Gene Therapy in Heart Failure

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Abstract

Heart failure is a significant burden to the global healthcare system and represents an underserved market for new pharmacologic strategies, especially therapies which can address root cause myocyte dysfunction. Modern drugs, surgeries, and state-of-the-art interventions are costly and do not improve

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survival outcome measures. Gene therapy is an attractive strategy, whereby selected gene targets and their associated regulatory mechanisms can be permanently managed therapeutically in a single treatment. This in theory could be sustainable for the patient's life. Despite the promise, however, gene therapy has numerous challenges that must be addressed together as a treatment plan comprising these key elements: myocyte physiologic target validation, gene target manipulation strategy, vector selection for the correct level of manipulation, and carefully utilizing an efficient delivery route that can be implemented in the clinic to efficiently transfer the therapy within safety limits. This chapter summarizes the key developments in cardiac gene therapy from the perspective of understanding each of these components of the treatment plan. The latest pharmacologic gene targets, gene therapy vectors, delivery routes, and strategies are reviewed.

Keywords

Gene therapy • Gene therapy vectors • Heart failure • Molecular targets • Routes of gene delivery

Abbreviations

AAV	Adeno-associated virus
AC6	Adenyl-cyclase type 6
Akt	Serine-threonine protein kinase
Bcl-2	B-cell lymphoma 2 gene
CCN family	Extracellular matrix-associated proteins
cDNA	Complementary DNA
CHF	Congestive heart failure
CVD	Cardiovascular diseases
dATP	Deoxy adenosine triphosphate
DNA	Deoxyribonucleic acid
GRK2 G	Protein-coupled receptor kinase 2
LacZ	Intracellular enzyme encoded beta-galactosidase
LVEF	Left ventricular ejection fraction
MCARD	Molecular cardiac surgery with recirculating delivery
microRNA	Small non-coding RNA
modRNA	Modified RNA
ODN	Antisense oligodeoxynucleotides
P-13	Chromosomally encoded integral outer membrane protein
PLN	Phospholamban
RNA	Ribonucleic acid
S100A1	Calcium-binding protein A1
SERCA2a	Sarcoplasmic reticulum calcium ATPase
siRNA	Short interfering RNA

SR	Sarcoplasmic reticulum
TGF beta	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
βARKct	Carboxyl terminus of beta-adrenergic receptor kinase

1 Introduction

Approximately 85 million Americans suffer from acute and chronic cardiovascular diseases (CVD). The associated mortality rates are stark, accounting for at least 33% of all deaths post 2010. This rate is 4.4 times more than cancer related morbidity and growing inline with record levels of obesity and diabetes. The total direct and indirect costs of CVD in the USA are estimated to be $315.4 billion, which is unsustainable (Go et al. 2013). These statistics clearly demonstrate that CVD are still the leading cause of mortality and morbidity in developed countries despite an increased awareness and focus on preventative measures.

Coronary artery disease is the prime risk factor and direct cause of CVD, prevalent in at least 65–75% of cases (St. John-Sutton et al. 1997). Coronary vessel disease progression typically results in single or successive myocardial infarction events, which permanently alter global function. Despite numerous interventions available to limit the initial postinfarction damage in the acute phase, the downward progression toward the congestive heart failure (CHF) condition is largely inevitable. Through the remodeling disease process strained myocytes are either lost, transition, or remain in a dysfunctional state typically out of sync with viable myocardium.

The CHF condition is the most expensive burden on the global healthcare system featuring accelerating annual costs alone exceeding $34 billion. The majority of these patients are destined for heart failure status despite significant efforts in accumulated, yet ineffective pharmacological interventions. All clinically available heart failure drugs, devices, and corrective interventional procedures only address secondary symptoms. Consequently, an additional 22 million CHF patients are diagnosed per year with 50% mortality within 5 years, despite cumulative failed therapeutic regimens.

Cardiac transplantation is the best option for end-stage CHF for long-term outcomes; however, more than 35% of patients unfortunately die waiting for a matched donor heart. Even more disturbing, as many as 60,000 patients per year could benefit from a transplant (Frazier and Myers 1998). Thus, given the shortcomings of the best available pharmacologic based therapies and donor hearts, there is a significant unmet need for newer, more efficacious, and more cost-effective treatments for severely afflicted patients.

There are two alternative treatment paradigms to improve long-term outcomes in both the early and late stage of CVD. These two paradigms are either a replacement (i.e., cell therapy) approach or a restorative genetic reprogramming of cells with gene therapy via vector transfer of nucleic acids. Although much more attention has

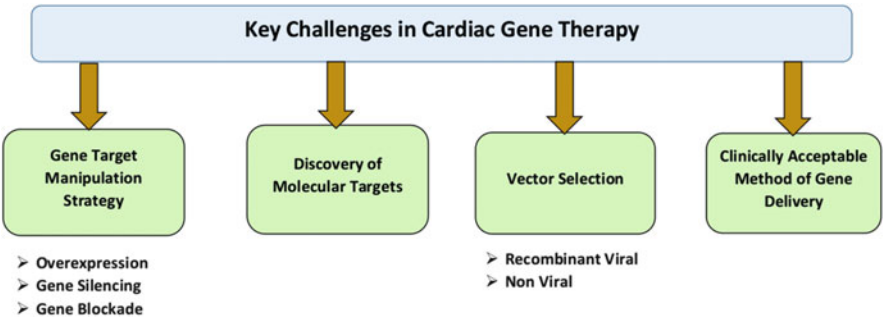

Fig. 1 Key challenges in cardiac gene therapy

been placed in cellular replacement strategies, these approaches do not address improving contractility function which is essential for increasing survival outcomes in CHF. The focus in late stage management is improving contractility, whereby the opportunity to arrest infarct expansion and global LV remodeling has past. Therefore, the transfer of therapeutic genes for the treatment has emerged as the more attractive strategy given its therapeutic impact on directly enhancing contractility.

The field of cardiovascular gene therapy has generated significant interest in recent times having completed a Phase II placebo controlled clinical trial in a robust heart failure population. A large base of extensive preclinical studies have provided solid proof of concept data indicating gene therapy's clinical potential, whereby the expression of selected transgenes in the myocardium enhances contractility, restores global function, and in some cases completely reverses CHF. Although promising, a number of challenges must be addressed in order to surpass regulatory clearance and ultimately reach the clinic.

The key translational challenges that must be addressed with this new therapeutic class are the following (Fig. 1), which will be extensively covered in the following text:

1. The validation of the specific target's genetic manipulation in the heart failure condition including overexpression and knockdown approaches
2. Selection of the best vector to manipulate the target that is both safe and results in efficacious expression
3. A clinically feasible method of delivery to safely achieve sufficient transfer in the myocardium
4. The discovery of novel targets that address root causes of myocyte function at the protein, RNA, and in some cases microRNA level
5. Synthesizing the said elements above such that the treatment is safe and effective as well as cost efficient for the healthcare provider.

