Gene Therapy Targets in Heart Failure: The Path to Translation

PWJ Raake[1], H Tscheschner[1], J Reinkober[1], J Ritterhoff[1], HA Katus[1], WJ Koch[2] and P Most[1]

Heart failure (HF) is the common end point of cardiac diseases. Despite the optimization of therapeutic strategies and the consequent overall reduction in HF-related mortality, the key underlying intracellular signal transduction abnormalities have not been addressed directly. In this regard, the gaps in modern HF therapy include derangement of β-adrenergic receptor (β-AR) signaling, Ca^{2+} disbalances, cardiac myocyte death, diastolic dysfunction, and monogenetic cardiomyopathies. In this review we discuss the potential of gene therapy to fill these gaps and rectify abnormalities in intracellular signaling. We also examine current vector technology and currently available vector-delivery strategies, and we delineate promising gene therapy structures. Finally, we analyze potential limitations related to the transfer of successful preclinical gene therapy approaches to HF treatment in the clinic, as well as impending strategies aimed at overcoming these limitations.

Heart diseases account for the highest morbidity and mortality rates in Western industrialized countries. The common denominator and final end point of heart diseases is the development of heart failure (HF). However, a significant gap is evident between current therapeutic approaches and key underlying biological processes relating to cardiac myocytes in the setting of chronic cardiac dysfunction.[1] Because there is no cure for HF short of heart transplantation,[2] and death occurs mainly from electrical abnormalities and contractile failure, one of the major therapeutic goals of modern cardiology is to design innovative strategies aimed at the prevention of lethal arrhythmias and restoration of cardiac performance.

Modern HF therapy is symptom-oriented, using pharmacological (β-blockers, angiotensin-converting enzyme inhibitors, angiotensin receptor II-antagonists, and diuretics), interventional (balloon angioplasty, intracoronary stent implantation, and percutaneous valve repair), electrophysiological (ablation of arrhythmic foci, cardioverter defibrillator implantation, cardiac resynchronization therapy), and surgical (ventricular assist device implantation, heart transplantation) principles. Despite extensive research and significant progress and success in reducing overall mortality rates, these therapeutic options do not deal with the key underlying intracellular signal transduction abnormalities that cause or perpetuate the development and progression of the disease. Gaps in modern pharmacological, interventional, and surgical HF therapy include deranged β-adrenergic receptor (β-AR) signaling, Ca^{2+}-imbalances, apoptosis, and diastolic dysfunction (see **Figure 1**). Promising novel technologies are needed to further optimize the care of patients with HF and to close the gaps in the therapeutic approach.

This review discusses the potential of gene therapy to fill the existing gaps and overcome the challenges that have not yet been satisfactorily addressed in modern HF therapy. We analyze the rationale for using gene therapy to treat the failing heart. Furthermore, we address strategies for manipulation of intracellular signaling and evaluate current vector technology and gene-delivery techniques. The gaps in modern HF therapy are addressed, and the current therapeutic constructs countering these challenges are presented. We discuss initial clinical evidence and delineate potential limitations of HF gene therapy that can be overcome by the application of basic pharmacological principles to this field.

BASICS

Why gene therapy?: the temptation to achieve direct modulation of intracellular signaling

Thus far, noninvasive treatment of HF has followed a systemic pharmacological approach. Standard therapy includes the use of β-AR antagonists, inhibitors of angiotensin II, aldosterone antagonists, and diuretics. Despite considerable improvements in therapy, HF-related mortality remains high. Furthermore, the use of systemic medications for HF causes unwanted

[1]Division of Cardiology, Department of Internal Medicine III, University of Heidelberg, Heidelberg, Germany; [2]Department of Medicine, Center for Translational Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania, USA. Correspondence: P Most (patrick.most@med.uni-heidelberg.de) or WJ Koch (walter.koch@jefferson.edu)

side effects. It is noteworthy that all the HF drugs currently available influence systemic signaling pathways (such as the renin–angiotensin–aldosterone system) or block extracellular membrane-bound receptors (such as β-ARs); these are doubtless the cornerstones of modern HF therapy. From a biochemical or pharmacological perspective, it is challenging to engineer compounds that can act effectively on intracellular targets; therefore, no pharmacological therapy is currently available that can operate directly inside the cell, where deranged signaling pathways merge and perpetuate the progress of the disease. Gene therapy offers the option to specifically target cardiac myocytes and introduce genetic material directly into the cell. The genetic information may be transcribed into a minipeptide, peptide, protein, or small interfering RNA (see below) that can directly affect and potentially correct the disturbed molecular pathways inside the failing cardiac myocytes. Furthermore, in the matter of genetic cardiomyopathies, there is no therapy available to treat the cause of the disease, and HF treatment is confined to alleviating the symptoms. If gene therapy could be used to replace defective transgenes or proteins, it would potentially constitute a cause-driven approach.

In conclusion, gene therapy is currently the only available approach that can effectively target molecular signaling within cardiac myocytes; this technology therefore has strong potential to complement and improve modern HF therapy.

Minipeptide, peptide, protein, or RNA interference (small interfering RNA or synthetic microRNAs): modes for manipulation of intracellular signal transduction

Gene therapy is aimed at directly targeting intracellular signaling pathways. We review several available tools offering divergent strategies: protein/enzyme replacement for downregulated or defective targets and inhibition of protein/enzyme function for maladaptively increased activity and/or levels.

Protein/enzyme replacement strategies. Proteins such as the Ca^{2+}-sensor S100A1 or enzymes such as the sarcoplasmic reticulum (SR) Ca^{2+} ATPase (SERC2a) (see "Positive inotropic therapies: Ca^{2+} cycling" below) are downregulated in the failing heart. Overexpression of these molecules in diverse HF models could improve cardiac function. Therefore, protein/enzyme replacement is a potential therapeutic option.

Inhibition of protein/enzyme function. Some signaling pathways lead to upregulation and/or activation of proteins/enzymes, creating a maladaptive response (e.g., G-protein-coupled receptor kinase 2 (GRK2); see "Resensitizing β-AR signaling: the β-AR–G-protein–adenylyl cyclase interactome" below) that is upregulated and more active in failing cardiac myocytes and contributes to cardiac deterioration.[1] In such a case, therapeutic strategies are aimed at knockdown or inhibition of these targets. Potential approaches include (i) expression of a dominant negative variant or an inhibitor (peptide or protein), allowing competitive target inhibition; (ii) the use of truncated proteins to express only the part of the protein that is relevant for its function or binding, aimed at direct inhibition of enzyme function or competitive disruption of protein–protein interactions; and (iii) the use of RNA interference technology. Small interfering RNA transfection with liposomes is transient. In cardiac gene therapy, a long-lasting and more stable effect is desired; for this purpose, introducing a miRNA or small hairpin RNA coding cassette into the genome may be of advantage.

Various molecular tools aimed at protein/enzyme replacement or inhibition of protein/enzyme function have already been established, and these can be further refined for an optimized gene therapy approach with the ultimate goal of correcting key underlying signaling abnormalities in failing cardiac myocytes.

