is mostly limited to the in vivo model used and cannot be extrapolated to other species, for example, from mouse to men. Repeated in vitro selection, on the other hand, is expected to deliver mutant rAAVs with optimal tropism toward isolated cardiomyocytes. Such selection neither predicts nor indicates in vivo cardiotropism, so that the ability of rAAV variants to overcome the endothelial barrier and interstitial space efficiently, as well as escape neutralizing antibodies, must be tested in vivo. Hence, transductional targeting is promising but of limited relevance when it comes to development of clinically applicable rAAVs and their cardiac specificity in humans.12,22

Previously developed replication-deficient Ads (rAds) that also transfact both dividing and nondividing cardiac cells are still predominantly used in current clinical gene therapy trials. To date 437 clinical trials were registered using rAds (http://www.abedia.com/wiley/vectors.php).1,21 The most prominent example in cardiac gene therapy is the Ad5.hAc6 Gene Transfer for Congestive Heart Failure trial. Because antegrade coronary delivery was chosen as clinical delivery mode, the investigators preferred rAd5 to rAAV because of its natural ability to cross the endothelial barrier efficiently.3 As a major advantage, rAds provide a packaging capacity of ≤30 kb, exceeding by far the AAV size. First- and second-generation rAds, nonetheless, bear the disadvantage of significant immunogenicity, which reflects in a transient expression because of immunogenic elimination.20 Because a rAd-triggered innate immune response resulted in a previous trial participant’s death,29 a potential immune reaction poses obstacle safety issue to the wide-spread use of rAds tipping the balance toward AAVs for clinical cardiac gene delivery.

Experimental and Clinical Modes of Myocardial Gene Delivery

The most optimal gene delivery system for cardiac therapeutic applications in humans will combine a clinically advantageous physical delivery route with the rAAV serotype that has the highest natural or engineered affinity for the targeted cardiac cell type. Additional use of a cell-type biased expression cassette (see Cardiac-Targeted and Regulated Expression Systems section for details) is expected to confine therapeutic gene expression to the targeted organ. Ideally, this procedure is safe and efficient when it comes to translation in humans. In the following section, we will briefly discuss delivery methods in small animals and focus on administration routes in preclinical large animal readily extrapolatable to humans or already used in phase II/III trials.

Myocardial Gene Delivery in Small Animal Models

Intravenous gene delivery via tail, jugular, or retro-orbital veins is the least invasive technique to administer a vector for myocardial gene delivery,17,18,26 but highly dependent on affinity of the vector toward cardiomyocytes. A recent study has shown that intravenous application of 1x1011 viral particles of rAAV9 in wild-type C57B/6 mice harboring the β-adrenergic receptor kinase C terminus (βARKct) cDNA driven by a cardiac-biased promoter resulted in homogeneous cardiac expression confined to cardiomyocytes.19 This approach is deemed highly useful for proof-of-concept but also for dose-dependency studies of DNA and RNA therapeutics in rodent models yielding necessary PK/PD data for definition of therapeutic window and threshold for adverse effects (see PK and PD of Cardiac Gene Therapeutics section for details). Although another study has reported tropism of AAV9 toward cardiac and skeletal muscle in nonhuman primates,26 improved transductional targeting is needed until this technique can be considered for use in human-relevant large animal HF models.

Intracoronary gene delivery in small animals has been used widely to enhance viral transduction efficiency in hearts via cardiac-directed application.27-30 The ascending aorta is cross-clamped, and injection of the vector solution into the aortic root or into the left ventricle via catheter enables antegrade intracoronary infusion. Reported myocardial transduction
rate, assessed by reporter genes, varied between 20% and 50% when $1 \times 10^{10}$ to $1 \times 10^{12}$ viral particles both of rAAV2, rAAV6, and rAd5 were used. Efficiency can further be increased by infusion of histamine, adenosine, and various other NO donors to enhance endothelial permeability.

Pericardial gene delivery has also been tested for rAd in small animal models and offers the advantage of targeted gene delivery to pericardial cells. The epicardial layer limits transduction of adjacent myocardium, and use of various pharmacological agents and proteolytic enzymes to overcome the barrier bears potential for cardiac damage. The technique thus yields insufficient cardiomyocyte transduction for proof-of-concept studies.

**Myocardial Gene Delivery in Translational Studies and Large Animal Models**

Antegrade coronary artery gene delivery is a minimally invasive percutaneous catheter-based injection into the coronary arteries (Figure 4A). The technique is clinical routine and, principally, gene delivery can be safely directed to each area of the heart. However, endothelial dysfunction, atherosclerosis, and occlusion of coronary arteries in humans with coronary artery disease might limit myocardial transduction efficiency at the cost of greater viral spill-over. Passage time of the delivered vector further depending on regional perfusion differences is usually short. A significant amount of the applied vector dosage is usually washed out into systemic circulation where it is subject to renal excretion or hepatic transduction. A first approach to enhance myocardial retention is temporary occlusion of the coronary artery and the corresponding coronary vein (Figure 4B and 4C), which increased myocardial gene expression significantly, but bears potential for cardiac damage. Some studies showed only a few β-gal-positive–stained cardiomyocytes after intracoronary Adβ-gal delivery in pigs at a dosage of $5 \times 10^{12}$ viral particles. Others reported on increased left ventricular (LV) function when AAV1-SERCA2a or Ad5-ACVI was delivered via coronary arteries at a dosage of $1 \times 10^{11}$ and $1.4 \times 10^{12}$ viral particles without reported myocardial transduction rates.

Various chemical agents have been tested to increase myocardial transduction, eventually advocating NO-dependent enhancement of vascular permeability and virus transmigration. Periprocedural intracoronary NO donor delivery, such as nitroglycerine, was routinely used in the CUPID and Ad5.hAC6 Gene Transfer for Congestive Heart Failure trials. In the quest for ideal myocardial transduction, a recirculating, closed-loop system between the coronary sinus and the left main coronary artery using a peristaltic pump (V-Focus) has been developed. Using V-Focus, AAV2-SERCA2a gene delivery in pacing-induced HF in adult sheep demonstrated dose-dependent ($1 \times 10^{10}$, $1 \times 10^{12}$, and $1 \times 10^{13}$ DNAse-resistant particles) beneficial functional and structural effects, but myocardial transduction rates were not available. The ambitious procedure might show its advantage, optimized vector dosage with efficient myocardial transduction, when handling can be further simplified and costs diminished to meet demands of a daily clinical routine. Because of superior safety and feasibility, clinical HF gene therapy trials testing SERCA2a and AC6 use percutaneous catheter-based antegrade vector injection into the coronary arteries. Naturally, evidence of myocardial transduction cannot be provided but results of phase Iia of the CUPID trial are encouraging. However, efficiency in patients with coronary artery disease is a matter of ongoing debate, and anticipated results of phase III of the CUPID trial might provide clarification.

Retrograde coronary venous gene delivery is based on percutaneous minimally invasive retrograde myocardial gene...
delivery via the coronary venous system (Figure 4E). The clinical use of this method is increasing because of the demand for cardiac resynchronization therapy. The anterior cardiac vein is sealed by a balloon, whereas the vector solution is retroinfused via catheter so that hibernating, viable myocardium can be targeted when corresponding coronary arteries are occluded. Passage time is significantly prolonged as compared with antegrade injection, and myocardial gene delivery was further enhanced when coronary arterial blood flow was temporarily blocked. When used in human-relevant large animal HF models, retrograde coronary venous delivery of $1.5 \times 10^{13}$ AAV9–S100A1 and $1.0 \times 10^{13}$ AAV6–βARKct particles was safe, rescued cardiac dysfunction, and reversed remodeling because of sufficient myocardial transduction.