2 Cardiac Gene Therapy Manipulation Strategies

A number of key gene therapeutic strategies have been investigated for improving outcomes in heart failure patients. The most commonly applied strategy features overexpression of a target gene, which involves either the replacement of a missing or restoring a gene's resultant protein levels. Dysfunctional genomic regulation in the heart can either be due to inherited genetic and or acquired with progressive cardiac disease. A common example of a purely inherited genetic is the X-linked recessive Becker's cardiomyopathy, which is an autosomal recessive gene defect such as those associated with alpha sarcoglycan deficiency. It is important to note that less research has been dedicated to this cause since a very low number of patients suffer from these defects. More commonly, however, ischemic cardiomyopathy induced CHF is characterized by certain genes that are consistently downregulated (e.g., SERCA2a). With the advent of biotechnology via rapid DNA/RNA profiling, these perturbations are readily identified against healthy controls. Moreover, viable mechanisms and interactions are in place but perturbed due to the degradation process secondary to the original infarction events. In the case of heart failure as covered later in this text, there are several key targets that have influential roles in driving contractility, energetics, survival, and structural integrity.

An alternative strategy to manipulating cardiac gene regulation is with gene silencing or blockade, which relates to the inactivation of dominant negative gene function involved in disease etiology or progression (Quarck and Holvoet 2004; Melo et al. 2005). By comparison with overexpression whose sole aim is to achieve a factor fold higher expression level, the degree of necessary efficacious interactions with this class is inherently much more complex since the expressed target must then interact further with subcomponents. Effective silencing often requires overexpression of the silencing element plus sufficient interaction with associated mechanisms of the disease mechanism, which may not be well established. Thus, due to the degree of conflicting interactions, these are much more difficult to execute in large animal model and human cardiovascular systems despite proof of concept in basic science experiments.

2.1 Target Overexpression Approaches

A gene's physiological function may be impaired or downregulated as a result of a mutation or a pathological process. Therefore, the restoration of function through exogenous DNA delivery to replace the deficient gene's action seems quite logical, but challenging to execute in vivo. In this case, full-length or partial cDNA encoding the deficient gene is delivered to the heart using a vector system capable of expressing the therapeutic protein (Quarck and Holvoet 2004). Several steps in the gene overexpression process may be modulated including the transcription, RNA splicing, translation, and posttranslational modification of a protein.

2.2 Silencing and Blockade Approaches

2.2.1 Antisense Oligodeoxynucleotides

Antisense oligodeoxynucleotides (ODN) are used as inhibitors of specific gene expression without any change in function of other genes. Single stranded ODN may be delivered either by direct administration (as a pharmacological agent) or by transfection with a vector encoding the ODN. The ODN binds to the target mRNA transcript and prevents translation. This mechanism of action is based on the presence of two forms of ODN: the RNase H-dependent ODN, which induces the degradation of mRNA, and the steric-blocker ODH, which physically blocks the progression of mRNA translation.

In CVD applications, the antisense ODH approach has been evaluated to prevent restenosis after balloon angioplasty (Quarck et al. 2001). In one study, treatment with antisense ODN directed against VEGF receptors could prevent VEGF-mediated arteriogenesis (Marchand et al. 2002). In another example, the systemic delivery of an antisense ODN induces silencing of miR-208a in the myocytes, thus improving cardiac function and survival in hypertensive-induced heart failure in rats (Montgomery et al. 2011).

2.2.2 Short Interfering

Gene silencing via siRNA technology is a promising strategy with great therapeutic potential despite continued problems with translation into the clinic due to delivery issues. siRNA is a short dsRNA molecule that induces sequence specific posttranscriptional gene modification. This mechanism is called RNA interference (RNAi). Recently, this strategy was used for the treatment of HF and the results showed that the restoration of cardiac function was most likely through the reduction of hypertrophy (Suckau et al. 2009). Once transferred into the cytoplasm, the siRNA incorporates into the nuclease complex, where they then disrupt the translation of the targeted genes. Successful left ventricular intracavitary delivery of DNA/siRNA complexes by means of sonoporation was demonstrated in murine hearts (Tsunoda et al. 2005). The incorporation of siRNA into terminally differentiated adult rat cardiac myocytes using adenovirus has also been reported (Rinne et al. 2006).

Due to sustainability challenges with blockage and silencing approaches, single gene target overexpression continues to dominate in the field since the posttranslational interactions and immune barriers are more lucid. Safe long-term overexpression provides the best possible chance to impact cardiac dysfunction provided sufficient levels of cDNA are present to drive therapeutic protein. siRNA, ODN, and other blockade strategies often have difficulty due to either insufficient delivery or other unknown mechanisms that counteract the intervention at the mRNA level. These strategies, however, are more attractive in angiogenesis applications where it is more desirable for the treatment duration to be short-term for repair. This is in direct contrast with the CHF patient's need in driving contractility.

3 The Molecular Basis of Congestive Heart Failure: Established and Novel Targets

Generally, the accepted clinical definition of CHF is the pathophysiologic state of impaired heart function that can result from any structural or functional cardiac disorder. These individually or compounded impair the ability of the ventricles to work as pump for maintaining metabolic requirements of the body's tissue and organs (Go et al. 2013). CHF is a progressive disease and irrespective of the cause, is accompanied by deterioration of cardiac function. From the pathophysiological molecular perspective, CHF includes alterations in the myocytes gene expression, qualitative changes in cardiac cell types, and composition of extracellular matrix. These alterations compounded eventually lead to structural changes of left ventricle geometry.

CHF can be categorized as systolic or diastolic. Systolic CHF is characterized by a reduction in left ventricular ejection fraction (LVEF), enlargement of the left ventricle, a reduction in contractility, and pulmonary congestion. In post-myocardial infarction (post-MI), the systolic function is primarily impaired. On the other hand, diastolic CHF with normal LVEF is currently diagnosed in approximately 40–45% of heart failure patients. This type of CHF can mainly be attributed to LV diastolic dysfunction. The contributing factors including impaired LV relaxation, decreased LV distension, and increased LV end-diastolic stiffness.