Vectors and techniques for gene delivery to the heart

A prerequisite for success in cardiac gene therapy is the specificity and efficiency of gene delivery to the heart. The target genomic material, promoter, (viral) vector, and vector delivery approach must all be orchestrated to optimize expression of the therapeutic transgenes. The **Supplementary Data** online provides an update on current vectors for cardiac gene therapy and discusses methods of applying these in cardiac therapy. The optimal delivery approach is a matter of debate and will certainly see modifications as technology improves; however, from the perspective of currently available technology, intramyocardial injection and intracoronary or coronary venous injection of adeno-associated viruses (AAVs) appear to be the most feasible approaches.[3] AAV serotypes 2, 6, and 9 offer the highest cardiac tropism[3] (see **Supplementary Data** online).

FILLING THE GAPS IN CURRENT THERAPY FOR HF: POTENTIAL GENE THERAPY TARGETS

The gaps in current pharmacological, interventional, and surgical HF therapies include deranged β-AR signaling, Ca^{2+} imbalances, diastolic dysfunction, cell death, and genetic cardiomyopathies. We delineate these gaps and analyze in detail the potential approaches, based on current gene therapy concepts, that can counter these limitations. The related molecular signaling pathways and potential gene therapy targets, which will be discussed in the following chapters, are shown in **Figure 2**.

Resensitizing β-AR signaling: the β-AR–G-protein–adenylyl cyclase interactome

In HF, cardiac myocyte β-AR signaling is deranged. β-AR catecholamine sensitivity is attenuated in end-stage HF in humans, paralleled by a lower density of β-ARs.[3] The molecular cause of this alteration was demonstrated to be an increase of a nodal intracellular protein kinase, GRK2 (formerly termed β-AR kinase 1).[1] GRK2 is a cytosolic enzyme that, upon receptor activation, binds the released Gβγ subunit of activated heterotrimeric G-proteins (Gβγ) at the plasma membrane.[2] GRK2 phosphorylates β-ARs, which subsequently bind inhibitory proteins called β-arrestins. β-ARs that are bound in this complex will not signal to the G-proteins, and therefore further signaling is terminated, for example, through receptor desensitization.[2]

In addition, β-ARs complexed with β-arrestins are internalized and thereby downregulated (mainly β_1-ARs).[1]

Abnormal β-AR inotropic responsiveness leads to an increased sympathetic drive with upregulation of tissue and plasma catecholamines in an attempt to stimulate myocardial contractile function.[1] This sustains a vicious circle as the chronic stimulation of cardiac β-ARs perpetuates a loss of signaling due, in part, to a marked upregulation of GRK2, which desensitizes β-ARs in the heart through phosphorylation.[1] Thus, a continual state of activation and abnormal signaling is created, and chronic catecholamine bombardment of the heart can contribute to further cardiac deterioration.[1] The successful use of β-AR blockers in clinical HF treatment demonstrates the effectiveness of an approach aimed at interrupting this vicious circle of sympathetic tone by blocking chronic β-AR activation; however, these agents do not directly address the molecular defects in failing cardiac myocytes.

βARKct. Recently, the pathologic nature of GRK2 was directly demonstrated in a study using infarct-inducible, cardiac-specific knockout (KO) mice in which GRK2 was deleted in ventricular myocytes after myocardial infarction. There was a significant improvement in overall animal survival and cardiac function.[4] This gave a clear indication that lowering of GRK2 is therapeutic in HF and that its upregulation is not a beneficial adaptive change because it was associated with compromised β-AR signaling and cardiac function.[4]

Because there is currently no suitable pharmacological small-molecule GRK2 inhibitor for targeted GRK2 inhibition in HF, a miniprotein inhibitor that blocks the membrane translocation and activation of GRK2 was engineered.[5] This miniprotein, known as the βARKct (C-terminal domain of GRK2), competes with endogenous GRK2 for membrane binding to the βγ subunits of activated heterotrimeric G-proteins (Gβγ) and has been shown to inhibit GRK2 activity on several receptors, including β-ARs.[5] Furthermore, transgenic or viral (adenovirus and AAV) expression of the βARKct to compromised myocardium has been found to rescue several models of experimental HF.[3,6–8] In addition, inhibition of GRK2 improves β-adrenergic signaling and contractile function in failing human myocytes.[9] However, efforts to obtain important mechanistic insights into the cardiac actions of GRK2, and GRK activity in general, are thwarted by the fact that βARKct targets Gβγ and may alter non-GRK functions.[3] In this regard, further underscoring the pathologic role of GRK2 activity in the failing heart, the true loss of GRK2 in genetically engineered conditional KO mice (with the KO being restricted to cardiac myocytes) is associated with increased HF survival, amelioration of cardiac dysfunction, and halting of adverse left-ventricular remodeling.[4] Therefore, the therapeutic mode of action of βARKct is most likely linked to direct inhibition of GRK2.

Although data related to cardiac βARKct expression, both in genetic mouse models of HF and in virus-mediated myocardial gene transfer, reveal positive results, the studies published to date have not involved any animal larger than a rabbit. To test whether βARKct and GRK2 inhibition represents a potential therapeutic approach to treating human patients with HF, we have recently completed a study in large animals (German farm pigs) more closely reflecting human physiology (Raake PW *et al.*). First, βARKct transgene delivery is feasible using AAV serotype as a vector and retroinfusion of the coronary veins as a delivery approach. With our established pig model of post–myocardial infarction systolic HF, we were able to demonstrate a sustained amelioration of preexisting cardiac dysfunction with AAV6-mediated βARKct delivery, with significant improvements in cardiac function, normalization of neurohumoral signaling, and repression of adverse cardiac remodeling and fetal gene expression. Therefore, translation of the βARKct approach to human HF patients appears to be warranted; however, data on long-term survival are necessary, as well as further improvements in vectors and gene delivery techniques.

Interestingly, all the data pertaining to βARKct-mediated HF rescue involve normalization of β-AR signaling, including receptor upregulation (to normal levels) and GRK2 lowering. Given that long-term β-AR antagonism (with pharmacological β-AR blockade) can promote receptor upregulation and GRK2 downregulation, improved β-AR signaling is also a natural consequence of clinical β-AR blocker use.[10] Therefore, the use of β-AR blockers and molecular GRK2 inhibition may act synergistically. Indeed, this has been shown in several small-animal models of GRK2 inhibition and HF.[6,8] Therefore, molecular GRK2 inhibition might complement the current pharmacological strategies aimed at normalization of β-AR signaling.

β_2-AR. The concept of simple re- or overexpression of downregulated β-ARs has led to intriguing results, reflecting the complexity of β-AR signaling in the normal heart as well as in the diseased heart. Transgenic (over-)expression (~30-fold) of human β_1-ARs in mice was associated with significant cardiomyopathy.[11] Although this result is not unexpected, it reflects the usefulness of β-AR blockade for treating the failing heart. However, Noma *et al.* recently reported that significant overexpression of the mouse β_1-AR was not linked to an adverse phenotype.[12] These results are potentially explained by the constitutive activity of the human β_1-AR, which is not present in the mouse β_1-AR.

