

Review

The function and significance of SERCA2a in congestive heart failure: an analysis of gene therapy trials

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Summary. Congestive heart failure (CHF) is a widespread disease that has a negative impact on health, worldwide. Despite advances in therapies, morbidity, mortality and hospital discharges due to CHF remain high. Advances in the understanding of the pathophysiological mechanisms of CHF and the development of gene transfer technology have made gene therapy a realistic potential therapeutic method for CHF. Among the various potential targets, sarco-endoplasmic reticulum Ca²⁺-ATPase 2a (SERCA2a), which is an important protein in the regulation of Ca²⁺ cycling, has piqued the interest of many researchers. Restoring decreased SERCA2a activity in CHF could improve cardiac contractions and energetics, as well as reducing myocardial fibrosis and ventricular arrhythmias, and these benefits have been confirmed by studies using both *in vivo* and *in vitro* models. Following these promising preclinical results, SERCA2a gene therapy advanced to clinical trials. However, results of the clinical trials were controversial, leading some to question whether SERCA2a is the right target for CHF treatment. In this review, we illustrate the function and significance of SERCA2a in CHF, and more importantly, analyze possible causes of the controversial clinical trials results, with the aim of stimulating future research on the relationship between SERCA2a and CHF.

Key words: SERCA2a, Congestive heart failure, Clinical trial, Gene therapy

Introduction

In the 2016 European Society of Cardiology guidelines, heart failure (HF) is defined as “a clinical syndrome caused by a structural or functional cardiac abnormality, resulting in a reduced cardiac output or elevated tachycardia pressures at rest or during stress” (Ponikowski et al., 2016). CHF is a clinical syndrome and its diagnosis relies on typical symptoms and signs.

Epidemiological studies have shown that CHF is a global health problem with high incidence, mortality and hospital discharges. The number of patients with CHF was more than 20 million worldwide in 2013 and the prevalence has increased over time (Roger, 2013). According to the 2016 American Heart Association statistical update (Mozaffarian et al., 2016), 1 in 9 deaths mentioned CHF on the death certificate, and the overall any-mention death rate remains as high as it was 10 years ago. With respect to hospital discharges for CHF, there was no significant difference between the years 2000 (1,023,000) and 2010 (1,008,000). In addition, the total cost attributed to CHF was \$30.7 billion in 2012 and is expected to increase by 127% in 2030.

With the development of cardiovascular molecular biology, great progress has been made in the understanding of the pathophysiological mechanisms of CHF. Common pathophysiological changes include volume overload, activation of the neurohormonal cascade, myocardial hypertrophy and abnormal Ca²⁺

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handling (Johnson, 2014). These changes happen as compensatory mechanisms when the heart is faced with injury, with the aim of assuring adequate ventricular blood ejection; however, continuous compensatory activities lead to deleterious cardiac remodeling (Mazurek and Jessup, 2015), which results in impaired cardiac contractile function and severe complications that may lead CHF.

According to the recently updated American Heart Association and European Society of Cardiology guidelines on HF therapy (Ponikowski et al., 2016; Yancy et al., 2016), the current approved therapies include angiotensin-converting enzyme inhibitors (ACEIs), beta-blockers, angiotensin II type I receptor blockers (ARBs), mineralocorticoid receptor antagonists (MRAs), as well as two new agents: angiotensin receptor-neprilysin inhibitor (ARNI) (valsartan/sacubitril) and Ivabradine, which mainly target cellular and molecular pathways involved in neurohormonal activation (including the rennin-angiotensin-aldosterone system, sympathetic nervous system, as well as neprilysin, endothelin and cytokines). Despite improvements in cardiac function and increased survival after CHF diagnosis (Roger, 2013), the current clinical approaches are not curative. Additionally, some patients are unresponsive to these agents. Therefore, it is necessary to develop novel therapeutic strategies. Advances in our understanding of both the pathophysiological mechanisms of CHF and gene transfer technology has made the gene therapy approach a promising potential alternative CHF treatment.