Interestingly, significant myocardial transfection was also achieved by retrograde delivery of plasmid DNA, which deserves further consideration.

Percutaneous intramyocardial injection describes a nonsurgical minimally invasive technique of intramyocardial gene delivery that requires a catheter with a retractable injection needle and imaging guidance modalities for determining the injection site. Among mapping systems, fluoroscopy, echocardiography, and MRI, the NOGA electromechanical mapping system is the most commonly used guiding system used in clinical angiogenesis trials. With therapeutic DNA/RNA formulations acting inside cardiomyocytes, the system might not allow sufficient transduction efficiency but might act favorably when a therapeutic paracrine factor can be used.

Surgical administration of vectors is convenient in patients undergoing open-heart surgery. Antegrade or retrograde gene delivery via coronary arteries during aortic cross-clamping for 30 minutes yielded highly efficient and selective myocardial gene expression. Refinement of selective retrograde coronary sinus infusion and recirculation of an AAV6–βARKct vector resulted in robust, although not quantified, gene expression and increased adrenergic reserve in sheep. In addition, the exposed heart is amenable to targeted intramyocardial injection of therapeutic vectors.

Cardiac-Targeted and Regulated Expression Systems

To date, no delivery system or AAV serotype with optimized cardiotropism can rule out vector leakage or transduction of noncardiac tissues, respectively. An efficient way to restrict therapeutic transgene expression is the use of cardiac-selective promoters referred to a transcriptional targeting: As concluded from numerous studies, engineered expression cassettes combining minimal fragments of genes that are exclusively expressed in cardiomyocytes with short enhancer sequences derived, that is, from viral promoters have shown most promising results with varying transgenes both in small and in large animal disease models. One example is the ventricle-specific myosin light chain-2 (MLC-2v) promoter ligated to a cytomegalovirus (CMV) enhancer fragment driving cardiac-selective expression both of S100A1 and βARKct without detectable noncardiac tissue expression after retrograde intravenous cardiac delivery. Another selective promoter uses a fragment of the cardiac α-actin enhancer sequence attached to the elongation factor 1α to achieve cardiac-specific expression of the same transgenes.

Importantly, after intracoronary and intravenous delivery, no unwarranted expression of S100A1 or βARKct was observed in noncardiac tissues.

Hence, transcriptional targeting further aids in decreasing the risks of systemic or side effects, rendering these systems suitable for human use. It is, however, important to note that in analogy to conventional drugs, the therapeutic window of a therapeutic gene product can vary widely. As seen with many transgenes, there is a considerable amount of empiricism in the choice of the promoter. Before its administration, a constitutively active one typically yields unpredictable expression levels that could lie either inside or outside the therapeutic window accounting for inefficiency and toxicity. Just as efficacy of conventional drugs relies on accurate understanding of dose–response relationship and PK/PD data in view of therapeutic window and fluctuations of disease severity, so does effectiveness of therapeutic gene delivery, thus demanding adjustable expression level and time course of the gene product. This type of control may be achieved by incorporation of engineered gene switches (Figure 5). Among the various available gene-switch platforms, the insect-derived ligand-inducible, ecydysoid receptor system, which is refractory to human endogenous steroids, typically shows low basal transgene expression, broad dose–response gradation, and high inducibility. It usually outperforms other systems such as the tetracycline- or rapamycin-regulated systems that have been used in the context of AAV vectors.

In addition, expression cassettes have been developed whose activity is governed by a disease condition. One such example is the hypoxia response element concatamer of the erythropoietin gene combined with a cardiac-specific promoter fragment that becomes active only under ischemic conditions. Other useful combinations currently under development comprise shorter versions of the brain natriuretic peptide promoter to control therapeutic transgene expression in the heart’s response to mechanical stress. In turn, brain natriuretic peptide–driven therapeutic genes capable to improve contractile performance and thereby reducing ventricular wall stress could establish a therapeutic feedback loop that automatically adjusts the level of transgene expression to changes in ventricular wall tension. In light of the limited packaging capacity of AAV vectors, targeted and inducible systems, however, still come at the price of reducing the allowable size of the therapeutic transgene to $0.8$ to $1$ kb. Options are currently being explored to overcome the limited coding capacity. One such possibility is that the AAV inverted terminal repeats of 2 genomes can anneal head to tail to form concatamers, almost doubling the capacity of the vector.

For future clinical use, spatial and temporal control of the expression of the therapeutic gene product should, therefore, not just be considered as merely desirable but rather as an indispensable instrument to adjust the intracellular dosage to treat effectively while staying within the safety range. Such clinical implicitness eventually constitutes the necessity for development of alternative vector systems beyond current AAV technology providing sufficient capacity for targeted and regulated expression systems. As we look forward to the
next generation of cardiac gene therapy studies, constitutively active, nontargeted expression is likely to lose ground in favor of more advanced technologies.

Translational Strategies for Efficient Preclinical Development of HF Gene Therapeutics

It was anticipated that therapeutic targets identified and tested in animal models with critical features of HF in humans have a higher likelihood of translating to patients (Figure 6). Given distinct advantages and shortcomings of small and large animal HF models, efficient preclinical translation rests on targeted use of model systems that best fit specific requirements of each developmental stage. Sound understanding of the molecular mode of action of a target is a conditio sine qua non before any translational attempts to validate a predicted therapeutic potential. All targets originating from reverse (bedside to bench and

Figure 5. Cardiac-biased controllable gene expression systems. Transcriptional control using a cardiomyocyte-biased controllable gene expression system (1). In the absence of the oral ligand (OFF-state), the cardiomyocyte specifically expressed ligand receptor (LR; blue) and activator domain (AD; orange) exist in an inactive conformation and transcription is kept off (2). Ingestion of the oral ligand leads to the ON State: in the presence of ligand (red), the 2 proteins (LR and AD) stably dimerize. The complex in an active conformation binds to the response element of the codelivered therapeutic gene and transcription is turned on. IRES indicates internal ribosomal entry site.

Figure 6. Translational strategy for preclinical heart failure (HF) gene therapy development. Integrative pipeline for preclinical development of HF and other cardiovascular gene therapy. For successful clinical implementation, experimental therapeutic proof-of-concept and molecular profile are ideally determined in molecular and small animal models (Basic Science). Human-relevant large animal disease models are key for translation toward use in humans enabling development and testing of clinically applicable delivery technology and target efficiency and safety. Data gleaned from this stage are essential for investigational drug (IND) status application and transition to clinical safety and exploratory dosing (phase I/II) trials in humans. Efficacy assessment (phase III) concludes successful clinical translation. Adapted with permission from Ritterhoff and Most. Authorization for this adaptation has been obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.
back) or forward (bench to bedside) translation require proof of their therapeutic potency in preclinical disease models. Despite differences to humans with respect to cardiac physiology (ie, higher heart rate, larger SERCA2a/sodium/calcium exchanger (Na+/Ca2+ exchanger) ratio, and difference in energy homeostasis, anatomy and molecular composition (ie, higher capillary density, smaller hearts, and different sarcomeric proteins), rodents will remain the cornerstone for proof-of-concept studies allowing for efficient testing of phenotype and outcome.54

Moreover, cost-effectiveness, smaller dosages of vectors, and various available cardiac disease models (ie, permanent myocardial infarction [MI], ischemia/reperfusion, transaortic constriction) together with advanced echocardiography, invasive hemodynamic assessment, and MRI technology enable efficient analyses. In addition, numerous high-throughput tools have been developed toward rodent models enabling large-scale collection of molecular data and holistic mechanism of action (see PK and PD of Cardiac Gene Therapeutics section for details) associated with the phenotype. Taken together, ease of therapeutic manipulation and insight into molecular mechanisms in greater animal numbers with more substantial statistical power makes them an indispensable part of a standardized translational platform concept to guide development of HF gene therapeutics.