With respect to β_2-ARs, a clear cardioprotective phenotype was observed with transgenic human β_2-AR overexpression. Mice showed amelioration of systolic and diastolic cardiac dysfunction, with no evidence of an adverse cardiac phenotype.[3,13] However, a dose-dependent effect has recently been described, with high (~200-fold) overexpression of this human receptor variant in mice being linked to the development of fibrotic heart disease at 40 weeks of age, whereas lower levels (~40-fold) of overexpression clearly incorporated the protective phenotype.[13] Furthermore, gene therapy (either as global delivery or through intracoronary injection) with an adenovirus expressing the β_2-AR was shown to confer cardioprotection in a rabbit model of myocardial infarction.[14,15]

Overall, the evidence suggests that β_2-AR gene therapy might improve cardiac function; however, more evidence is required from studies in larger animals, and long-term survival

observations should be undertaken before promoting this novel approach to the direct restoration of desensitized β-AR signaling.

Adenylyl cyclase VI. Currently, there are nine known adenylyl cyclase isoforms (adenylyl cyclases I to IX) that translate the increased catecholaminergic β-AR-G-protein signal into an intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) response. Adenylyl cyclase types V and VI are the most predominant forms in cardiac myocytes.[16] Diminished adenylyl cyclase activity parallels the downregulation and desensitization of β-ARs in HF. Overexpression of adenylyl cyclase VI in the failing heart is an appealing concept because it does not involve any constitutive activity as in the case of β-ARs or G-proteins; rather, it gives rise to increased cAMP-generation with β-AR stimulation, leaving basal cAMP levels unchanged.[3]

Previously, a transgenic adenylyl cyclase VI mouse was crossed with a transgenic Gαq mouse as a model of HF. In this model, overexpression of adenylyl cyclase VI was linked to amelioration of cardiac dysfunction, reduction in diastolic dysfunction, and improved survival rates.[17] Interestingly, the overexpression of adenylyl cyclase V in the presence of Gαq led to opposing effects, resulting in accelerated cardiac deterioration.[18] This fundamental difference was never explained mechanistically; however, it is worth mentioning that adenylyl cyclase V confers a constitutive basal activity and is associated with increased basal cAMP generation, which is not the case with adenylyl cyclase VI overexpression.

Because the Gαq does not reflect the most predominant form of HF in Western industrialized countries, this construct was further tested in a post–myocardial infarction HF model. Interestingly, transgenic adenylyl cyclase VI overexpression (transgenic mice) resulted in improved cardiac function and survival at 1 week after myocardial infarction.[19]

The concept of adenylyl cyclase VI overexpression was applied to a clinically relevant, large-animal, rapid pacing–induced HF model (swine) in which an adenovirus encoding adenylyl cyclase VI was injected directly into the coronary arteries.[20] This resulted in a moderate level of improvement in cardiac function and an increase in intracardiac cAMP generation.

Overall, adenylyl cyclase VI overexpression is an interesting concept with clinical potential and is one of the few constructs being evaluated in a clinically relevant large-animal model; however, because the data for adenylyl cyclase VI overexpression have been published almost exclusively by a single research group, independent affirmation is required.

Positive inotropic therapies: Ca^{2+} cycling

Calcium cycling during excitation contraction coupling in the heart is the prerequisite for force development in systole and relaxation in diastole. Because normal calcium cycling is deranged in HF, many current therapeutic agents are aimed at correcting calcium cycling and thereby restoring excitation contraction coupling and inotropic response of cardiac myocytes.[21]

In HF, calcium cycling and excitation contraction coupling are severely disturbed at different levels: ryanodine receptor (RyR) open probability is enhanced in diastole ("leaky RyR"), whereas SERCA2a expression levels are diminished, along with a simultaneously increasing phospholamban (PLN)/SERCA2a ratio; these circumstances result in an overall decrease in diastolic SR Ca^{2+} uptake, contributing to diastolic dysfunction. Additionally, Ca^{2+} extrusion from the cytosol is enhanced by increased expression of the membranous sodium-Ca^{2+}-exchanger. This amplifies the overall diminishment of SR Ca^{2+} content and induces diminished Ca^{2+} transients, contributing to systolic dysfunction. Additionally, the positive force–frequency relationship becomes inverted. In addition to the dysfunctional contractile performance of the heart, deranged Ca^{2+} handling can also result in life-threatening arrhythmias and contribute to adverse remodeling.[22]

The restoration of normal Ca^{2+} handling in HF has therefore been the target of many therapeutic interventions. To date, only electrophysiological cardiac resynchronization therapy has been able to achieve successful restoration of heart rhythm to some extent. All pharmacological approaches aimed at normalization of intracellular Ca^{2+} handling (e.g., using Ca^{2+}-sensitizers such as levosimendan) have failed to reduce overall mortality significantly, despite achieving short-term improvements in cardiac function.[23] In addition, these approaches are associated with unwanted effects on cardiac energetics and intracellular Ca^{2+}-levels, rendering them unviable. Therefore, there is an imminent need for more specific, tailored, and targeted interventions to correct intracellular Ca^{2+} handling. Here we focus on promising candidates for use in potential gene therapy for HF, specifically aimed at correcting deranged intracellular Ca^{2+} handling.

SERCA2a. SERCA2a is the major isoform of SR Ca^{2+}-ATPases in the adult heart; it accounts for 40% of the total SR proteins. SERCA2a is responsible for SR Ca^{2+} uptake in diastole, and its activity is tightly regulated by the small protein PLN, which, in its dephosphorylated form, reduces SERCA2a Ca^{2+} affinity and thereby decreases SR Ca^{2+} uptake.[21,22]

The observed association between decrease in SERCA2a expression levels and reduction in SR Ca^{2+} uptake in chronic HF has prompted the selection of SERCA2a as a promising target for HF gene therapy. Early *in vitro* studies were performed in 1999 in failing human cardiac myocytes, confirming normalization of contractility and Ca^{2+} handling consequent to adenoviral SERCA2a overexpression.[24]

The results of numerous studies in rodent HF models also indicate that SERCA2a is a promising target for gene therapy. SERCA2a-KO mice showed an exaggerated response to transverse aortic constriction (TAC) as compared to control mice, whereas SERCA2a-transgenic mice showed superior survival and preserved contractile reserve in response to TAC.[25,26] In a rat TAC model, intracoronary AdV-SERCA2a administration enhanced overall survival and improved contractile performance.[27] These data were later challenged because SERCA2a-transgenic rats showed increased mortality 24 h after myocardial

infarction, probably caused by ventricular arrhythmias; at the end of 6 months, no beneficial effects were attributable to SERCA2a overexpression in this model. Furthermore, SERCA2a overexpression in failing canine cardiac myocytes promoted loss of β-AR responsiveness.[28] Despite these early concerns, compelling evidence for SERCA2a gene therapy comes from a large model of mitral regurgitation HF in pigs. Antegrade intracoronary infusion of an AAV-1 expressing SERCA2a under a nonspecific (cytomegalovirus) promoter preserved systolic function and reduced ventricular remodeling for up to 2 months after vector delivery.[29]

Recently, the first human clinical HF gene therapy trial using the SERCA2a construct—the Efficacy and Safety Study of Genetically Targeted Enzyme Replacement Therapy for Advanced Heart Failure (CUPID)—completed phase I/II. The CUPID trial was aimed at demonstrating the safety and efficiency of intracoronary AAV-1 SERCA2a delivery in HF patients. All the patients included had to be equipped with an implantable cardioverter defibrillator, thereby addressing the concerns regarding inhomogeneous SERCA2a expression and consequent ventricular arrhythmias.[30] In the phase II study, AAV-1 SERCA2a gene transfer was shown to be superior to placebo treatment, improving cardiac function as well as overall performance.[30] Currently, enrollment for Phase III has started; this phase of the trial is aimed at confirming the efficacy of SERCA2a gene therapy in a larger HF patient population.