Gene transfer technology was initially used to explore the pathophysiological mechanisms of CHF and to identify molecular targets that could lead to the development of new agents. However, a series of experimental studies revealed significant therapeutic efficacies of gene transfer for CHF, and investigators subsequently focused on identifying ways to directly apply gene transfer as a therapeutic strategy for CHF. The main components of gene transfer technology are the vectors, delivery method and targets. This general process is shown in Fig. 1 (Braunwald, 2015). The therapeutic DNA is cloned into a vector, which is subsequently delivered to dysfunctional myocytes by surgical- or catheter-based delivery methods. After vector delivery, transcription and translation begin, allowing the molecular target to be produced and play a part in restoring cardiac function.

Every molecule that is involved in signaling pathways involved in the pathophysiology of CHF is a potential target for gene therapy. Currently, there are several highlighted molecular targets, including micro-RNAs, which mostly regulate cardiac function through altering the expression of various genes that have functional roles in cardiac homeostasis (Dassanayaka and Jones, 2015). Other targets include β -adrenergic receptors, adenylyl cyclase 6, G-protein-coupled receptor kinase 2 and other genes that are components of the β -adrenergic signaling pathway (Braunwald, 2015).

Another focus of CHF gene therapy studies have been targets involved in Ca^{2+} -dependent signaling. Many proteins are known to regulate Ca^{2+} cycling (Tilemann et al., 2012; Marks, 2013), including L-type calcium channel, ryanodine receptor (RyR), SERCA2a, phospholamban (PLB), sarcolemmal $\text{Na}^{2+}/\text{Ca}^{2+}$ exchanger (NCX), S100A1 and small ubiquitin-Like modifier type 1 (SUMO1). Other potential targets are upstream regulators of Ca^{2+} cycling (Hoshijima, 2005), including components of the calcium/calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC) signaling pathways. Among these potential targets, SERCA2a has received significant attention because of its crucial role in regulating calcium cycling (Park and Oh, 2013) and has advanced to several clinical trials. However, the outcomes of these clinical trials were debatable. In this review we will highlight experimental and clinical studies focused on SERCA2a, analyze the results of the SERCA2a clinical trials and discuss the future direction of SERCA2a-focused research in CHF.

Calcium cycling and SERCA2a

Ca^{2+} is the most important ion during the excitation-contraction coupling process in myocytes (Bers, 2002). During this process, Ca^{2+} enters the myocyte through the L-type Ca^{2+} channel following depolarization of the sarcolemma. Subsequently, Ca^{2+} stored in the sarcoplasmic reticulum (SR) is released into the cytoplasm through RyR because of trigger action caused by Ca^{2+} entry, leading to an increased free intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Finally, cardiac contraction is initiated by the combination of Ca^{2+} and myofilament protein troponin C. Cardiac relaxation subsequently begins as a result of quick $[\text{Ca}^{2+}]_i$ decline, which is attributable to four pathways involving sarcolemmal Ca^{2+} -ATPase, SERCA2a, NCX and the mitochondrial Ca^{2+} uniporter. Through this process, Ca^{2+} cycling exerts great impact on cardiac systolic and diastolic activities, and abnormal Ca^{2+} handling contributes to cardiac dysfunction in cardiovascular diseases.

In the early 1980s, a series of experimental results in animals and humans suggested that abnormal Ca^{2+} cycling was a primary cause of cardiac dysfunction in HF (Gwathmey and Morgan, 1985; Gwathmey et al., 1987). Well-known mechanisms of abnormal Ca^{2+} cycling include Ca^{2+} leakages from the SR and decreased Ca^{2+} uptake, which led to cytosolic Ca^{2+} overload and decreased SR Ca^{2+} content (Gorski et al., 2015). Every Ca^{2+} -handling protein may contribute to Ca^{2+} homeostasis in a cooperative or independent manner. Among these proteins, SERCA2a aroused particular interest, as SERCA2a participates in reducing $[\text{Ca}^{2+}]_i$ during cardiac relaxation. Actually, almost 80% of $[\text{Ca}^{2+}]_i$ is transported from the cytoplasm to SR via SERCA2a (Frank et al., 2003). The function of SERCA2a-mediated SR Ca^{2+} uptake is to decrease

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cytoplasmic Ca^{2+} , which is necessary for cardiac relaxation and the restoration of Ca^{2+} in the SR that is needed for cardiac contraction. Thus, SERCA2a not only regulates cardiac contractile activation, but also contributes to cardiac relaxation.