Human failing and normal cardiomyocytes are other highly valuable models to assess the therapeutic efficacy of a target.20 Although difficult isolation procedure and scarcity of explant ed human hearts pose a challenge for sufficient cell numbers and quality, the in vitro model remains the only option to test molecular therapeutics in diseased human myocardium. Importantly, human cardiomyocytes provide features not present in rodent cardiomyocytes, such as force-frequency response and β-adrenergic receptor (β-AR) compositions, strengthening the predictive value of a positively evaluated therapeutic target. With a sound understanding of its therapeutic mode of action and efficiency in small animal models, a target is principally amenable for translation to large animal HF models.54

Because of closer approximation of human cardiovascular physiology and pathology, large animal models are the critical step in the translation of advanced drug, cell, and gene therapeutics to clinical practice.55,56 They pose practical questions as to scalability of a particular therapeutic, dosage, and delivery method. Studies in large animal models usually require 10- to 100-fold greater amounts of therapeutic viral particles and provide preclinical data with respect to feasibility, efficacy, and biological safety for novel therapies before FDA-approved commencement of clinical trials.57 Pigs have emerged as the species of choice in the field of cardiac gene therapy for several reasons.58,59 Although pig genome mapping has successfully spurred the evolution of high-throughput omics technologies, a broader use of the model is still hampered by a lack in basic molecular biology tools (ie, antibodies approved or designed for the use in pigs). In addition, new research facilities often lack the considerable space and resources demanded by large animal facilities, and to perform preclinical studies requires a multidisciplinary team of scientists and clinicians. Ideally, research and clinical facilities provide the teams at this stage with access to state-of-the-art clinical and molecular diagnostic and analytic tools, ranging from hemodynamic (ie, MRI, pressure-volume-loop measurements, and echocardiography), electrophysiological (ie, 3-dimensional cardiac mapping, telemetric ECG recordings) to metabolic (ie, positron-emission tomography [PET]-computed tomography [CT], single photon emission computed tomography, and metabolic assay platform) and systems biology tools. Assembling such a powerful toolkit is necessary to advance the informative value of the costly model beyond integrative cardiovascular physiology.

The NIH GTRP (http://www.gtrp.org) provides a framework to promote clinical translation of advanced HF gene therapies to enter the application process for an investigational drug status. As a unique catalyst of gene-based therapies, the GTRP provides professional support in the area of required good manufacturing/laboratory practice testing of therapies, pharmacology–toxicology studies, good manufacturing practice–grade vector production, and regulatory support in accompanying interactions with the FDA. The next steps include design, planning, and cost calculation of forthcoming clinical trials, selection of competent good manufacturing practice/ good laboratory practice certified contract-research companies, and eventually development of a business model to seek sufficient funding by a financial or industrial partner.

**DNA/RNA-Based HF Therapeutics Targeting Cardiac Inotropy**

Scientists and clinicians expect that an understanding of the disease mechanisms and ensuing molecular defects can deliver novel therapeutic targets. Cardiac hypertrophy, being an independent clinical risk factor for cardiac death, has been the subject of intensive research throughout the past 2 decades.57–59 Although numerous advocated targets derived from molecular in- depth analysis of proteins and mRNA regulatory RNA intermediates as for phospholamban (PLN), GRK2, and its indirect suppression by regulatory RNA intermediates as for phospholamban (PLN), GRK2, and its indirect suppression by regulatory RNA intermediates as for phospholamban (PLN). Figure 7 illustrates underlying molecular mechanisms in failing cardiomyocytes. If corrected by cardiomyocyte-directed DNA/RNA delivery, the order of in vivo therapeutic effects is (1) regeneration of contractile performance of the failing heart and, because of improved peripheral perfusion, (2) subsequent reversal of neurohormonal overdrive and cardiac remodeling as demonstrated in numerous large animal HF models.
The central question eventually emerging from these concepts is, What makes a molecular HF target amenable to DNA/RNA-based treatment? A major prerequisite is that its abnormal function must be correctable through the chronic expression of the therapeutic DNA/RNA. But unlike pharmacological regimes where effective doses are freely adjustable, and it is advisable to start with liberal empiricism in the choice of vector expression system results in unforeseeable and fixed levels of expression of the therapeutic DNA/RNA product.5 Vice versa, the therapeutic window of a DNA/RNA in the diseased heart is mostly unknown, unless gene-dosage studies are performed.

In other words, precise understanding of the DNA/RNA dose–response relationship seems mandatory to avoid premature dismissal of an otherwise effective gene product.19,55 Failure to test all emerging targets categorically and systematically is a major factor why the progress of HF gene therapy has been much slower than promised, and only a handful of the many reported potential targets have been developed toward clinically applicable treatments. An important lesson from the few successes is that only a refined translational pipeline integrating previously discussed data and focus on therapeutic inotropic targets with proven clinical applicability highlights critical steps of their development.

**β-Adrenergic System as Target for Gene Therapy**

More than 20 years ago, diminished β1-AR density was demonstrated in explanted failing human hearts.59 Ensuing studies unveiled a loss of cAMP-dependent cardiac reserve mechanisms as a common defect in failing myocardium irrespective of the underlying cause because of β1-ARs internalization and desensitization.60 Clinical attempts aiming at improved cardiac contractility by administering chronic catecholamine or phosphodiesterase inhibitor therapy resulted in increased patient mortality; quite unexpected at this time.22 Now, comprehensive evidence suggests that pharmacological blockade of cardiac β-AR stimulation is actually life saving by shielding the vulnerable heart against noxious sympathoadrenergic overdrive.

Because βAR blockade has become a cornerstone of clinical practice, extensive research has been undertaken into the maladaptive molecular consequences of chronically activated β-ARs in the failing heart.61 Evidence gleaned from numerous experimental studies indicates that phosphorylation of the β1-AR cytoplasmic domain through GRK2, previously known as β-AR kinase 1 (βARK1), is a key event in β-AR desensitization.62 This leads to internalization of the receptor complex followed by attenuated G-protein coupling to AC, which entails diminished cAMP generation capacity. Although counterintuitive at first glance, inhibition of GRK224 and improvement of AC activity1 surfaced as a potent strategy amenable to genetically targeted therapy (Figure 7) to restore β-AR–dependent cardiac performance without adverse effects.