In conclusion, SERCA2a is a promising target for future enzyme replacement gene therapy aimed at restoration of disturbed cardiac myocyte Ca^{2+} handling in HF in humans. Despite fears of increased vulnerability to ventricular arrhythmias, no such incident was reported in the first-in-human phase I/II trials; nonetheless, safety concerns will demand implantation of an implantable cardioverter defibrillator prior to SERCA2a gene therapy.

PLN. PLN is another promising target for novel gene therapy strategies. PLN regulates SERCA2a activity. Dephosphorylated PLN binds SERCA2a, thereby inhibiting its activity. PLN can be phosphorylated both at serine16 by protein kinase A (PKA) and at threonine17 by Ca^{2+}-calmodulin kinase II upon β-AR stimulation, thus liberating SERCA2a and increasing Ca^{2+} uptake into the SR.[31] In failing rat myocardium, diminished SERCA2a activity is associated with decreased serine16 phosphorylation levels of PLN. This finding underscores the importance of PLN for Ca^{2+} handling.

Early studies with PLN KO and transgenic mutant PLN mice were able to show the importance of PLN as a central regulator of SERCA2a activity; mice expressing the constitutive active PLN variant showed deranged Ca^{2+} handling and developed cardiomyopathies, whereas KO mice showed improved cardiac performance and enhanced SR Ca^{2+} uptake.[32,33]

Crossbreeding the PLN KO mouse with genetic cardiomyopathy mouse models (MLP-KO mouse, calsequestrin-overexpressing mouse) resulted in rescue of cardiac function.[34] The application of the TAC model in the PLN KO mouse augmented cardiac function to a moderate extent.[35]

However, no effects of PLN knockdown were observed in murine HF models of Gαq overexpression, tumor necrosis-α overexpression, or tropomodulin overexpression. Interestingly, in all the models, PLN knockdown caused the expected effects on intracellular Ca^{2+} handling and contractility at the cellular level, without altering global cardiac function and without remodeling.[36–38] This is interesting for two reasons. First, positive outcomes in one HF model do not predict outcomes in other HF models. Second, the data from genetic mouse models should be interpreted with caution.

With the aim of creating a gene therapy approach targeting PLN, a dominant negative mutant PLN molecule (phosphomimetic mutation at serine 16 = PLN-S16E, the site of PKA activation) was designed. AAV gene therapy with PLN-S16E halted cardiac dysfunction in a hamster cardiomyopathy model as well as in rats after myocardial infarction.[3,31,39] In addition, intracoronary delivery of an AdV expressing PLN-S16E reversed advanced HF in a large-animal model.[40] Furthermore, RNA interference was used successfully to downregulate PLN expression; small interfering RNA knockdown of PLN allowed for improvements in intracellular Ca^{2+} handling and single-cell contractility in cardiac myocytes isolated from failing human hearts.[41] When an AAV serotype 9 expressing a small hairpin RNA targeting PLN was injected into the aortic root in HF rats, there was a reduction in PLN content by about 75%; thereby, suppression of SR Ca^{2+} ATPase in the HF groups was rescued, and systolic and diastolic functions were normalized.[42]

Despite significant evidence from these preclinical models, the effect of PLN inhibition in human HF is not predictable. Several published cases of genetic cardiomyopathies involve mutations or deletions in the PLN coding regions with resulting PLN loss-of-function or PLN knockdown.[43] Some of these human mutations with consequential cardiomyopathy were reproduced in mice.[44]

Overall, significant evidence regarding PLN comes from preclinical rodent and large-animal studies. However, neutral results from genetic cardiomyopathy mouse models and from human patients with genetic cardiomyopathies caused by PLN loss-of-function or knockdown mutations perfectly demonstrate the difficulties in extrapolating these data to human conditions. Further investigations are required before definitive conclusions can be reached.

S100A1. The small protein S100A1 belongs to the family of S100 EF-hand calcium-binding proteins and is expressed predominantly in cardiac myocytes.[45,46] The finding that S100A1 protein and transcript levels were diminished in human ischemic and dilated cardiomyopathies led to the unveiling of S100A1's unique role as a central regulator of cardiac myocyte function.[47]

In normal cardiac myocytes, S100A1 can be found at the SR, in the mitochondria, and within the sarcomere. It has a dual role in RyR function: it enhances open probability in systole and reduces the occurrence of diastolic Ca^{2+} sparks. In addition, S100A1 enhances Ca^{2+} uptake into the SR in diastole by increasing SERCA activity.[45,46,48]

Adenoviral overexpression of S100A1 in neonatal rat cardiac myocytes showed proof of concept of the therapeutic properties of S100A1 and resulted in enhanced systolic force development, enhanced cytosolic Ca^{2+} transients, and accelerated diastolic Ca^{2+} clearance. These unique features are independent of β-AR signaling, given that no changes in intracellular cAMP levels or PKA activity are observed; furthermore, the effects of S100A1 are preserved under β-AR stimulation.[45,49]

Various animal and human disease models have provided insights into S100A1's unique therapeutic spectrum. S100A1 KO and transgenic (STG) mice presented opposing phenotypes after myocardial infarction. Although KO mice showed no overt phenotype when stress-free, myocardial infarction caused higher mortality in these mice as compared to wild-type mice, whereas STG mice showed superior survival rates. On the cellular level, KO mice exhibited abnormal Ca^{2+} cycling and increased left-ventricular remodeling, whereas the STG mice revealed improved Ca^{2+} cycling in line with cessation of adverse left-ventricular remodeling.[50] These results were confirmed in a post-cryomyocardial infarction rat HF model; intracoronary delivery (during aortic cross-clamping) of an AAV6-expressing human S100A1 under the control of a cardiac myocyte-specific promoter (α-cardiac actin enhancer/elongation factor 1 promoter) significantly improved left-ventricular function and reversed adverse remodeling.[51] The rescue effect was evident down to the cellular level, at which intracellular Ca^{2+}-transients were enhanced and single-myocyte contractility was almost normalized.[51] This study is of clinical importance because synergistic effects of S100A1 gene therapy and β-AR blockade (standard HF therapy) were observed.[51]

Important features of S100A1 are its pleiotropic effects, increasing SERCA2a activity and Ca^{2+} transients on the one hand and stabilizing the RyR and reducing diastolic SR Ca^{2+} leaks on the other.[52] This latter effect reduces vulnerability for ventricular arrhythmias; this is obviously a favorable outcome in HF, in which mortality arises not only from pump failure but, to a significant extent, from lethal arrhythmias.[52]

Recent evidence from a large-animal HF trial (Pleger S *et al.*) and from an *in vitro* study of failing human cardiac myocytes[53] further underscores the therapeutic relevance of S100A1 gene therapy.