Hasenfuss et al. (1994) investigated the relationship between cardiac contraction and SERCA protein levels in myocytes isolated from normal and failing human hearts with different etiologies, finding that the various types of myocardium were predictive of different contractile performance, which was in parallel to different SERCA protein levels in failing or healthy hearts. Their study provided the first experimental evidence of the significance of SERCA protein levels to cardiac function. This opinion was further confirmed by Periasamy et al. (1999), who showed that heterozygous

mice with only one wild type copy of SERCA had a significant reduction of SERCA2a protein levels and Ca^{2+} -uptake activity. Importantly, cardiac contraction and relaxation in these mice were also found to be decreased. This study indicated that reduced SERCA2a expression and activity was a cause of impaired cardiac function.

Altered SERCA2a expression and decreased SERCA2a activity in CHF

Three genes, ATP2A1, ATP2A2 and ATP2A3, encode for SERCA1, SERCA2 and SERCA3, respectively (Periasamy and Kalyanasundaram, 2007). An analysis of SERCA expression patterns revealed tissue-specific expression profiles and suggested that

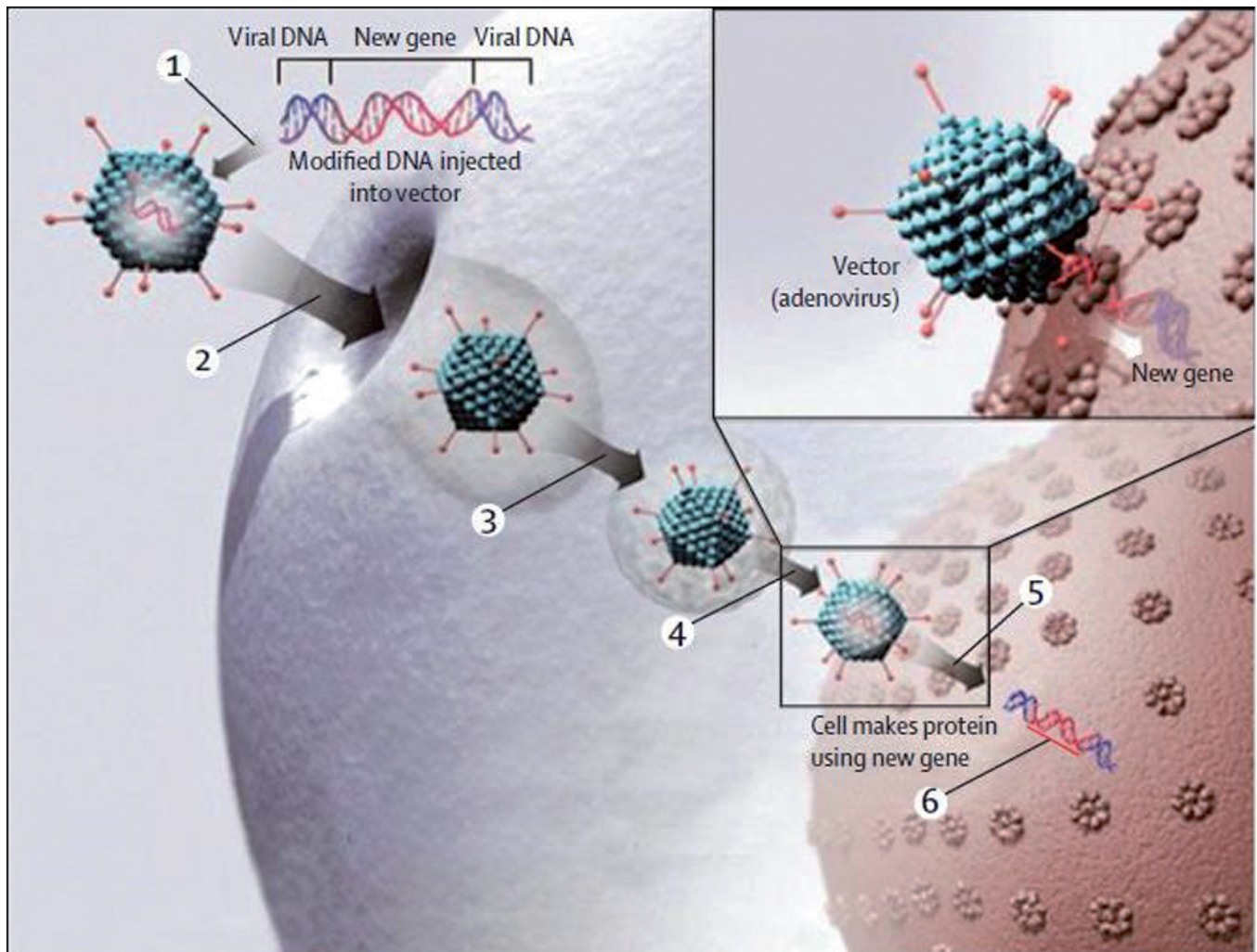


Fig. 1. Progress of gene transfer. In 1, the new gene is injected to the viral vector. In 2, the vector is delivered to the myocardiocyte. In 3, 4 and 5, the vector traverses the cytoplasm and enters the nuclear. In 6, the gene merges into the DNA of the targeting myocardiocyte. Source: Braunwald E. (2015). The war against heart failure: the Lancet lecture. *Lancet*. 385, 812-824. (The citing of this figure was permitted by Elsevier publisher. License No. 4002930962697.)

SERCA2a, which is one of the SERCA2 isoforms, is the “cardiac isoform” because of its predominant cardiac myocytes expression and significant impact on contractile function (Lipskaia et al., 2014).

Whether there are alterations in SERCA2a protein levels in myocytes isolated from patients with CHF has become a matter of debate. In some studies, decreased SERCA2a protein levels were observed (Hasenfuss et al., 1994; Meyer et al., 1995), while other studies found unchanged SERCA2a protein levels (Schwinger et al., 1995; Flesch et al., 1996). However, impaired SERCA2a activity has always been found in conjunction with reduced SERCA2a mRNA expression in many studies investigating human HF of different etiologies (Hasenfuss et al., 1994; Schwinger et al., 1995; Flesch et al., 1996). Differences in disease etiology, the condition of the patient, drug treatments and laboratory methods used could contribute to the inconsistencies in these reports. Furthermore, unchanged SERCA2a protein levels in some patient-derived samples indicate that there may be a compensatory up-regulation of SERCA2a expression at the translational level in response to the pathological changes of CHF. The heterogeneity of SERCA2a expression and activity is also reasonable, as SERCA2a expression and activity are regulated by complex mechanisms (Vafiadaki et al., 2009; Nai et al., 2015; Lee et al., 2016).

Regulating SERCA2a expression and activity

SERCA2a expression and activity can be regulated by its binding partners (e.g. Phospholamban; PLB, S100A1 and sarcolipin), posttranslational modifications (e.g. SUMOylation) and activation of different signaling pathways (Fig. 2). Among these factors, PLB, which is a downstream mediator of the adrenergic pathway, plays an important role in regulating SERCA2a activity by controlling the affinity of SERCA2a for Ca^{2+} . This level of SERCA2a regulation was recently reviewed by Vafiadaki et al. (2009). After adrenergic stimulation, CaMKII and cAMP dependent protein kinase (PKA) are activated and phosphorylate PLB, resulting in increased SERCA2a Ca^{2+} affinity. CaMKII activation can also increase SERCA2a activity through direct SERCA2a phosphorylation. Furthermore, inhibitor-1 (I-1) activation and protein phosphatase1 (PP1) inhibition can also increase PLB phosphorylation via the PKC pathway. SUMO1 can also alter SERCA2a expression and activity via post-translational modification (Lee et al., 2016), and these effects were further confirmed by SUMO1 gene transfer *in vivo* and *in vitro*, suggesting that targeting SERCA2a SUMOylation may be a novel therapeutic approach for CHF treatment. Additionally, the PI3K/Akt pathway, known for its cardioprotective role in controlling hypertrophy and preventing CHF progression (Aoyagi and Matsui, 2011), is another signaling pathway that can regulate SERCA2a expression and activity. In our previous study (Nai et al., 2015), we found that luteolin could increase SERCA2a

expression and activity via PI3K/Akt signaling in a rat ischemia/reperfusion model. More recently, we showed that luteolin exerted similar functions via the PI3K/Akt signaling pathway in rat CHF models.