In the last decade there has been tremendous increase in knowledge concerning the molecular mechanisms underlying both types of heart failure. The most recent developments indicate that the human heart in CHF is subjected to numerous important gene, cell, and organ level molecular changes which include these primary alterations: (1) in excitation–contraction coupling leading to changes in the contractile properties of the myocyte; (2) cytoskeletal proteins such as sarcomeric, membrane-associated, and proteins of the intercalated disc; (3) myosin heavy chain expression; (4) the maladaptive progression of β-adrenergic desensitization; (5) occurrence of hypertrophy, and myocytolysis with myofibrillar degeneration and progressive loss of myofilaments; and (6) abnormal myocardial energetics secondary to mitochondrial dysfunction (Shah and Mann 2011).

These changes in turn cause myocyte loss due to necrosis, apoptosis and autophagy, and alterations in composition of the extracellular matrix including enhanced matrix degradation and myocardial interstitial and perivascular fibrosis (Mann et al. 2012). Pharmacological management of CHF, such as angiotensin converting enzyme inhibitors, β-adrenergic receptor blockade, calcium-channel blockers, diuretics, and inhibition of renin-angiotensin-aldosterone system. These have provided benefit to decrease morbidity and mortality. However, this treatment is symptom-oriented and cannot stop the disease progression and reversal CHF to healthy state, thus increasing the value of a gene therapy solution which directly focuses on permanently treating the mechanism. Here we summarize the current state of main existing molecular targets in cardiac gene therapy (Fig. 2).

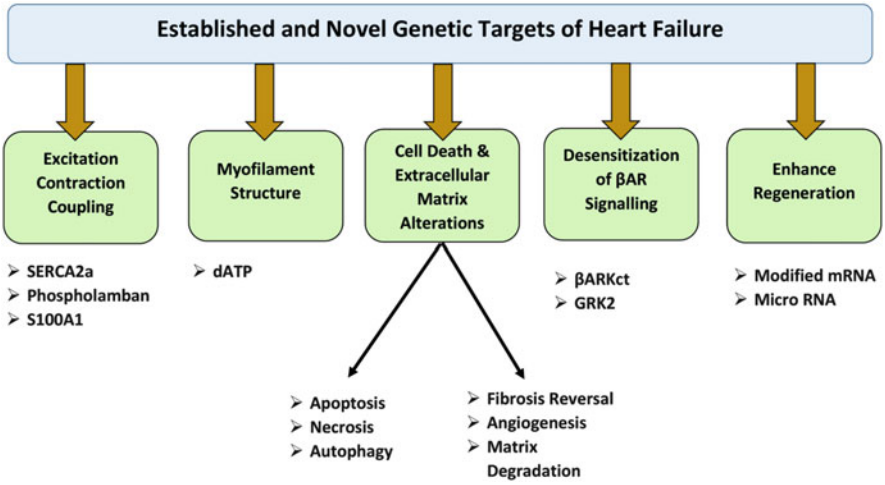

Fig. 2 Established and novel genetic targets in heart failure. *SERCA2a* sarcoplasmic reticulum calcium ATPase, *S100A1* calcium-binding protein A1, *dATP* deoxy adenosine triphosphate, *βARKct* carboxyl terminus of beta-adrenergic receptor kinase, *GRK2* G-protein-coupled receptor kinase 2

3.1 Excitation–Contraction Coupling

To understand the molecular defects in heart failure, we need to briefly describe the processes occurring in cardiac excitation–contraction coupling. During the cardiac action potential, Ca^{2+} enters the cell through depolarization-activated Ca^{2+} channels as an inward Ca^{2+} current, which contributes to the action potential plateau. This action lead to triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors located at the SR membrane. This, in turn, allows Ca^{2+} to bind to the myofilament protein troponin C, resulting in sarcomere shortening and muscle contraction. For relaxation to occur, it is necessary to have a decrease in intracellular Ca^{2+} concentration, thus allowing Ca^{2+} to dissociate from troponin. This requires Ca^{2+} transport out of the cytosol by pathways involving SR Ca^{2+} ATPase, sarcolemmal Na+/Ca^{2+} exchange, sarcolemmal Ca^{2+} ATPase, or mitochondrial Ca^{2+}. (Hajjar et al. 2000).

3.1.1 SERCA2a

Deficient SR Ca^{2+} uptake during myocyte relaxation has been identified in failing hearts from both humans and animals and is associated with a decrease in the expression and activity of the sarcoplasmic reticulum calcium ATPase (*SERCA2a*). This protein is a Ca^{2+} ATP-dependent pump of the sarcoplasmic reticulum that has a critical role in Ca^{2+} regulation. The importance of *SERCA2a* in HF has been studies extensively in rodents and large animal models and it was tested in clinical trials. Gene transfer of *SERCA2a* restored the calcium transit and significantly improved contraction and relaxation velocity in all experimental models of heart

failure. The mechanism of improved heart function induced by *SERCA2a* overexpression includes an enhanced SR Ca^{2+} uptake during diastole, increased SR Ca^{2+} content, and more efficient Ca^{2+} efflux during systole. Moreover, *SERCA2a* gene delivery with normalization of intracellular Ca^{2+} prevents mitochondrial dysfunction and decreases energy cost for regulation. This results in the restoration of cardiac energetics by improving mechano-energetic efficiency and by enhancing energy supply (Hajjar et al. 1998). On basis of these promising results, the CUPID 2 clinical trial was designed to assess whether gene transfer with SERCA2a improves outcome in patients with moderate to severe heart failure. CUPID 2 was the largest gene transfer study done in patients with HF so far. However, the results of AAV1/SERCA2a at the dose and delivery mode tested did not support the positive findings seen in a pilot trial (Greenberg et al. 2016).

3.1.2 Phospholamban Inhibition

Phospholamban (PLN) regulates the homeostasis of SR Ca^{2+}, mediating slower cytosolic Ca^{2+} decay in cardiomyocytes, which translates into diastolic relaxation. It is hypothesized that the suppression of the inhibitory effect of PLN is a promising approach to improve cardiac function. Indeed, AAV-mediated overexpression of a mutant form of PLN improved LV function and mitigated adverse remodeling (Tsuji et al. 2009). Silencing of PLN expression after tachycardia-induced CHF in sheep increased ejection fraction and decreased LV end-diastolic area (Kaye et al. 2007).

3.1.3 S100A1

In cardiomyocytes, S100A1 primarily plays a role in increasing SERCA2a activity. This is achieved via diminishing diastolic SR Ca^{2+} leak, reinforcing function of the ryanodine receptors during systole, leading to an overall gain in SR Ca^{2+} cycling. S100A1 regulates SERCA2A/Phospholamban function, resulting in an amplification of SR Ca^{2+} release and uptake as well. The molecular level of S100A1 is reduced at the development of CHF (Most et al. 2007). This confirms that S100A1 may be a promising factor in the treatment of CHF. A study in a postinfarction pig model with CHF revealed improvement in contractility and also restoration of high-energy phosphate homeostasis (Pleger et al. 2011).