In summary, S100A1 expression is reduced in the failing heart, and replacement of S100A1 enables restoration of Ca^{2+} cycling and excitation contraction coupling and reduction of diastolic SR Ca^{2+} sparks. These pleiotropic effects of S100A1 target the cornerstones of HF mortality, namely, pump failure and lethal arrhythmias. Its unique profile makes S100A1 a truly promising target for future HF gene therapy efforts.

Protein phosphatase-1 (PP1) and inhibitor-1 (I-1). Another key player in the modulation of Ca^{2+} cycling and cardiac contractility is PP1. The threonine/serine phosphatase PP1, localized to SR membranes, is a negative regulator of β-AR signaling. In particular, it mediates restoration of cardiac function to basal levels after β-AR stimulation by dephosphorylating key phosphoproteins such as PLN.[54] Despite the tight secondary control of PLN and SERCA2a, inhibition of PP1 activity by the endogenous inhibitors types 1 and 2 (I-1 and I-2) is required for fine-tuning cardiac contractility and Ca^{2+} homeostasis. β-AR signaling activates I-1, a small PKA substrate, and thereby promotes attenuation of PP1 activity. In animal models and human HF, PP1 activity and expression were found to be increased and I-1 protein expression was found to be decreased. Therefore, both of these enzymes are potential targets for future HF therapy.[55]

Transgenic overexpression of an active I-1 form in mice subjected to TAC completely restored cardiac function and partially reversed remodeling.[56] Carr *et al.* demonstrated improvement in contractility to nonfailure levels after intracoronary adenoviral delivery of a truncated form of I-1 (I-1c) in a rat model of preexisting HF due to pressure overload.[57] These results were confirmed in a recent study involving hamsters with cardiomyopathy. The study demonstrated that stabilization of cardiac function could be achieved by PP1 inhibition with an AdV expressing I-2.[58] Interestingly, these beneficial effects were associated with selective phosphorylation of PLN, leaving the phosphorylation levels of RyR (another PP1 target) unchanged.[58] This is important because increased RyR phosphorylation potentially leads to diastolic SR Ca^{2+} leakiness and arrhythmogenic vulnerability.[59]

However, recently published data challenge these findings. It was shown that I-1 KO mice (Ppp1r1a KO mice) displayed only slightly reduced catecholamine sensitivity and were even partially protected against acute and chronic catecholamine toxicity.[60] Furthermore, this protection was related to reduced phosphorylation levels not only of PLN but also of RyR, thereby casting doubt on the assumed specificity of I-1 for PLN.

Overall, these data add to the complexity of tight intracellular regulation of Ca^{2+} handling and SERCA2a by PP1 and its inhibitor I-1. Future studies, potentially involving larger-animal models, should further investigate the potential of therapeutic approaches targeting PP1 and I-1, with the ultimate goal of increasing SERCA2a activity and thus improving systolic and diastolic function.

Diastolic dysfunction

Diastolic dysfunction, in contrast to systolic dysfunction, presents with preserved ejection fraction (systolic function) and reduced diastolic left-ventricular filling. Diastolic dysfunction can occur either as an isolated dysfunction or in combination with systolic dysfunction. Diastolic dysfunction is characterized mainly by prolonged relaxation, increased filling pressures, and decreased contraction velocity, leading to impaired relaxation and culminating in the inability of the ventricle to maintain adequate preload.

At the molecular level, increased cytosolic diastolic calcium concentrations were found. These may be explained principally in terms of reduced SERCA2a activity, which limits Ca^{2+} reuptake of the SR in failing human myocardium; in addition, leaky RyRs further augment diastolic Ca^{2+} levels. Also, increasing amounts of collagen in the interstitial layer (interstitial fibrosis) and elongated cardiac myocytes cause diastolic stiffness of the ventricles (adverse remodeling).[61]

To date, only angiotensin-converting enzyme inhibitors have been confirmed as ameliorating diastolic dysfunction, mainly by halting adverse remodeling and reducing interstitial fibrosis. No specific pharmacological approach addresses deranged diastolic Ca^{2+} fluxes that contribute to diastolic dysfunction. Therefore, a need for novel molecular therapies to address this gap in modern HF therapy is evident. Three molecular approaches, targeting molecular mechanisms of diastolic dysfunction with a focus on diastolic Ca^{2+} handling, are reviewed briefly in this section.

Parvalbumin. Parvalbumin, an 11-kDa small cytosolic protein belonging to the superfamily of E-F hand Ca^{2+}/Mg^{2+} binding proteins, is normally absent in the heart. It is known to function as a soluble relaxing factor in fast-twitch skeletal muscle fibers by acting as a delayed Ca^{2+} sink. In contrast to SERCA2a, which consumes ATP to drive Ca^{2+} into the SR, parvalbumin acts through an energy-independent mechanism to accelerate relaxation by removing cytosolic Ca^{2+} in diastole. This characteristic makes parvalbumin an attractive target for gene therapy in HF.

An excellent model for human diastolic dysfunction is the hypothyroid rat model, which is uncomplicated by structural defects that could obscure the Ca^{2+} response. AdV gene transfer of parvalbumin in this model shortened the process of prolonged cardiac myocyte relaxation, thereby ameliorating diastolic dysfunction.[62] In line with this finding, the crossbreeding of parvalbumin transgenic mice with a mutant α-tropomyosin A63V line accelerated diastolic relaxation parameters.

The translation of these intriguing results into clinical application is potentially hampered by the Ca^{2+} binding kinetics of parvalbumin; this factor could impose a restriction on the therapeutic range because higher parvalbumin concentrations in the cytosol would bind systolic Ca^{2+} and impair systolic function.

This limitation was recognized from the results of a study involving parvalbumin (AdV) delivery to cardiac myocytes from a dog TAC model. Low parvalbumin expression improved diastolic parameters whereas at higher concentrations contraction kinetics was diminished.

In conclusion, parvalbumin is the only candidate therapeutic agent that has been confirmed to exclusively target diastolic dysfunction. One of its key features is energy independence; therefore, it does not further exacerbate the energy imbalance in the failing cardiac myocyte. However, its narrow therapeutic range demands intensive preclinical testing.