**Inhibition of GRK2**

Failing human myocardium exhibits enhanced GRK2 expression and activity.60 A critical role of GRK2 in cardiac β-AR responsiveness was first shown by cardiac-targeted GRK2 overexpression in mice resulting in chronic β-AR desensitization.63 Cardiac-directed GRK2 deletion improved post-MI contractile performance basally and, as expected, in response to β-AR stimulation. Most importantly, post-MI outcome was improved by GRK2 ablation providing a clear rationale for the design of a GRK2 inhibitor, potentially suitable for gene-based therapy.64

GRK2 requires Gβγ-dependent association with the cell membrane before β-AR phosphorylation. Because interaction with membrane-bound Gβγ subunits occurs via the C-terminal domain of the kinase, this prompted design of an engineered cDNA encoding the C terminus of GRK2, named βARKct, to compete with Gβγ binding and subsequently prevent β-AR phosphorylation (Figure 7).63 Proof-of-concept for augmented β-AR responsiveness was demonstrated in transgenic mice with cardiac-restricted βARKct expression.63 Ensuing functional rescue and improved survival of various genetic murine HF models by crossbreeding them with βARKct transgenic mice promoted βARKct as a therapeutic GRK2 inhibitor.65

**Figure 7. Overview of selected inotropic targets for heart failure (HF) gene therapy.** See DNA/RNA-Based HF Therapeutics Targeting Cardiac Inotropy section for detailed mechanistic information (illustration credit: Ben Smith). AC indicates adenylyl cyclase; β-AR, β-adrenergic receptor; βARKct, β-adrenergic receptor kinase C terminus; GRK2, G-protein–coupled receptor kinase 2; NCX, sodium/calcium exchanger (Na+/Ca2+ exchanger); PKA, protein kinase A; PLB, phospholamban; RyR2, ryanodine receptor 2; SERCA2a, sarco(end)plasmic reticulum Ca2+ ATPase 2a; and VDCC, voltage-dependent calcium channel.
A DNA-based strategy was first tested in a rabbit model of post-MI HF with intracoronary delivery of an Ad harboring the CMV promoter–driven βARKct cDNA (AdβARKct) and it resulted in significantly improved β-AR responsiveness as well as AC-mediated cAMP production after 2 weeks.64 Subsequent experiments using AAV6 for cardiac-restricted βARKct gene therapy in an ischemic rat HF model demonstrated long-term therapeutic efficacy that was additive to pharmacological β-blocker treatment.53 A total of 2.5×10^{11} infectious particles of AAV6-βARKct were delivered via coronary arteries preceded by intracoronary application of a NO donor to facilitate vascular permeability and myocardial transduction. Achieving ca. 40% to 50% myocardial transduction rate, AAV6-βARKct not only restored cardiac β-AR density and signaling after 8 weeks but curtailed sympathetically overdrive, most likely because of sustained enhancement of myocardial performance. Equivalent to β-blocker treatment, AAV6-βARKct therapy exerted robust antihypertrophic actions in injured hearts supporting the notion that recovery of cardiac function is sufficient to reverse cardiac remodeling. Cardiomyocyte-targeted βARKct expression was assured by the use of a biased engineered expression cassette combining the cardiac actin enhancer with the elongation factor 1 promoter (Card-EF1). The relevance of these studies was supported by improved β-AR responsiveness in AdβARKct-treated isolated human failing cardiomyocytes that were derived from patients with end-stage HF undergoing cardiac transplantation.67 Mechanistic studies detailed that βARKct’s immediate inotropic actions do not rely on enhanced cAMP production in normal or failing cardiomyocytes. It exerts its beneficial effects early on through a Gβγ-dependent but cAMP-independent enhancement of cardiomyocyte Ca^{2+} handling.18 As the ultimate step toward clinical translation, the long-term therapeutic impact and feasibility of AAV6-βARKct gene therapy was assessed in a human-relevant porcine model of ischemic cardiomyopathy.50 Cardiac-directed delivery of 1.5×10^{11} AAV6-βARKct particles 2 weeks after MI via the anterior coronary vein resulted in βARKct expression in the LV anterior wall, septum, and regions adjacent to the infarcted area. Chronic βARKct expression led to significant enhancement both of systolic and diastolic LV performance, reversed myocardial remodeling, and normalized catecholamine plasma levels after a 2-month follow-up. The use of an engineered cardiac-biased expression cassette coupling the CMV mini-enhancer to the MLC ventricle 2 promoter fragment (CMV-MLCV2) enabled cardiac-restricted expression of βARKct that was without negative impact or toxicity on peripheral organ functions and the hematopoietic system.

This systematic evaluation of βARKct’s therapeutic potency along a refined translational sequence provided striking evidence for its efficacy and safety in human-relevant HF models (Table). Because of a lack of quantitative data, an important question still remains whether the therapeutic effects seen in different HF models using various vectors and expression cassettes actually result from similar expression levels. Future studies evaluating the therapeutic profile of the Gβγ-sequester and GRK2 inhibitor should establish standards to quantify absolute βARKct expression levels in myocardium. Defining βARKct/GRK2 or βARKct/GAPDH ratios might help to determine effective gene dosages and lay the groundwork for an understanding of βARKct’s PK and PD. With growing understanding of its molecular actions that might actually extend far beyond GRK2 inhibition, βARKct-DNA–based therapy seems ready to enter phase I/II trials to evaluate its therapeutic potential in human HF.

### Improving AC6 Activity

As previously alluded, cAMP signaling plays a pivotal role in cardiac contractility.16,65 Pharmacological attempts to increase cAMP signaling in failing myocardium rendered patient hearts more susceptible to lethal tachyarrhythmias and increased mortality. Similarly, cardiac-directed expression of β1-AR, Gαq, and protein kinase A, presumably because of sustained cAMP elevations, is deleterious and associated with cardiomyopathy in mice.3 It, therefore, surprises at first glance that expression of AC6, the enzyme catalyzing cAMP formation, exerts beneficial effects on the failing heart (Figure 7), but AC6 protein elevations do not effect basal cAMP production, and, additionally, may increase cardiac performance through cAMP-independent mechanisms.3

Proof-of-concept for AC6 to increase cardiac responsiveness beneficially to β-AR stimulation is derived from

### Table. Preclinical and Clinical Key Steps in HF Gene Therapy Development

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<tr>
<th>(1) Target Formulation</th>
<th>(2) Heart Failure Model (Preclinical)</th>
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<th>(4) Pharmacodynamic/Pharmacokinetic</th>
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<tr>
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Summary of (1) DNA-based inotropic target formulations to restore systolic cardiac performance, (2) proof-of-concept in different preclinical heart failure (HF) models, (3) current therapeutic systems using viral vector, expression element, and effective administration routes, (4) pharmacodynamic and pharmacokinetic aspects for clinical application (dose-dependency, long-term efficacy, biosafety), and (5) clinical trials. ACVI indicates adenyl cyclase VI; βARKct, β-adrenergic receptor kinase C terminus; CMV, cytomegalovirus; cardiac, cardiac-biased promoter; MCARD, molecular cardiac surgery with recirculating delivery; rAd, replication-deficient adenovirus; rAAV, recombinant adeno-associated virus; and SERCA2a, sarco(endo)plasmic reticulum Ca^{2+} ATPase 2a.
transgenic mice with both constitutive and conditional cardiac-directed AC6 overexpression.68,69 These hearts displayed increased cAMP-generating capacity along with enhanced LV contractile function and relaxation in response to β-AR agonists. Basal performance and cAMP levels were not affected, and expression even at high levels (≥20-fold) for >20 months neither resulted in cardiac abnormalities nor affected longevity in mice. These results indicated a potentially favorable therapeutic window.