3.2 Targeting the Myofilaments

The use of 2-deoxy adenosine triphosphate (dATP) instead of adenosine triphosphate (ATP) as the energy source improves muscle contractility by enhancing crossbridge binding and cycling kinetics (Regnier et al 2004; Nowakowski et al. 2013). It was developed as an approach to elevate dATP in vivo by increasing the expression of the enzyme ribonucleotide reductase. This results in increased levels of 2-deoxy ATP (dATP). It was shown that increasing dATP in intact cardiomyocytes via adenovirus-mediated transfection increased contractile magnitude and kinetics (Moussavi-Harami et al. 2015). dATP can increase contraction

and the rate of crossbridge cycling in cardiac muscle from patients with end-stage heart failure, without retarding relaxation. It was also found that raising cultured rat cardiomyocyte dATP levels to only ~1% by adenoviral vector-mediated overexpression of ribonucleotide reductase under control of the constitutive cytomegalovirus promoter, significantly enhances the magnitude and rate of contraction, as well as the rate of relaxation, without altering intracellular Ca^{2+} transient amplitude (Kolwicz et al. 2016). Based on these results, overexpression of RNR and increased dATP content appear to have significant potential as a therapeutic strategy with the myofilament approach for treating heart failure.

3.3 Inhibiting Apoptosis

3.3.1 Bcl-2

Apoptosis regulates the balance between pro-death and pro-survival cell signals. The Bcl-2 family of proteins has emerged as a key component of the cell death process. Bcl-2 can prevent the opening of mitochondrial transition pores and the resultant release of cytochrome *c* that directly triggers apoptosis. Also, Bcl-2 can block cytokine-mediated apoptosis via the nuclear factor-kB signaling pathway. In an animal model of HF, group treated with Bcl-2 had superior preservation of LV geometry with less ventricular dilatation and wall thinning. There was also reduced apoptosis compared with the controls (Chatterjee et al. 2002).

3.3.2 Akt, P-I3

The Akt proto-oncogene, a serine/threonine protein kinase, controls multiple responses in the cardiac cells including the ability to inactivate pro-apoptotic proteins like Bad and caspase 9. Act is activated by the products of PI-3 kinase reaction and eventually led to inhibition of cardiac myocytes apoptotic death. Intracoronary delivery of adenoviral construct encoding Akt in small animal models revealed reduced infarct size and protected cardiac myocytes from apoptosis (Miao et al. 2000).

3.4 Desensitization of βAR-Signaling

3.4.1 βARKct

The βAR system is a hallmark signaling pathway for the regulation of cellular communication in cardiac contractility and is therefore an attractive molecular target (Koch et al. 1995). Long-term neuro-hormonal activation in CHF induces the desensitization of β-adrenergic signaling transduction including β-adrenergic receptor (βAR) down regulation, upregulation of GRK2 (βAR kinase), and increased inhibitory G-protein alpha-subunit function (Raake et al. 2008). Extensive research studies revealed that the βAR system includes two main components: β-receptors and the GRK2. GRK2 is a cytosolic enzyme that, upon receptor activation, binds the released Gβγ subunit of activated heterotrimeric G-proteins

at the plasma membrane allowing for phosphorylation of βARs, which subsequently bind inhibitory proteins called β-arrestins (Brinks et al. 2010). βARs that are bound to this complex undergo receptor desensitization. Abnormal βAR responsiveness leads to upregulation of sympathetic drive with chronic catecholamine release that contributes to further cardiac deterioration.

The clinical use of β-blockers in pharmacological management of HF has shown great success by blocking chronic βAR activation. However, β-blockers do not act on the molecular pathways of the CHF. Therefore, gene treatment is much more promising for long-term management. A GRK inhibitor was developed to effectively reverse βAR desensitization permanently via overexpression. It consists of the carboxyl terminus of βARK, known as βARKct which directly competes with GRK2. Many studies in preclinical models of CHF have demonstrated that myocardial overexpression with βARKct reversed ventricular remodeling and lowered sympathetic outflow of catecholamines. This implies that the βARKct overexpression and targeting GRK2 inhibition are very fruitful therapeutic targets in CHF (Raake et al. 2013).

3.5 Activation of Cardiac Adenyl-Cyclase Expression

Adenyl-cyclase (AC6) regulates the transfer of adenosine triphosphate to cyclic adenosine monophosphate. This sequence initiates many cardiac intracellular and extracellular signaling pathways. CHF is associated with decreased expression and activity of AC6, and a desensitization of β-adrenergic receptors. Cardiac-directed expression of AC6 in genetic animal models of CHF increases impaired LV function, enhances cAMP capacity in response to βAR stimulation, normalizes PKA activity, increases phospholamban phosphorylation, and increases sarcoplasmic reticulum (SR) Ca2+ uptake (Tang et al. 2011).

3.6 Enhance Regeneration

CHF results in comparatively large-scale loss of myocardium. As opposed to the theory of the postmitotic organ differentiation, studies in animal models have shown that myocytes can divide and express growth-related genes after myocardial infarction (Bergmann et al. 2009). The evaluation of cardiac cell cycle events, as well as genetic and metabolic fate mapping, have provided new data that CMs are not terminally differentiated cells. Studies performed in the mice and zebrafish suggest that cell turnover during normal conditions and after injury leads to appearance new mononucleated and polynucleated CMs with their proliferative capacity. Also, molecular markers associated with mitosis were described in the presence of human myocardium (Anversa et al. 2002).

Furthermore, it has been proven that the majority of new CMs are derived from preexisting CMs through cell division rather than activation of undifferentiated stem or progenitor cells (Kikuchi et al. 2010). These data altogether demonstrate

that mammalian hearts maintain a regenerative capacity throughout life, providing a rationale for the development of a new direction to restore function after significant myocardial damage. Postnatal mammalian CMs are being studied in two major ways that may be utilized independently or together: exogenous cell transplantation and stimulation of endogenous regenerative processes (Katz et al. 2016a).

3.6.1 Modified RNA

Recently, a new approach to manipulate the gene program of the adult cardiomyocyte has been reported via the generation of chemically modified mRNA (modRNA). The use of modified RNA technology in delivering paracrine factors into a damaged region in the heart has important implications for cardiogenesis, and the pathways that might trigger heart regeneration. It was demonstrated that modRNA is an efficient approach for transient, high level, and localized gene transfer into the heart. Moreover, intramyocardial injection of modRNA encoding human vascular endothelial growth factor-A resulted in the expansion and directed differentiation of endogenous heart progenitors in a murine HF model. VEGF-A/modRNA markedly improved heart function and enhanced long-term survival of recipients. This improvement was in part due to mobilization of epicardial progenitor cells and redirection of their differentiation toward cardiovascular cell types (Zangi et al. 2013).