SERCA2a. As outlined above, SERCA2a is responsible for SR Ca^{2+} uptake during diastole, and its activity is tightly regulated by its direct inhibitor, PLN.[22] Not only does SERCA2a modulate contractility by increasing SR Ca^{2+} levels, but it also renders Ca^{2+} transients faster through quicker SR Ca^{2+} uptake, thereby shortening the relaxation process. This was studied in failing human cardiac myocytes that presented with normalized relaxation velocities following AdV SERCA2a overexpression.[24] Furthermore, in a rat HF model with SERCA2a overexpression, not only was systolic function recovered, but diastolic parameters were also significantly improved.[27,63] Therefore, SERCA2a gene therapy affects systolic function in addition to ameliorating diastolic dysfunction.

S100A1. As discussed in detail above (see "S100A1" under "Positive inotropic therapies: Ca^{2+} cycling"), S100A1 is an important regulator of Ca^{2+} homeostasis, among other agents that regulate SERCA2a activity. Compelling data from various studies demonstrate enhanced SERCA2a activity during relaxation after S100A1 overexpression, with enhancements in diastolic function similar to those seen with SERCA2a overexpression.[48,64]

Furthermore, S100A1 modulates sarcomeric stiffness as it binds to the giant protein titin.[65] Titin is important for the generation of passive tension and myocardial stiffness within the sarcomere. The PEVK (proline-, glutamate-, valine-, and lysine-rich) domain of titin interacts with F-actin, hampering its movement toward myosin. After Ca^{2+} binding, S100A1 binds to the PEVK domain, liberating actin and facilitating actin–myosin interaction. This shows that S100A1 is an important regulator of titin. During systole, S100A1 entraps titin, thereby reducing passive tension; during diastole, it enables titin to stabilize the sarcomere by preventing excessive actin slippage. This effect on sarcomeric compliance was demonstrated in skinned left-ventricular muscle strips, in which S100A1 attenuated passive tension in a Ca^{2+}-dependent manner.[45]

Despite these promising results, the effect of S100A1 on diastolic performance warrants further investigation, especially in preclinical animal models of diastolic dysfunction. However, the pleiotropic actions of S100A1 on systolic function and diastolic Ca^{2+} sparks (potentially lowering the risk for lethal arrhythmias) and its favorable effects in ameliorating diastolic dysfunction render this protein uniquely valuable for future molecular HF therapies.

Cell survival

The contractile capabilities of single myocytes and the number of viable cells in the heart that contribute to cardiac force define global cardiac function. HF is associated with loss of cardiac myocytes, as is evident from the upregulation of apoptotic markers in failing adult cardiac myocytes.[66] Because loss of adult cardiac myocytes cannot be compensated for in HF, at least not to the full extent of the loss, this may contribute to cardiac dysfunction. Halting cell death under stressed conditions is therefore a novel potential approach to hindering HF progression. We briefly discuss two strategies with effects on cell-survival pathways: β_2-AR and βARKct.

Cardiac β-ARs deliver their signals via G-proteins. Whereas β_1-ARs couple exclusively to G_S, activating the AC-PKA interactome, β_2-ARs couple with G_S as well as with the inhibitory G_i subunit. The G_i pathway of β_2-AR is connected to the phosphatidylinositol-3′-kinase/Akt cell-survival pathway.[67] This can be inferred from the finding that pretreatment with a specific β_2-AR agonist decreased apoptotic cell death and improved left-ventricular function in isolated rat hearts subjected to ischemia/reperfusion injury. Furthermore, in isolated hypoxic cardiac myocytes (from a cyanotic heart disease rabbit model), transfection with β_2-AR improved survival and diminished apoptosis.[68]

However, the actual mechanism may be more complex, given that β_2-AR (through G_S) also stimulates pathways analogous to those stimulated by β_1-AR; moreover, G_i-signaling seems to prevail under the delineated conditions.[69] The opposing roles of the two adrenergic receptors and the heterogeneity of β_2-AR signaling may account for the contradictory results seen with low- and high-level β_2-AR overexpression in various genetic models (see "Resensitizing β-AR signaling: the β-AR–G-protein–adenylyl cyclase interactome" above). Targeting a downstream mediator of the phosphatidylinositol-3′-kinase/Akt cell-survival pathway might therefore allow for a more specific intervention.

GRK2, an endogenous regulator of β-AR signaling, interacts with and inhibits the pro-survival pathway kinase Akt.[70] GRK2 is upregulated in HF, and inhibition of GRK2 by the miniprotein βARKct was shown to rescue HF in several animal models (see "βARKct" above).[3,6–8] Recently, a putative role for GRK2 in cardiac myocyte death has found support in the hypothesis that inhibition of GRK2 by βARKct in mice subjected to ischemia/reperfusion could diminish apoptosis by restoring Akt activation, thereby increasing cytosolic NO production.[70]

Obviously, extensive research focusing on cell-survival pathways and the involvement of β_2-AR and GRK2 in different models of HF is imminent. However, targeting the downstream effectors of β_2-AR and the phosphatidylinositol-3′-kinase/Akt cell-survival pathway is a potentially promising new concept.

Genetic cardiomyopathies

Genetic cardiomyopathies are an excellent target for gene therapy for several reasons. Monogenetic cardiomyopathies, in particular, are less complex than most final HF states. Given that a single-nucleotide polymorphism occurs rarely and is often distributed only within a single family, pharmaceutical interest in tailoring specific therapies for such rare polymorphisms is low to nonexistent. However, being of monogenetic origin, these cardiomyopathies offer excellent strategies for gene therapy because replacing the defective or knocked-down proteins will potentially offer a therapy based on the cause of the disease.

A first attempt with AAV-mediated delta-sarcoglycan gene transfer into hearts of TO2 hamsters (a genetic cardiomyopathy inbred hamster line) was shown to rescue animals from developing dilative cardiomyopathy and to lead to long-term improvements in cardiac function.[71] In another gene therapy approach, delta-sarcoglycan KO mice were used; these animals develop cardiomyopathy and muscular dystrophy similar to human patients with defects in the gene encoding this protein. Goehringer *et al.* developed an AAV serotype 9 gene therapy to substitute delta-sarcoglycan; 6 months after intravenous injection of this vector, immunohistochemistry revealed almost complete reconstitution of the sarcoglycan subcomplex in heart muscle and stabilization of left-ventricular function.[72] Genetic cardiomyopathies caused by larger proteins remain a challenge for gene therapy. One such example is Duchenne muscular dystrophy, characterized by dystrophin defects that cause skeletal muscle weakness and cardiac dysfunction. Various mutations were found, causing truncated dystrophin proteins or knockdown, with severe forms leading to cardiomyopathy, among other manifestations of the disease.[73] Systemic administration of a dystrophin minigene (termed micro-dystrophin) improved left-ventricular remodeling and prevented dobutamine cardiomyopathy.[74] In an alternative approach involving exon skipping, antisense technology is used to skip defective exons during mRNA splicing, leading to the production of a truncated protein and thereby restoring dystrophin function.[75]

Although gene therapy offers a potential approach targeted at the cause of the disease, attempts to treat monocausal genetic cardiomyopathies are rare because of the technical challenges involved. Genetic screening in specialized cardiomyopathy centers (which are currently available in Europe and the United States) will yield novel candidate genes; this in turn will result in a growing number of cases of dilative cardiomyopathy being etiologically linked to genetic disorders; improvements in molecular technology will facilitate tailored gene therapy approaches specifically for the individual patient or family with the disease. Gene therapy of genetic cardiomyopathies therefore has great potential to offer the first complete applications of personalized medicine. An overview of gene therapy targets and preclinical and clinical evidence is presented in **Table 1**.