Advancement to murine HF models first unveiled AC6’s therapeutic potential; crossbreeding AC6 with G_{s} transgenic mice that mimic clinical aspects of HF reversed decreased cAMP generation capacity, enhanced LV contractile performance as well as cardiac structural and electric remodeling, and increased survival.34 Correction of defects in β-AR–stimulated cAMP production and sarcoplasmic reticulum (SR) Ca^{2+} uptake were of mechanistic importance for these functional improvements. In addition, AC6 seems to involve activating transcription factor 3 and pleckstrin homology domain leucine-rich protein phosphatase 2 for cAMP-independent actions.3 An AC6 mutant devoid of catalytic CAMP formation still decreased phospholamban (PLN) expression through activating transcription factor 3 activation and increased PLN phosphorylation at serin-16 through inhibition of the Akt phosphatase pleckstrin homology domain leucine-rich protein phosphatase 2, both reliving PLN-mediated attenuation of SERCA2 activity. AC6 apparently uses indirect SERCA2 activation for its therapeutic efficacy in HF. More mechanistic studies are clearly warranted to unearth underlying molecular mechanisms fully.

These results prompted testing of DNA-based strategies using AC6 expression in small and large animal HF models by means of cardiac-directed gene delivery. Intracoronary delivery of 2.5×10^{10} viral particles of AdAC6 to failing hearts of G_{s} transgenic mice showed improvement in LV systolic and diastolic contractile indices after 14 days.31 Subsequent testing of AdAC6 gene delivery in a porcine model showed >30% myocardial transduction rate when 1.4×10^{12} viral particles of AdAC6 were delivered via intracoronary infusion preceded by histamine application.70 Driven by a CMV promoter, LV AC6 protein content increased by ≥2-fold. Enhanced LV basal and β-AR–stimulated function persisted for ≥18 weeks including elevated cAMP production capacity. These results were recapitulated in a second large animal model of pacing-induced HF. Plasma brain natriuretic peptide concentrations were lower because of AC6 treatment but hearts showed no signs of reversed remodeling at that time.43 Interestingly, there is a previous report detailing antibifilotic effects by AC6 overexpression in fibroblasts with the use of an unbiased CMV promoter,71 so that the collateral effects of AC6 on cardiac fibroblast functions in failing hearts deserve future considerations.

AC6-DNA–based HF therapy was subsequently tested in human-relevant large animal models yielding promising results with respect to efficacy and safety.3 Despite the lack of quantitative data to describe PK/PD aspects of AdAC6 HF therapy, a current clinical phase I/II trial applies catheter-based intracoronary AdAC6 delivery to human failing hearts (Table). Cardiac inflammation previously reported for the use of Ad in myocardial gene delivery was not seen in AdAC6 large animal studies, but AdAC6 was applied at an =10- to 100-fold lower dosage per gram heart weight.3 This might explain persisting effects on cardiac performance for ≥18 weeks. Although failing hearts are generally more prone toward β-AR–mediated arrhythmias, there was no indication for this in preclinical studies using AdAC6. Results of the ongoing phase I/II trial will soon inform about safety and finding of efficient dosages.

**Calcium (Ca^{2+}) Cycling as a Target System for HF Gene Therapy**

Disturbed cardiomyocyte Ca^{2+} cycling emerged as hallmark of HF as failing human cardiomyocytes display characteristic alterations of their intracellular Ca^{2+} cycle.72 This includes decreased systolic Ca^{2+} levels followed by delayed diastolic Ca^{2+} decay and elevated resting Ca^{2+} concentrations. Failing hearts subsequently generate a weak systolic contraction and prolonged diastolic relaxation with elevated stiffness impairing chamber filling. Pharmacological approaches such as levosimendan seeking to overcome systolic Ca^{2+} short by enhancing Ca^{2+} sensitivity of thin filaments resulted in improved systolic force development but came at the cost of impaired diastolic Ca^{2+} dissociation that aggravated cardiac muscle relaxation and enhanced propensity toward malignant tachyarrhythmias.73,74

Impaired activity of the SERCA2a/PLN complex and the EF-hand Ca^{2+} sensor protein S100A1 have emerged as key defects in abnormal cardiac Ca^{2+} cycling.54,75,76 In view of their critical function, restoring both SERCA2a and S100A1 activity in failing hearts poses promising therapeutic DNA-based strategies to improve cardiac contractile performance (Figure 7).

**Restoring SERCA2a Function**

Failing human myocardium exhibits decreased SR Ca^{2+} uptake because of diminished SERCA2a expression.77 The major function of the SR Ca^{2+} pump in cardiomyocytes is diastolic SR Ca^{2+} reuptake and maintenance of SR Ca^{2+} load. Although the former is a crucial determinant of cardiac relaxation, the latter critically contributes to systolic performance. The important biological role in cardiac contraction regulation was shown specifically in heterozygous SERCA2a gene-ablated mice.78,79 An ≥2-fold decrease in cardiac SERCA2a expression significantly impaired cardiac contraction and relaxation. Vice versa, rodents with only a 1.6-fold cardiac-directed SERCA2a overexpression demonstrated increased global cardiac function without signs of long-term pathological consequences, and isolated cardiomyocytes yielded improved Ca^{2+} handling and enhanced SR Ca^{2+} load.80

Pathophysiological relevance of diminished cardiac SERCA2a levels was shown when heterozygous SERCA2a gene-ablated mice were subjected to experimental cardiac injury and hemodynamic stress.81 Mice with decreased SERCA2a expression both displayed increased MI size and worsened post-MI performance, and accelerated transition to HF in response to hemodynamic overload. Increased susceptibility of SERCA2a transgenic rats toward lethal ventricular...
arrhythmias after MI raised concerns about potential proarrhythmogenic side effects of the molecular inotrope, although surviving animals had improved cardiac performance.82

The above-named studies provided a clear rationale to test targeted restoration of SERCA2a expression in diseased myocardium. In human failing cardiomyocytes, adenoviral-mediated SERCA2a-DNA (Ad-SERCA2a) delivery greatly improved cell shortening and Ca2+ handling.83 In rat hearts with chronic hemodynamic overload, antegrade coronary delivery of Ad-SERCA2a restored diminished SERCA2a protein to normal levels when a nonselective promoter element was used.84 LV systolic and diastolic performance was significantly enhanced and improvement in survival and energy potential of overloaded hearts was seen up to several weeks. Likewise, restoration of global SERCA2a protein expression in overloaded rat hearts was achieved using an AAV1-SERCA2a vector.85 Subsequently, LV function improved and oxygen cost of LV contractility within a 45-day follow-up was significantly reduced.

These beneficial effects prompted testing of long-term efficacy and safety in large animal models. Percutaneous catheter-based intracoronary delivery of 1×1012 DNase-resistant particles of AAV1-SERCA2a resulted in restoration of global LV SERCA2a protein levels in a pig model of experimental LV volume overload.42 Cardiac systolic and diastolic functional indices and structural remodeling were significantly improved within a 2-month follow-up. No myocardial toxicity and alterations in blood cell counts and clinical chemistry were reported, indicating safety of the viral-based HF therapy in a clinical-like setting. Potential extracardiac SERCA2a expression because of the use of a CMV promoter was not investigated. Prior concerns of an arrhythmogenic potential were weakened in Ad-SERCA2a–treated pigs showing reduced incidence of ventricular arrhythmias after ischemia/reperfusion injury.