3.7 Activation of Cytoprotective Mechanisms

Reactive oxygen species and oxidative stress have been implicated in a number of pathological processes that contribute to HF, including vasoconstriction, cardiac hypertrophy, apoptosis, fibrosis, inflammation, and myocardial stunning (Zablocki and Sadoshima 2013). Angiotensin II and norepinephrine, two mediators whose production/release is abnormally upregulated in failing hearts, promote oxidative stress by activating NAD(P)H oxidase and feeding the hydrogen peroxide-generating enzyme mono amino oxidase. The selective VEGFR-1 ligands VEGF-B and PlGF prevent mitochondrial superoxide and cytosolic hydrogen peroxide overproduction in cultured neonatal cardiomyocytes exposed to angiotensin II. Moreover, VEGF-B could mitigate hydrogen peroxide overproduction in cultured cardiomyocytes exposed to norepinephrine. These results suggest that a mechanism underlying the therapeutic action of VEGF-B, in vivo, might consist of antagonizing the pro-oxidant effects of angiotensin II and norepinephrine (Woitek et al. 2015).

3.8 Reverse Established Cardiac Fibrosis

One of the most important contributors to the development of cardiac fibrosis is the transforming growth factor beta (TGFβ1)-SMAD signaling cascade, which stimulates collagen expression and other downstream profibrotic targets and is

markedly upregulated in HF (Dobaczewski et al. 2011). A growing body of evidence from studies conducted in the heart indicates that CHF-activated TGFβ1-SMAD signaling system is phosphorylated and subsequently translocated to the nucleus to regulate target gene transcription (Rosenkranz 2004).

3.8.1 CCN Family

A group of matricellular proteins known as the CCN family (CCN1 to CCN6) are participated with many cellular functions including TGFβ1-SMAD system (Holbourn et al. 2008). CCN5 was significantly decreased in the myocardium of patients with severe CHF. A study evaluated the effects of adeno-associated virus (AAV)-mediated overexpression of CCN5 on established fibrosis with concomitant cardiac dysfunction. CCN5 was found to reverse fibrosis, as shown by its effects on collagen contents and the cardiac myofibroblasts. CCN5 inhibited endothelial to mesenchymal transition mediated by TGF-beta signaling and transdifferentiation of fibroblasts into myofibroblasts and thus CCN5 can reverse established fibrosis (Jeong et al. 2016).

3.8.2 SERCA2a

In another study it was demonstrated that SERCA2a gene delivery disrupts activation of the TGFβ1/SMAD signaling cascade, inhibiting de novo collagen synthesis and downregulating angiotensin II and its receptor. Moreover, it was found that TGFβ1 signaling genes are dramatically upregulated in CHF in all myocardial regions (Katz et al. 2016b).

4 Cardiac Gene Therapy Vectors: The Evolution

Gene therapy continues researching improvements with delivery, specifically in addressing the rate limiting translational gaps from animal models to clinical applications. Here, a key focus is often placed on host considerations such as anatomical, physiologic, disease, and immune barriers. Mammalian cells were not designed to uptake foreign genetic material, thus the delivery challenge is recognized as the greatest impediment once the target is validated. Choosing the right vector for the intended cardiovascular application is the most important decision, since safe and efficacious therapeutic genetic manipulation is absolutely demanded for successful gene therapy.

The availability of vectors for gene transfer has improved dramatically attributable to both increased available basic science research and the emerging clinical trial data. The ideal vector should have the following characteristics for the intended CVD application: (1) Cardiotrophic, since the myocyte microenvironment contains a disproportional population of endothelial cells, (2) Result in long-term expression (i.e., a must for contractility genes and enough to induce repair in the case of regeneration), (3) Minimize the risk of innate and adaptive immune responses, and (4) Possess a large coding capacity to incorporate the gene and enhancing promoters (Gaffney et al. 2007).

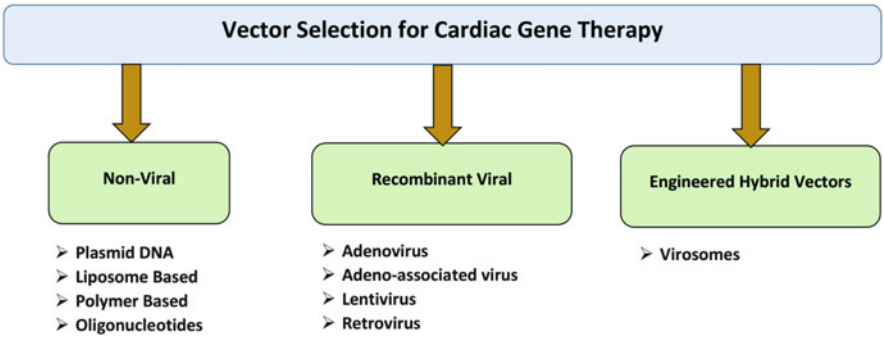

Fig. 3 Vector selection for cardiac gene therapy

The key challenges for a cardiac gene therapy vector are as follows: (1) Escaping the neutralizing effects of specific antibodies and non-specific adsorption to other blood and off target cellular components, (2) Overcoming the endothelial barrier and penetrating the vascular wall for diffusion through the extracellular matrix, and (3) Uptake into the cell at the level of the plasma membrane and efficient trafficking to the nucleus, and (4) Synthesis by the host of the complimentary DNA strand for single stranded delivery vectors followed by transcription and translation of the transgene (Müller et al. 2008). Various vectors have been used to achieve myocardial gene transfer, modified or selected to enhance the probability of overcoming each of these challenges. All vectors can be classified into two main categories, either non-viral, recombinant viral, or engineered hybrid (Fig. 3).

4.1 Non-Viral Vectors

Non-viral vectors are grouped as plasmid DNA, liposome-DNA complexes (lipoplexes), and polymer-DNA complexes (polyplexes). Oligonucleotides are also considered non-viral vectors (Felgner et al. 1997). In 1990, Lin and associates injected plasmid DNA into the left ventricle and demonstrated that the lacZ gene could be introduced and expressed in cardiac myocytes (Lin et al. 1990). Although non-viral vectors have the major advantage of production in large quantities at low cost while at the same time possess fewer toxic or immunological problems, their transfer efficiency is generally poor independent of delivery route (Nabel 1995). Nevertheless, a large number of human cardiac clinical trials are based on plasmid-mediated gene transfer investigating angiogenesis in myocardial ischemia (Losordo et al. 1998; Vale et al. 2001; Kastrup et al. 2005). A major advantage of this approach is that it avoids many of the biosafety concerns associated with viral vectors. However, the level of transgene expression and the efficiency of gene transfer (percent of target cells expressing the transgene) are low and expression is restricted to the zone of the injection site. DNA complexes are relatively more efficient, but not at the level of viral vector driven expression (Qin et al. 1998).