Restoring SERCA2a activity in CHF

Decreased SERCA2a activity is common in CHF; therefore, restoring SERCA2a activity has received special attention. Benefitting from advances in gene transfer technology, overexpressing SERCA2a in myocytes has become the primary strategy for restoring SERCA2a activity in CHF.

SERCA2a overexpression in vivo and in vitro

Massive human and animal studies have confirmed that SERCA2a overexpression improves the contractile function of failing hearts. For example, Hajjar et al. (1997) used adenoviral (Ad) vectors to transfer the SERCA2a gene into isolated rat myocytes. In this study, they found that SERCA2a overexpression improved cardiac contraction in a dose-dependent manner. Similar effects of *in vivo* exogenous SERCA2a overexpression in studies using rat CHF models induced by different methods further confirmed these results (Miyamoto et al., 2000; Niwano et al., 2008), as did studies in large animal models (Byrne et al., 2008). Furthermore, Kawase et al. (2008) applied a swine volume overload-induced CHF model to test whether SERCA2a overexpression exerted long-term influence on cardiac function, finding that SERCA2a restoration still positively impacted cardiac function after 4 months. This long-term impact is considered a unique superiority of SERCA2a overexpression, as CHF is a chronic disease. Alongside these animal models, SERCA2a transduction of myocytes isolated from failing human hearts showed similar results (del Monte et al., 1999).

Along with ameliorating cardiac contractile functions, SERCA2a overexpression can also improve cardiac energetics. A continuous supply of energy is important for normal cardiac activity; however, less energy is available in failing hearts because ATP levels are depleted during CHF by 25%–30% (Gorski et al., 2015). Inotropic agents, which are usually used to improve contractile function in CHF patients, result in increased mortality because of their increasing energetic demands. With the energy derived from one ATP hydrolysis, SERCA2a can only transport two Ca^{2+} (Periasamy and Kalyanasundaram, 2007). Therefore, it is possible that SERCA2a overexpression produces similar side effects as inotropic agents. However, in a rat CHF model, it was demonstrated that in contrast to the inotropic agents, SERCA2a overexpression improved the phosphocreatine/ATP ratio, resulting in improved survival without adversely affecting energetics (del Monte et al., 2001).

Recently, Sikkil et al. (2014) reviewed the effects of

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SERCA2a overexpression on preventing arrhythmia in various animal models. They explicated that SERCA2a overexpression had unexpected beneficial effects on reducing ventricular arrhythmias, as the enhanced SR Ca^{2+} load that resulted from SERCA2a gene therapy could potentially increase spontaneous SR Ca^{2+} leakages, which are responsible for arrhythmias. The mechanisms underlying the anti-arrhythmic effects remained unclear. More recently, in a study utilizing a sheep CHF model induced by large myocardial infarctions, Katz et al. (2016) reported that increased SERCA2a expression mitigated the progression of fibrosis in CHF via disrupting transforming growth

factor beta (TGF β) signaling. These favorable results indicate that directly increasing SERCA2a expression by gene transfer technology represents a novel CHF treatment.

SERCA2a gene transfer clinical trials

Owing to the promising results of the previous animal and human studies, SERCA2a gene transfer was applied in several clinical trials (Table 1).

Calcium Up-regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID), which was an initial phase 1/2 study that investigated the