PRECLINICAL AND CLINICAL TRIALS

IND status and pharmacology of HF gene therapeutics

In the United States, when a gene therapeutic agent has shown great promise in treating experimental HF, it can be tested in a clinical trial for its therapeutic benefit in humans only after an investigational new drug (IND) designation has been obtained from the US Food and Drug Administration. The application is required to be based on preclinical data, typically from animal studies, that show the drug to be safe enough to be tested in controlled clinical trials. Clinical trials are the ultimate means of demonstrating the safety and efficacy of a drug and are a prerequisite for the agency's approval for clinical use (http://www.gtrp.org). Hence, for a gene therapeutic agent to avoid becoming "lost in translation," an academically considered "promising" candidate with secured intellectual property must be transformed into a "validated" therapeutic target with the potential to attract pharmaceutical partnership in order for the final steps of preclinical development to be completed.

To this end, it is important that the IND application contain preclinical data from animal pharmacology and toxicology studies to permit assessment as to whether the drug is reasonably safe to be tested in humans. As with any other biological drug entering the clinical stage, it is necessary to establish precisely what an HF gene therapeutic does to the human body (pharmacodynamics) and what the human body does to it (pharmacokinetics). This includes thorough assessment of the drug dosages that can treat HF effectively while staying within the safety range (therapeutic window); it also involves investigation of absorption/administration, distribution, metabolism, elimination, and toxicity, tested—ideally, in conformity with the guidelines of good laboratory practice, employing a gene-based therapeutic produced in accordance with good manufacturing practice. Once comprehensive data have been obtained from proof-of-concept studies in small-animal models, one must consider employing large-animal disease models that more closely approximate human size and

Table 1 Molecular targets for cardiac gene therapy in heart failure, supporting animal models, and state of clinical trials

Construct	Vector	Rodent	Large animal	Human CM	Clinical trial
SERCA2a	AdV/AAV	+	+	+	CUPID trial (phase I/II/III)
S100A1	AdV/AAV	+	+	+	
sh-Phospholamban	AAV	+	+	+	
Phospholamban-S16E	AdV	+	+		
βARKct (GRK2 inhibitor)	AdV/AAV	+	+	+	
β_2-AR	AdV	+			
Adenylyl cyclase VI	AdV	+	+		
Parvalbumin	AdV	+			

AAV, adeno-associated virus; AdV, adenovirus; β_2-AR, β-adrenergic receptor; βARKct, C-terminus of adrenergic kinase; CM, cardiac myocytes; HF, heart failure; SERCA2a, sarcoplasmic reticulum Ca^{2+}-ATPase; sh, short hairpin.

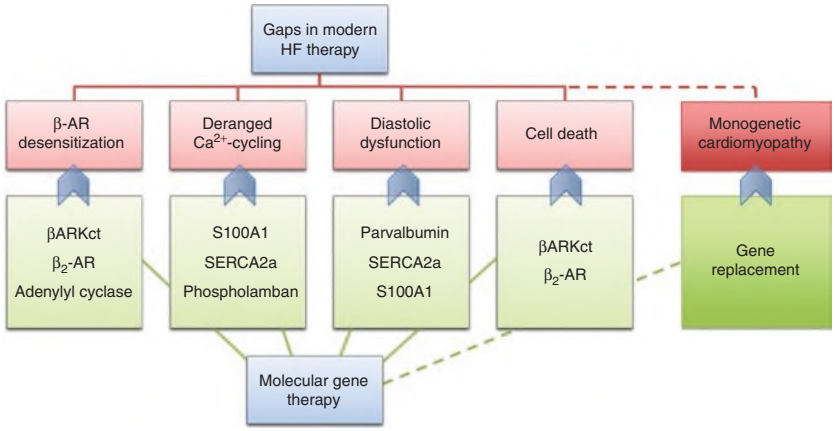

Figure 1 Gaps in modern heart failure therapy and potential gene therapy targets for closing these gaps. β-AR, β-adrenergic receptor; βARKct, C-terminal domain of GRK2; SERCA2a, sarcoplasmic reticulum $Ca2^+$ ATPase.

cardiovascular pathology to interrogate the pharmacology of the HF gene therapeutic in order to obtain the information necessary for be extrapolation to humans.

Characteristics of HF gene therapeutics that challenge classical pharmacological considerations

It is far less straightforward for an HF gene therapeutic to meet these requirements than for a single chemical compound. It is difficult to predict the biological effect of a defined gene dose because therapeutic gene expression results from a complex interplay among gene dose, myocardial transduction efficiency, promoter activity, and decay of the therapeutic protein within the targeted cardiac cell type, i.e., cardiac myocytes. Hence, transduction efficiency of the viral vector at the desired target site in combination with activity of the expression cassette deserves careful consideration to determine the safety range of the therapeutic gene product inside and outside target cells. Another potential shortcoming is that the state of the art in cardiovascular gene therapy does not yet provide the opportunity to regulate or even terminate expression of the therapeutic gene. Because disease severity can fluctuate, the adverse effects of gene-based treatment might result from "relative" overdosing; the expression levels of the therapeutic gene might therefore require acute or chronic attenuation during the course of therapy.

Gene therapeutics for HF: different stages of clinical translation

Despite the underlying complexities, AAV- and adenoviral-based gene addition studies employing SERCA2a, S100A1, βARKct, and AC VI, as well as strategies to silence PLN in cardiac myocytes, have been successfully tested in human-relevant large-animal HF models for efficacy and safety.[20,29,36–38] Although some of the targets lack comprehensive dose-dependency relationships, these studies emerged from comprehensive proof-of-concept studies in small-animal HF models and human failing cardiac myocytes.[5,14,39,53] They clearly indicate the feasibility and efficacy of various therapeutic genes in treating the disease effectively within their safety ranges under near-clinical conditions. The stepped translational strategy might convey a predictive value when SERCA2a, a molecular target in an advanced stage of testing, proceeds beyond phase I testing in humans, using AAV technology in combination with an unbiased expression cassette. Preliminary data from the CUPID trial are encouraging and serve to prepare the ground for identification of targets with unique molecular profiles and clear indicators of biosafety, such as S100A1 and βARKct. Employing advanced AAV methodology in combination with cardiac myocyte–biased expression cassettes, AAV-S100A1 and AAV-βARKct gene therapeutics are currently seeking IND status, with the umbrella of the National Institutes of Health Gene Therapy Resource Program initiative providing comprehensive methodological and regulatory support for the IND application process.