Despite significant reformulation efforts with a variety of chemical and biomimetic polymeric strategies, the key problem is that the nucleic acid content typically remains unstable and is often cleared or biologically inactivated following delivery. This is especially the case for transvascular delivery systems which impose significant contact with blood. An additional disadvantage of these vectors is their short biological half-life due to intracellular degradation and non-specific binding (Kizana and Alexander 2003).

The demonstration of plasmid gene transfer opened a new era of cardiovascular therapy. Despite numerous efforts to enhance efficiency through modification, direct myocardial plasmid injection basically remains a proof of concept tool, although there are a growing number of clinical trials employing plasmid gene transfer (Wasala et al. 2011). Despite their attractiveness in terms of cost and better risk/reward profile, the delivery problem becomes clear when comparing the data obtained between in vivo and in vitro efficacy. In most cases the scale and model system data require very large, unrealistic levels of DNA to achieve measurable benefit that cannot be administered clinically.

4.2 Viral Vectors

Since successful cardiac gene therapy for chronic patients demands efficient myocardial transduction long-term, viral vectors are the prime choice due to their unmatched performance. Since 2000, the performance of viral vectors in terms of efficiency has improved significantly due to key investments in basic and preclinical research evaluations. These key investigations have resulted in innovations that have addressed several rate limiting problems including cell specific targeting and at the same time reduced immunogenicity concerns via recombinant tropism selection. Moreover, the growing body of long-term clinical trial safety data has bolstered the case that only viral vectors appear to meet the demands of cardiovascular performance that could be executed in the clinic (Vinge et al. 2008; Hinkel et al. 2011).

Compared to non-viral vectors, viruses simply have an evolutionary advantage in their interactions with the cellular surface receptors, directly leading to more efficient intracellular trafficking of packaged DNA to the nucleus. This is important to note since many constructs can successfully enter the target cell but fail to achieve any expression due to the complex trafficking interactions. Furthermore, their protein capsid protects the message from degradation in lysosomes (Rapti et al. 2011; Ding et al. 2005). Some viral vectors are able to integrate into the host genome, whereas others remain episomal. Integrating viruses result in persistent transgene expression while viruses in episomal form lead to long-term expression in predominantly non-dividing tissues (e.g., adult myocardium) but only transient expression in rapidly dividing tissues (e.g., the hematopoietic system). It should be noted that for some disorders, short-term expression in a relatively small proportion of cells would be sufficient or even desirable (e.g., angiogenesis

post-myocardial infarction) whereas other pathologies might require long-term expression (e.g., autosomal recessive cardiomyopathy).

4.2.1 Lentiviruses

These vectors were initially developed for HIV therapy. Lentiviral vectors can infect non-dividing cells, cause long-term expression, and do not typically induce an inflammatory or immune response. The major limitation is the risk for mutagenesis and oncogenesis, thus limiting their desirability for cardiovascular applications (Wasala et al. 2011). The new generation of lentiviruses, containing an mRNA and a nuclear import sequence, have been used for successful myocardial transduction, although expression is usually short-term (Zhao et al. 2002; Bonci et al. 2003). Fleury et al. in a study with rat cardiomyocytes in vivo succeeded in obtaining persistent GFP transfer for up to 10 weeks (Fleury et al. 2003). In another study the transduction efficiency of lentiviral vector-mediated SERCA2 gene transfer was about 40% and the positive physiological effect persisted 6 months later (Niwano et al. 2008).

4.2.2 Adenoviruses

Adenoviral vectors have historically been the most frequently used transfer system in experimental studies. This is attributed to the vector's advantages of the ability to transduce non-dividing cells, ease of manufacture in very high titers, rapid peak onset of transgene expression, and a large transgene cloning capacity. However, their use is limited clinically due to the resultant transient gene expression. In addition, adenoviral vector particles are highly immunogenic and cause inflammatory and toxic reactions in the host. This is due to the fact that the adenovirus stimulates both the innate and adaptive immune systems. Using a rat model, it was confirmed that adenovirus was several orders of magnitude more efficient in transducing myocytes than plasmid DNA expressing the same construct (Guzman et al. 1993). Another study featuring direct intramyocardial injection of replication-deficient adenovirus demonstrated gene expression in a large animal model. However, the authors noted a robust T-cell-mediated immune response against the vector and limited distribution of the reporter gene (French et al. 1994).

Simultaneously, several groups confirmed the possibility to achieve significant cardiac gene expression after catheter-mediated delivery of adenovirus encoding phospholamban and the β2-adrenergic receptor (Hajjar et al. 1998; Maurice et al. 1999). Despite sophisticated modifications in an attempt to attenuate the host immune response to the adenovirus, the risk is too high to further advocate the use of this delivery vector for clinical cardiovascular applications of chronic nature.

4.2.3 Adeno-Associated Viruses

The adeno-associated virus (AAV) is a small (20 nm), non-enveloped virus that belongs to the dependovirus genus of the parvovirus family. AAVs have a single stranded DNA genome. The viral genome is approximately 4.7 kb in length, and is composed of two major open-reading frames which encode a Rep (replication) and

Cap (capsid) proteins (Berns and Giraud 1996). For an infection to occur, wildtype AAV requires co-infection with a helper virus such as adenovirus. This allows the viral genome to replicate episomally, and leads to synthesis of the AAV proteins. AAV is one of the smallest viruses, with a capsid mean diameter of 22 nm.

The first AAV2 infectious clone was created in 1982 by Samulski and colleagues (1992). One of the major advantages of AAV vectors is that in multiple animal models and humans, it has been demonstrated that after reaching a steady state level, AAV expression may last for years with an absence of a significant immune response to the transgene (Rivera et al. 2005). Moreover, AAV vectors can be engineered to provide a wide range of cell type tropism with the ability to transduce both dividing and non-dividing cells. Due to their biological properties and advantages over other viral vector systems, AAV has gained great popularity in the last decade in many clinical trials. Seventy-five clinical trials using AAV have been initiated over the past 15 years although only approximately 10% indicated for CVD (Coura Rdos and Nardi 2007).