In a sheep model of experimental LV volume overload, 5×1012 particles of AAV6-SERCA2a were intracoronarily delivered during balloon-mediated occlusion of cardiac venous outflow and prior adenosine application.39 AAV6-SERCA2a application restored global cardiac SERCA2a protein levels, resulting in better LV contractile indices and remodeling parameters within a 3-month follow-up. Myocardial transduction rate and potential extracardiac SERCA2a expression, as well as AAV6 biodistribution, were not reported.85 In addition, the usage of a cardiac-directed recirculating gene delivery methodology elicited dose-dependent increases in LV function and reversed remodeling in a pacing-induced sheep HF model when incremental amounts of AAV1-SERCA2a (1×1010, 1×1011, and 1×1012 DNase-resistant particles) were applied.39 Neither clinical chemistry parameters nor blood cell counts were altered. Despite report of greatest therapeutic efficacy in the high-dosage group, the study did not investigate myocardial and extramyocardial distribution of SERCA2a protein to underline their results.

On the basis of sufficient data with respect to therapeutic efficacy, safety, and technical feasibility for cardiac-directed AAV-based gene delivery (Table), the CUPID trial has evaluated safety and efficacy of AAV1-SERCA2a in human chronic HF.86 Participants with advanced ischemic HF (New York Heart Association [NYHA] class III) enrolled in this trial received a single antegrade intracoronary infusion of AAV1-SERCA2a using a noncardioselective CMV promoter. CUPID phase I showed an acceptable safety profile;2 and previously published functional results and cumulative clinical event rates of phase II that randomized 39 patients with HF to 3 different dosages of intracoronary AAV1-SERCA2a (6×1011, 3×1012, and 1×1013 DNase-resistant particles) versus placebo were encouraging.84 In light of limited statistical power, predefined multiple-efficacy domain analysis was applied showing improvement in the high-dosage AAV1-SERCA2a group versus control patients. Such domains take into consideration a series of parameters (symptoms, 6-minutes walk distance test, maximal oxygen consumption, brain natriuretic peptide levels, and echocardiographic measures). Greatly anticipated results of phase III will eventually allow further assessment of efficacy and effect morbidity. Other ongoing trials are currently recruiting patients to investigate effects of SERCA2a gene therapy on LV remodeling and on LV assist device therapy in patients with advanced HF.23

Besides, targeted manipulation of SERCA2a expression is not the sole means to modulate SR Ca2+ uptake. Suppression of PLN was used as indirect approach to increase SERCA2a activity.86 Using RNA interference, an AAV9 vector generated stable production of a regulatory RNA sequence after being injected into the tail vein of rats. In this study, small hairpin RNA (shRNA) silenced cardiac PLN expression by ≥75%. More than 3 months, rats with transaortic banding–induced HF treated with AAV9-shPLN displayed improved cardiac function, reversed cardiac dilation and hypertrophy, and reduced cardiac fibrosis. Expression of shPLN was driven by a noncardioselective CMV promoter. Apparently, no adverse effects on microRNA and liver function were seen. Because a PLN null mutation is associated with cardiomyopathy in humans,87 forthcoming genetic strategies modulating SERCA2a activity by targeting PLN function are to be viewed with extreme caution. For further information on alternative strategies to target SERCA2 protein turnover and PLN phosphorylation, we refer to a previously published excellent review in this journal.75

Improving S100A1 Activity

S100A1, an EF-hand Ca2+-sensor protein, is mostly expressed in cardiomyocytes.86 Failing human hearts show diminished expression of the EF-hand Ca2+-sensor raising interest in of S100A1’s role in cardiac muscle.89,90 A function in cardiac contractility regulation was first demonstrated by cardiac-targeted transgenic and viral-mediated S100A1 overexpression in mice and isolated cardiomyocytes, respectively, revealing inotropic and lusitropic effects.80,92 S100A1-mediated chronic gain-infection is additive to β-AR stimulation but occurs cAMP independent.90–93 S100A1 controls a Ca2+-driven network in cardiomyocytes, which is key to the heart’s ability to contract and relax, to maintain cardiac mitochondrial energy homeostasis, and to withstand maladaptive growth and tachyarhythmia.54,76,88 S100A1’s pleiotropic actions are mediated via direct binding and activity modulation of previously identified target proteins such as the ryanodine receptor 2,38,46,91–94 cardiac titin,27,98 and numerous mitochondrial enzymes involved in cardiac energy homeostasis.99
S100A1/ryanodine receptor 2 binding improves excitation–contraction (ec) coupling gain and prevents diastolic SR Ca\(^{2+}\) leak. Interaction with the SERCA2a/PLN complex improves SR Ca\(^{2+}\) uptake and decreases diastolic Ca\(^{2+}\) levels. Decreased stiffness and improved diastolic Ca\(^{2+}\) dissociation from cardiac myofilaments involves S100A1 binding to cardiac titin and attenuation of cardiac actin/titin interaction. S100A1 is also located in mitochondria improving ATP generation caused by F1-ATPase stimulation besides activity modulation of further mitochondrial enzymes.

In gene-ablated mice, cardiac S100A1 deletion worsened post-MI contractile performance basally and in response to β-AR stimulation, and post-MI survival was severely impaired.\(^{93}\) S100A1 overexpressing mice on the other hand, although having the same MI size, showed the opposite phenotype providing a strong rationale for therapeutic S100A1 restoration, potentially suitable for gene-based strategies. First proof-of-concept was demonstrated in an experimental rat HF model potentially suitable for gene-based strategies. First proof-of-concept was demonstrated in an experimental rat HF model.

First evidence for the same therapeutic efficacy of long-term cardiac-specific S100A1 restoration was demonstrated using intracoronary delivery of an AA V6, enabling sustained myocardial S100A1 expression with 40% to 50% transduction efficiency and reconstituted phosphocreatine/ATP ratio. Overall, this study unveiled a new therapeutic paradigm conveyed by S100A1 combining regeneration of contractile performance with reversal of maladaptive growth and energetic deficit. Further evidence for the same therapeutic efficacy of long-term cardiac-specific S100A1 restoration was demonstrated using intracoronary delivery of an AA V6, enabling sustained myocardial S100A1 expression with 40% to 50% transduction efficiency.

During the past 2 decades, PK/PD data and models both for gene therapy and development of companion diagnostics for technology and development of companion diagnostics for cardiac retrograde delivery restoration to human failing cardiomyocytes by Ad5-mediated human S100A1 cDNA delivery corroborated the previous findings entirely.\(^{90}\)

Systematic analysis of S100A1’s therapeutic potency and mechanisms yielded strong evidence for its efficacy and safety in human-relevant HF models (Table). Notably, a moderate S100A1 gene dosage is already sufficient for normalized expression yielding significant therapeutic effects and the inotropic effect is not associated with increases in heart rate or arrhythmias. S100A1’s therapeutic window tested to date in failing cardiomyocytes and hearts ranges from expression restoration to ≤10-fold overexpression without occurrence of adverse effects. Refinement of cardiac retrograde delivery technology and development of companion diagnostics for AA V9-S100A1 therapy is expected to facilitate translation to phase I/II trials.