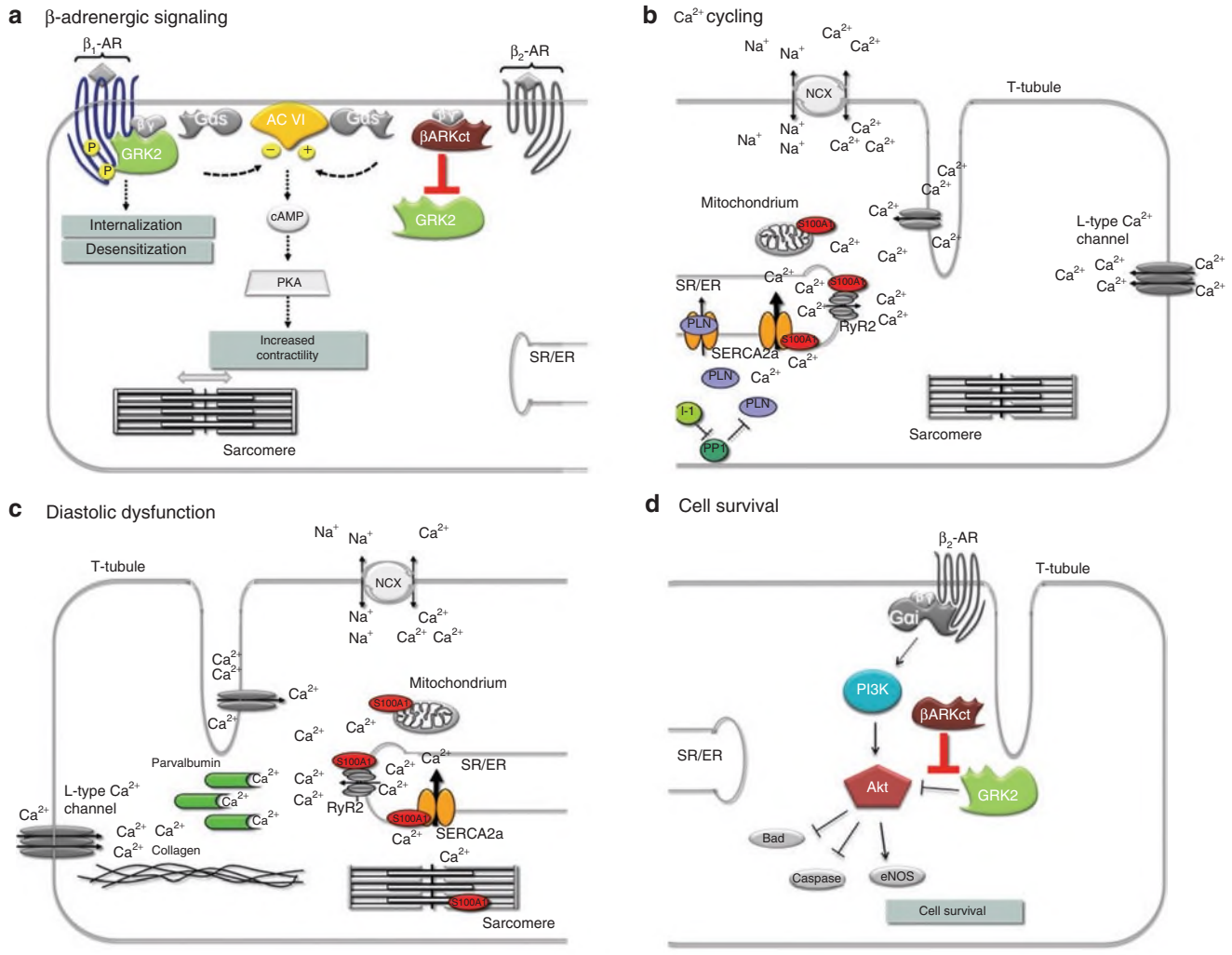

Figure 2 Gaps in conventional treatment of heart failure include deranged β-AR signaling, distorted Ca^{2+} cycling with depletion of SR Ca^{2+} levels, diastolic dysfunction, and cell death. For each of these gaps, the molecular signaling pathways involved and potential gene therapy targets (depicted in color) are shown. (**a**) β-AR signaling: overexpression of the β_2-AR, overexpression of AC VI, and inhibition of GRK2 by its C-terminal domain βARKct are all aimed at resensitizing the β-AR–G-protein–AC interactome. (**b**) Ca^{2+} cycling: overexpression of SERCA2a, S100A1, or a dominant negative form of PLN or an shPLN are aimed at normalization of intracellular Ca^{2+} handling. S100A1 also stabilizes the RyR, thereby reducing arrhythmogenic vulnerability. I-1 overexpression inhibits PP1, thereby also activating SERCA2a. (**c**) Diastolic dysfunction: overexpression of SERCA2a and S100A reduce diastolic cytosolic Ca^{2+} levels and increase SR Ca^{2+} reuptake, thereby improving diastolic relaxation parameters. Overexpression of parvalbumin, an intracellular Ca^{2+} sink, leads to an energy-independent selective amelioration of diastolic dysfunction. (**d**) Cell survival: β_2-AR exerts antiapoptotic effects through the G_i-protein-mediated activation of the phosphatidylinositol-3′-kinase (PI3K)/Akt cell-survival pathway. Overexpression of a constitutively active form of Akt or PI3K and the abolishment of GRK2-mediated inhibition of Akt activate the survival pathway in heart failure. AC VI, adenylyl cyclase VI; β-AR, β-adrenergic receptor; βARKct, C-terminal domain of GRK2; GRK2, G-protein-coupled receptor kinase 2; I-1, inhibitor-1; PI3K, phosphatidylinositol-3′-kinase; PLN, phospholamban; PP1, protein phosphatase type 1; RyR, ryanodine receptor; SERCA2a, sarcoplasmic reticulum Ca^{2+} ATPase; shPLN, short hairpin PLN; SR, sarcoplasmic reticulum.

CONCLUSION

Gene therapy has the potential to close the gaps in HF therapy by directly rectifying intracellular signaling abnormalities and thereby offering a more cause-related therapeutic approach. From the current perspective, transvascular and intramyocardial delivery of AAV vectors seem to be the most feasible approaches to achieving effective cardiac transduction. Promising targets are aimed at facilitating β-AR signaling, balancing out deranged Ca^{2+}-cycling, preventing cardiac myocyte death, and ameliorating diastolic dysfunction. However, the translation of these techniques to clinical use is hampered by the complexity of viral vectors and intracellular targets and demands extensive preclinical optimization. In this context, it is evident that a purely academic exercise of establishing a therapeutic proof of concept in an appropriate small-animal HF model on the basis of a relevant clinical observation might end abruptly, faced with methodological and regulatory hurdles and no access to a translational developmental pipeline. However, if gene therapy is to finally emerge as a viable clinical option in HF, it is necessary to build on the current momentum provided by the ongoing CUPID trial and to take advantage of the unique National Institutes of Health–Gene Therapy Resource Program initiative. At the same

time, we need to foster the development of controllable, personalized, gene-based treatment for HF, employing a molecular target with a favorable and unique therapeutic profile.

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