The process of AAV endocytosis and intracellular trafficking is complex and cannot be underscored in understanding problems with clinical outcomes. Despite the availability and diversity of AAV vectors, several biological barriers appear to limit the effectiveness of AAV mediated gene therapy (Ziello et al. 2010; Coura Rdos and Nardi 2008). Understanding the fundamental basis of these barriers has led to the establishment of methods to improve the efficiency of rAAV-mediated gene delivery. Clarification of the processes by which a virus first enters and traffics through a cell helps to understand the life cycle of the virus and its ability to transduce cardiac muscle. The transport activity of AAV is mainly determined by selective receptor-mediated vesicle transcytosis (Di Pasquale and Chiorini 2006). This intracellular route does not appear to alter the properties of the AAV.

AAV transport to the myocyte's microenvironment can be abrupted by neutralizing antibodies, temperature, and physical and chemical inhibitors through a time and dose-dependent process. In vivo studies have noted that several serotypes of AAV are able to cross vascular endothelium with different efficiencies (Wang et al. 2005). It is known that AAV2 has a relatively poor tropism for vascular cells, although reasonable levels of transduction have been achieved in cardiac myocytes (Melo et al. 2002). Local delivery of AAV2 led to transduction of underlying vascular smooth muscle cells and sequestration of AAV in the extracellular matrix around endothelial cells thus preventing cell binding and entry. The potential of the AAV6 vector for cardiac gene therapy was achieved through the use of VEGF to increase vascular permeability. To date, AAV1, 6, and 9 are considered the best choices for CVD (Gregorovic et al. 2004).

5 Route of Administration Methods for Cardiac Gene Delivery

An important prerequisite for introducing cardiac gene therapy (i.e., following vector/gene construct) into clinical practice is the development of safe and efficient gene delivery techniques. During the last two decades, the field has witnessed the development of several experimental gene delivery strategies with potential therapeutic value for the transition from the preclinical phase to clinical trials. Yet, efforts will need to resolve several problems that exist with delivering sufficient quantities of therapeutic vector in order to establish the efficacious expression profile within safety limits (Katz et al. 2010).

The key challenges with delivery are as follows: sufficient delivery to the myocytes and not collaterals organ systems, improved efficacy in the heart, prevention of injury from the procedure itself, the creation of devices to accommodate the techniques, and avoiding deleterious immune responses. Although cardiac tissue-specific promoters may mitigate collateral organ gene expression, only a true cardiac specific gene delivery method can diminish the biodistribution of vector capsids to extra cardiac organs.

Systemic exposure of vector in the blood can result in unsafe levels of exposure to antigen presenting cells. Antigen presenting cells provide another mechanism to increase the potential for a T-cell-mediated immune response to the vector capsid and or packaged transgene. Ideally, the most optimal gene delivery system should be combined with an appropriate vector, whereby the selections transduce cardiomyocytes but avoid all collateral and immune cells.

Cardiac gene delivery methods can be classified into two main categories: direct or transvascular system approaches. Each of these categories has subsets of particular approaches that range from minimally invasive to very invasive access points (Fig. 4). Direct delivery methods administer vector directly into the cardiac muscle. Typically, this is achieved via intramuscular needle injection or other physical methods that penetrate the heart while depositing vector. On the other hand, the more commonly employed transvascular system approach seeks to leverage the heart's vast network of arterial, venous, and capillary transport system for broad distribution to adjacent cardiac tissue from the site of administration. Here, vector is infused through a designed access point in the coronary anatomy with a specific time interval, pressure, and flow rate. The end goal is to diffuse the vector through the endothelial barriers, where they become bioavailable in the cardiomyocyte compartments.

5.1 Direct Myocardial Delivery

Direct gene delivery methods have been utilized for more than two decades and many authors continue to reference and utilize them in their preclinical cardiac gene therapy studies. Numerous methods have also successfully been translated for use in clinical trials, which range in the degree of invasiveness. The most relevant of

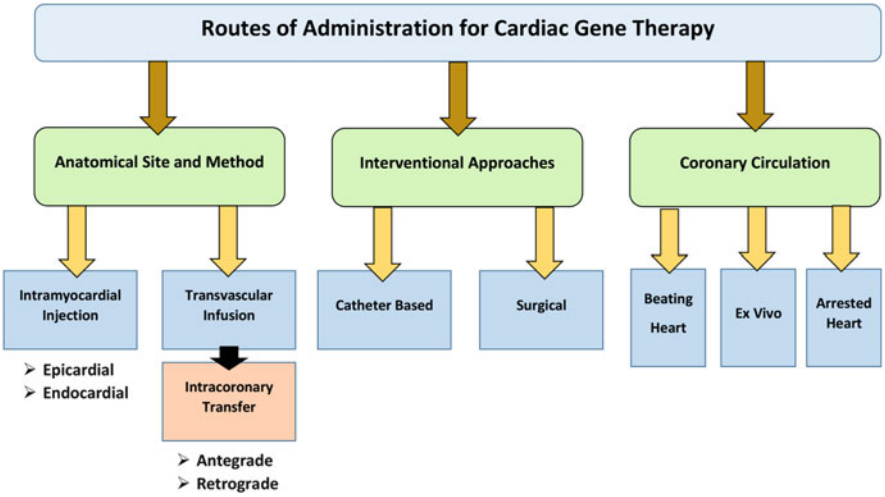

Fig. 4 Routes of administration for cardiac gene delivery

them are usually classified as either an open technique, that is to include the surgical opening of the chest or a closed technique, i.e., transcutaneous or minimally invasive.

5.1.1 Intramyocardial Injection

The majority of successful preclinical studies have involved direct administration of vector via standard needle. This technique allows for the application of a high concentration of vector directly at the target site with excellent specificity and control. Several groups have demonstrated the feasibility of delivering transgenes to the heart via direct intramyocardial injection of plasmid DNA (Acsadi et al. 2002; Buttrick et al. 1992; von Harsdorf et al. 1993). Although these studies have been encouraging because plasmid DNA may be expressed for up to 6 months by cardiomyocytes adjacent to the area of injection, estimates of the number of myocytes that can be transfected in vivo consistently have been as low as 60–100 cells per injection (Acsadi et al. 2002). This low efficiency has made it difficult to measure the physiological effects of gene expression in myocytes, making it unlikely that clinically significant effects will result since there is an ultimate limit to the number of injections that can be performed without permanent injury (von Harsdorf et al. 1993).

The low transduction efficiency of plasmid DNA vectors leads to the search for improved gene transfer efficiency with direct injection of an adenovirus vector. Hearts transfected with an adenovirus vector containing the β-galactosidase gene showed significantly increased β-galactosidase enzymatic activity compared with hearts injected with β-galactosidase plasmid. Unfortunately, the gene expression persisted for only 1 week after injection and it included acute inflammatory response, which the authors considered to be related to the injury produced by

direct injection as well as a cellular immune response against the adenovirus itself (Guzman et al. 1993).