For the discussion of DNA/RNA-based strategies aimed at manipulation of (1) stem cell homing and survival for cardiac regeneration, (2) angiogenesis, and (3) oxidative stress and resistance to cell death, we refer to already published excellent reviews.\(^{7–11}\)

**PK and PD of Cardiac Gene Therapeutics**

During the past 2 decades, PK/PD data and models both for efficacy and for safety have been developed in almost all therapeutic areas, showing a clear benefit in the drug development process. There are areas, however, where PK/PD is still an exception, and one of those areas is gene therapy.\(^{100,101}\) Cardiac therapeutic gene delivery is still reminiscent of a biological blackbox where the input and output are defined between the administered dose of a therapeutic vector and its biological effect, that is, on cardiac contractile performance or remodeling, respectively, whereas the inner workings of the system remain enigmatic.\(^5\) To date, most preclinical studies that reported improvement of experimental HF using cardiac therapeutic gene delivery were limited to a single vector dosage and 1-time estimation of cardiac transgene expression levels, mainly at the study end point. The current lack of understanding the PK/PD underlying therapeutic effects will eventually delay translation and design of proper clinical trials.\(^5\) Systematic acquisition of quantitative PK/PD data during preclinical development is inevitable to assess whether the therapeutic product was sufficient to normalize global cardiac S100A1 protein levels. Within 2 weeks, basal in vivo systolic and diastolic contractile performance of the heart was restored and β-AR responsiveness improved significantly, despite persistent signaling abnormalities. Approximately 40% to 50% myocardial transduction efficiency yielding inhomogeneous S100A1 expression distribution was sufficient to generate this therapeutic effect. Accordingly, intracellular Ca\(^{2+}\)-transient amplitudes of AdS100A1-infected cardiomyocytes isolated from treated hearts were found to be either restored or increased above normal levels. S100A1 also decreased cytoplasmic Na\(^+\) levels that further contributed to improved mitochondrial ATP generation and reconstituted phosphocreatine/ATP ratio. Overall, this study unveiled a new therapeutic paradigm conveyed by S100A1 combining regeneration of contractile performance with reversal of maladaptive growth and energetic deficit. Further evidence for the same therapeutic efficacy of long-term cardiac-specific S100A1 restoration was demonstrated using intracoronary delivery of an AA V6, enabling sustained myocardial S100A1 expression with 40% to 50% transduction efficiency.

A total of 2.5×10\(^{11}\) infectious viral particles of AA V6-S100A1 expressing the transgene under control of the cardiac-biased CARD-EF1 promoter restored overall cardiac S100A1 protein levels and prevented extracardiac S100A1 expression. Both systolic and diastolic contractile indices in S100A1-treated failing hearts were significantly improved within an 8-week follow-up period. Notably, the inotropic effect of S100A1 was preserved under concurrent β-AR blocker therapy. In contrast to β-AR blocker therapy alone, which only slowed down disease progression, AA V6-S100A1 therapy reversed contractile dysfunction. Both therapies were equally effective and when combined synergistic in protecting injured hearts from cardiac remodeling, but only S100A1-treated hearts showed improved β-AR responsiveness.

Using an experimental porcine post-MI HF model, cardiocardiac-directed delivery of 1.5×10\(^{10}\) AA V9-S100A1 particles 2 weeks after MI via the anterior coronary vein resulted in wide-spread S100A1 expression.\(^{84}\) The use of the cardiac-biased CMV\(_{α-MEF1}\) MLCV2 expression cassette restricted S100A1 expression to the heart ranging from normal to 5- to 6-fold overexpression in targeted areas. Restoring S100A1 in failing pig hearts yielded significant long-term improvement both of systolic and diastolic cardiac function after 3 months. Functional regeneration was mirrored by increased Ca\(^{2+}\) transient amplitudes in isolated cardiomyocytes from S100A1-treated hearts. AA V9-S100A1 treatment, furthermore, reversed myocardial remodeling and normalized the decreased phosphocreatine/ATP ratio indicative of improved cardiac energy homeostasis. Comprehensive biomarker assessment showed unchanged clinical chemistry, blood count, and coagulation markers in addition to normal peripheral organ histology and indicated safety of AA V9-S100A1 therapy. In support of its efficacy in human myocardium, effects of S100A1 restoration in human failing cardiomyocytes by Ad5-mediated human S100A1 cDNA delivery corroborated the previous findings entirely.\(^{60}\)

For the discussion of DNA/RNA-based strategies aimed at manipulation of (1) stem cell homing and survival for cardiac regeneration, (2) angiogenesis, and (3) oxidative stress and resistance to cell death, we refer to already published excellent reviews.\(^{7–11}\)
is reasonably safe for initial testing in humans, and a prerequisite for investigational new drug application to obtain legal drug status.100,101

In the case of PK, referring to “what the body does with the drug,” quantitative characterization of the biotransformation, biodistribution, and excretion would allow for describing and understanding the effects elicited by the gene therapy system.3 Here, biotransformation is defined as the process during which the therapeutic gene sequence packaged in any vector is converted into a potentially therapeutic gene product in the target cell. Although the vector has to overcome several biological barriers after intravascular or intramyocardial delivery that could be described by conventional PK models, biotransformation of a therapeutic gene product uses the innate cellular transcriptional and translational machinery referring to a new concept of intracellular PK.100,101 The process thus integrates (1) efficiency of vector uptake, (2) (in the case of AAV) dynamics of nuclear trafficking and escape from vector degradation, (3) mRNA transcription rate and stability, (4) eventual translation, and (5) degradation of the gene product. For gene therapy to be successful, it is essential to describe the net effect of intracellular biotransformation systematically in terms of its time course and level of cardiac transgene expression. Stable long-term expression seems to be mandatory for the treatment of chronic HF. Defining the kinetics and dose–response relationship among the therapeutic vector system, transgene expression, and therapeutic efficacy is thus necessary to determine whether the particular combination is appropriate and safe.5

With cardiac gene transfer where vector delivery to myocardial tissue involves direct physical methods, the potential for the wider distribution of the vector exists, and this may result in untoward effects on distant organs. Available biodistribution and excretion data from preclinical studies in small and large HF animal models have assessed either viral transduction or transgene expression of noncardiac tissues.30,46 But they were mostly limited to nonsystematic analysis of a single vector dosage and lacked comprehensive organ and tissue assessment including reproductive organ systems and body fluids. Noninvasive imaging of transgene expression particularly in large animal models approximating human size and weight may change the way transgene expression is defined.102 Taking advantage of advances in molecular imaging might allow for PET- or SPECT-based serial in vivo imaging of transgene expression and biodistribution alike. A suitable example of this is imaging of either cardiac-targeted thymidine kinase or sodium iodine symporter gene expression with the use of labeled and radioactive substrates used in clinical routine.103,104 Application of the clinically established technology in large animal preclinical studies is expected to advance our understanding of the kinetics of expression and biodistribution greatly.