French et al. first demonstrated in a porcine model these important points relevant to needle injection: (1) Direct intramyocardial injection of replication-deficient adenovirus is 140,000 times more efficient than injection of an equal number of genome copies of recombinant plasmid DNA, (2) The impact of this procedure on cardiac function appears to be negligible provided the number of injections is reasonable and across myocardial surface area, (3) The amount of recombinant protein produced increases with the amount of virus, but plateaus, (4) The expression of recombinant genes following intramyocardial injection is similar in the left and right ventricles; and (5) The percentage of cardiomyocytes expressing β-galactosidase in the needle track adjacent to the injection, but rarely are lacZ positive cells detected farther than 5 mm from any given injection site (French et al. 1994).

Large animal model studies generally report robust expression within weeks 1–4 with a decline in follow-up timepoints. For example, in a canine study using adenovirus encoding chloramphenicol acetyl transferase, peak gene expression was noted at 2 days and decreased by an order of magnitude 14 days after direct single myocardial administration. In this study, there was not significant transduction of distant organs and no documented changes in global or regional LV function (Magovern et al. 1996). However, the feasibility of adenovirus-mediated gene transfer has been limited by the cellular immune response which causes myocardial inflammation and results in transient recombinant gene expression (Barr et al. 1994).

In summary, the direct gene delivery approach was the first among others that helped establish the therapeutic efficacy of cardiac gene therapy. Furthermore, the use of this method in some experimental models resulted in successful therapeutic myocardial angiogenesis, and focal treatment of cardiac arrhythmias through effects on cellular electrophysiology; thus, making this platform widely used. Finally, this approach has been successfully utilized in Phase I/II clinical trials demonstrating its potential therapeutic relevance. This branch of delivery continues to evolve with some new concepts such as liquid jet injection, sonoporation, and electroporation applications being tested in preclinical evaluations.

5.2 Transvascular Gene Delivery

Transvascular delivery approaches are more widely practised due to the high frequency, reliability, and safety of interventional catheter based procedures. Effective therapy demands a gene delivery method capable of globally transducing the myocardium while minimizing the systemic exposure to collateral organs (Hajjar et al. 2000; Donahue et al. 1997). This addresses the key rate limiting problem with direct delivery, where only a high concentration of expression is limited to the injection site. Transvascular applications can result in a more homogenous profile with a greater area of myocardium transduced from the original

infusion site. This paradigm is particularly valid in heart failure gene therapy where most authors agree that gene transfer should be as diffuse and homogeneous as possible to access maximum failing myocardium. Furthermore, a diverse number of configurations without sacrificing the degree of invasiveness are available with modification of the catheter including antegrade (arterial infusion via left/right main branches), retrograde (greater cardiac vein, coronary sinus), and both via concomitant blockage and delivery to increase local perfusion delivery gradients.

Additional transvascular delivery procedures that achieve more robust delivery profiles at the cost of invasiveness include surgical approaches that use cardiopulmonary bypass. Bridges group have pioneered the use of molecular cardiac surgery with recirculating delivery (MCARD) which features complete cardiac isolation of the heart with retrograde delivery to maximize vector transfer to the heart with minimal collateral circulation in the context of open heart surgery (Fargnoli et al. 2013; Katz et al. 2014).

5.2.1 Antegrade Intracoronary Delivery

The most preferred gene delivery route at present involves percutaneous catheter based vector infusion into the coronary arteries. The benefits of this technique include its minimal invasiveness, the possibility of transgene delivery to all four myocardial chambers, and the delivery of vector genomes using endovascular coronary catheterization. Early reports using simple antegrade intracoronary delivery achieved very limited myocardial transduction efficiency (Magovern et al. 1996; Hayase et al. 2005; Kaplitt et al. 1996). The low degree and variability in transduction was due to a number of factors including animal species, biocompatibility of catheter and vector, different pharmacological agents used to permeabilize the vasculature, and vector-related variables such as vector serotype and dose (Ding et al. 2004).

The critical parameters influencing the efficiency of intracoronary perfusion included exposure time, high coronary flow rate, perfusion pressure, the use of crystalloid solution as opposed to whole blood, virus concentration, and temperature (Donahue et al. 1997). Further investigations supported that the most critical variable for intracoronary gene transfer is the short residence time of vector within the coronary circulation of a beating heart (Boekstegers and Kupatt 2004). Increasing perfusion pressure and flow augments myocardial expression perhaps by increasing the fenestration width between capillary endothelial cells, permitting better viral transendothelial transfer and enhancing virus–myocyte interaction (Wright et al. 2001).

5.2.2 Advanced Selective Retrograde Intracoronary and Surgical Approaches

The feasibility of percutaneous retrograde gene delivery by selective pressure-regulated retroinfusion of the coronary veins has been demonstrated by Boekstegers et al. This was achieved with a custom device consisting of a pump unit, extracorporeal circuit, and retroinfusion catheter coupled with a suction device. The authors demonstrated advantages of retrograde delivery compared to antegrade and

confirmed the results from several groups that blocking the venous outflow and coronary ischemia can significantly increase viral transfection of the myocardium (Boekstegers and Kupatt 2004).

Unlike previous studies that utilized a single-pass perfusion technique, Bridges group was the first to create an isolated "closed loop" recirculating model of vector-mediated cardiac gene delivery in the large animal heart using cardiopulmonary bypass with an antegrade delivery approach, allowing for vector recirculation for 20 min. Later, they used CPB with high-pressure retrograde coronary sinus infusion with multiple-pass recirculation of vector through the heart and washed out of the cardiac circuit prior to weaning from CPB, which limited extracardiac gene expression. They were able to show an increase of several orders of magnitude in cardiac marker gene activities compared with controls. Furthermore, there was minimal gene expression in the liver and other collateral organs (Fargnoli et al. 2013; Katz et al. 2014). These results validate this surgical technique as a potentially clinically translatable approach for cardiac gene therapy in carefully selected cardiac surgical patients.

6 Future Perspectives

The outlook for cardiac gene therapy is very bright since robust safety data is now available from numerous Phase I and II trials. A large pool of patients has completed trials without any major adverse events, specifically with the adeno-associated virus and plasmid DNA vectors. The ideal cardiac gene treatment plan, depending on target manipulation strategy, is to safely administer the least amount of product that would result in improved survival outcome for chronic heart failure patients. Currently, efficient delivery remains a key issue as doses are high. Improvements in vector design, medical device based delivery technologies, and genetic discovery are necessary to advance the field toward its goal for first FDA approved treatment.

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