With respect to PD, defined as “what the drug does to the body,” a major concern in the field of gene therapy is the immune response to vector delivery, as well as vector- and transgene-associated toxicity that requires special consideration in clinical studies.5,100,101 Viral vectors such as Ads have the potential of inducing significant immunologic responses that limit transgene expression and may cause collateral tissue damage or autoimmune response. Hence, defining the mechanisms of immunologic responses and the maximally tolerated dose for each vector and delivery route should be a priority in preclinical development.12–14,101 AAV vectors have attracted the interest of gene therapists worldwide because of several features, but chief among these is the virus apparent lack of pathogenicity. Further development of wild-type AAV as therapeutic vectors has eliminated its ability to integrate into the host genome at a specific site (designated AAVS1) in the human chromosome 19 by removal of the rep and cap from the DNA of the vector.

Innate immune response to AAV vectors occurs in the form of transient increase of inflammatory cytokines and some liver infiltration of neutrophils, which together with macrophages seems to sequester a large percentage of circulating AAV particles.12,13,100 Given the rather modest innate response, AAV instigates robust humoral immunity both in animal models and in humans, where 80% to 90% of individuals are thought to be seropositive for AAV2 antibodies. Of those, ≈30% showed neutralizing ability and they can drastically mitigate vector transduction efficiency.105 Importantly, the humoral immune response to 1 serotype can give rise to a complex profile of serum antibodies capable of binding and neutralizing various AAV serotypes. In addition to persistent AAV-specific antibody levels, it seems from prime-boost studies in animals and from clinical trials that the B-cell memory is also strong. In a clinical trial using an AAV2-based vector to treat hemophilia B, targeted destruction of transduced liver cells resulted from a T-cell–mediated response to this AAV serotype.106 Apparently, the T lymphocyte response against AAV2 is susceptible to prednisolone and it is currently unclear whether the cytotoxic response equals to all serotypes. Of similar interest is the recent observation that the humoral response to AAVs seems to be T-cell dependent; anti-CD4 antibodies inhibiting T-cell function prevented antibody formation and allowed vector readministration.107 Notably, only 1 patient in the CUPID trial that uses AAV1 showed transient positivity in an enzyme linked immunospot assay indicating a cellular immune response against the AAV1 capsid.

Because AAVs are currently among the most frequently used viral vectors in clinical trials, and still little is known about variability of AAV-induced immunity, clinical trials should make every attempt to pool available data and enable a deeper understanding of AAV–host interactions for the safe and efficient use of AAV as a gene transfer vector.107 Although in its infancy, unraveling PK/PD data for gene-based therapeutics is expected to provide a clear benefit in the gene-based drug development process and safety for clinical use.5,100,101

**Systems Biology in HF Gene Therapy**

Although the goal of gene therapy in preclinical models is rather focused on the impact of the protein encoded by the delivered gene on phenotypic outcome in disease models, our aim must be understanding the effects of this change in protein expression on the overall molecular pathways involved to define quantitative and predictive biomarkers.5 This reflects a shift from a 1-gene-per-disease mindset to understanding of complex multigene disease processes, where phenotypic outcome is dependent on the behavior of interrelated networks...
and systems as a whole. Systems biology, including high-throughput experimental and computational studies that account for the complexity of host–disease therapy interactions, holds significant promise in aiding the development and optimization of gene therapy. With respect to target selection, a particular strength of systems biology studies is the ability to predict effects on the system that are not directly an intended target of the therapy and thereby identify hidden drivers of therapeutic success or failure. The idea behind this approach is that activity within a signaling network is probably more important than merely altered expression of a specific protein.

Typically, gene delivery studies use elevated levels of the therapeutic protein as confirmation that the treatment is working. However, the expression of the encoded protein is only the start of a process of the tissue responding to the intervention, and the protein will interact with many endogenous molecules and cause multifocal effects. It is the impact of these effects on the desired therapeutic outcome that may define the efficacy of the treatment and enable development of predictive biomarkers as companion diagnostics. Systems biology can aid in predicting changes and effects by incorporating dynamic information on the expressed protein and the overall network in which it is involved to deliver a holistic understanding of its mechanism of action and therapeutic efficacy. Potential approaches could pursue, for example, serial assessment of changes in the transcriptome (also including regulatory RNAs) of a target cell corresponding to the treatment over time. As already shown in other disciplines, in silico simulation can then integrate the time-resolved information and provide a computational model of the regulatory gene network that shapes the disease phenotype.

Within this framework, therapeutic factors can be considered as beneficial system perturbations, which is key to understanding how a particular pathway reacts. Thus, systems biology could assist in evaluating success and consequences of gene therapy and provide a bridge between therapeutic design and outcome. In addition, side effects and interactions of gene therapy with other drugs would also be identifiable and this could enable us to design around them. However, the systematic use of this approach is still underused relative to its potential.

Study Population, End Points, and Cautionary Tales

Considering the appropriate target population for first clinical testing of a novel HF therapy is of utmost importance. In the CUPID trial, patients had to fulfill the criteria of stable, severely depressed LV contractile function (left ventricular ejection fraction <30%) rendering the patient highly symptomatic (NYHA functional state III or IV), despite stable (>6 months) optimized HF drug treatment including β-blockers, angiotensin converting enzyme inhibitors, and aldosterone receptor antagonists. Importantly, this study only included patients with HF of ischemic origin, although the large animal models used in preclinical testing of SERCA2a did not reflect this population properly. As a precaution because of the proclaimed arrhythmogenic potential of a therapeutic intervention with Ca2+ cycling, all patients included in this study underwent implantation of an implanted cardioverter defibrillator device. Such accommodations may also be a lesson learned from a study using cell therapy with fatal arrhythmia being an effect.

Because the additional value of DNA/RNA-based therapies are tested against optimized HF medication in phase I/II trials, appropriate controls are mandatory. After initial feasibility and dose-escalating studies, future credibility of cardiac gene therapy to treat HF will depend on placebo groups and blinding as essential features. Myocardial biopsy, alike after cardiac transplantation, may be 1 possibility to learn about PD and potential inflammatory adverse effects. As one of the potentially most constraining factors, patients need to be screened for neutralizing antibodies against the administered AAV serotype to exclude the possibility of reduced therapeutic effectiveness. Vehicle-related side effects that also need to be thoroughly investigated might include inflammation, viral shedding, and associated insertional mutagenesis. This may pose a major issue for broad application of gene therapy and needs to be addressed by further vector optimization.

To eliminate potential side effects it is worth the effort to use controllable or ON/OFF promoter constructs. Moreover, dissemination of vector distribution and target gene expression within the germline needs to be monitored and might be controlled by ON/OFF constructs.

As always with novel treatments, ethical considerations and responsibility need to provide the balance when weighing the possibility to improve treatment of HF patients against the remaining uncertainty of an innovative and still only partially understood gene therapy approach.

Conclusions and Perspective

Originating from a visionary therapeutic concept, translation research paved the way for first clinical trials that evaluate safety and efficacy of molecular-targeted inotropic therapies with the aim to restore cardiac performance and interrupt the vicious cycle of HF. Although phase I/II trials already indicate safety, phase III results detailing therapeutic efficacy are eagerly awaited. With more therapeutic innovations to come, rigorous evolution in critical areas such as dynamic gene expression control, PK and PD of therapeutics, and holistic understanding of therapeutic modes of action and companion diagnostic development mark the entry to the next generation HF gene therapy, which might eventually become clinical reality.

Disclosures

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