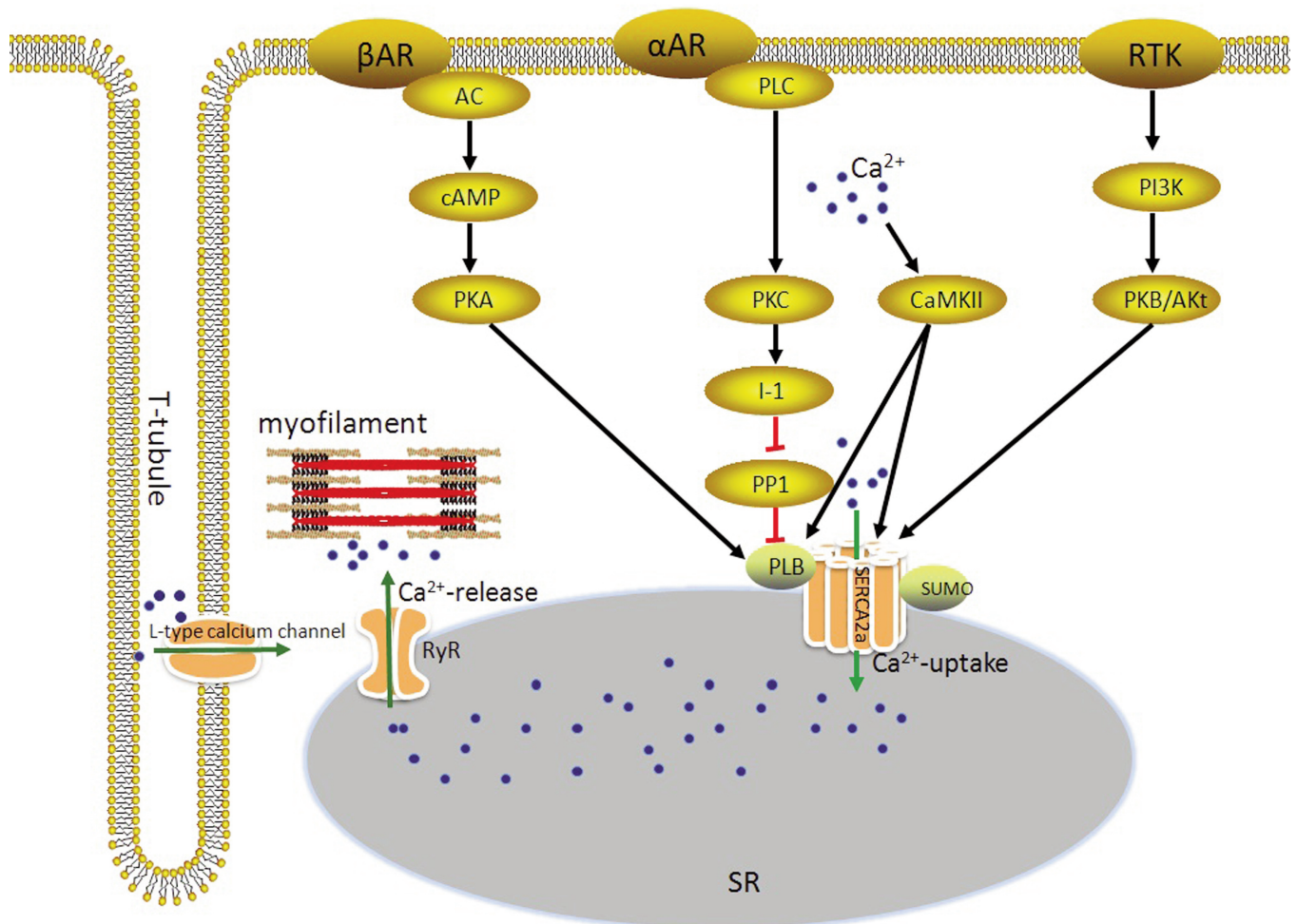


Fig. 2. Regulation of SERCA2a expression and activity. SERCA2a expression and activity can not only be regulated by direct phosphorylation via CaMKII pathway and PI3K/Akt pathway, but also be indirectly mediated via PKA, PKC and CaMKII pathways. Furthermore, sumoylation of SERCA2a is another important way in regulating SERCA2a expression. aAR, α -adrenergic receptor; β AR, β -adrenergic receptor; AC, adenylyl cyclase; CaMKII, calcium/calmodulin-dependent kinase II; cAMP, cyclic adenosine monophosphate; I-1, inhibitor-1; PI3K, phosphoinositide 3-kinase; PKA, PKB, PKC, protein kinase A, B, C; PLB, phospholamban; PLC, phospholipase C; PP1, protein phosphatase1; RTK, receptor tyrosine kinase; RyR, ryanodine receptor; SERCA2a, sarcoplasmic reticulum Ca^{2+} -ATPase 2a; SR, sarcoplasmic reticulum; SUMO, small ubiquitin-like modifier; T-tubule, transverse tubule.

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effects of SERCA2a restoration using gene transfer technology in CHF patients, was conducted in 2007 by Hajjar et al. (2008). For the trial, the SERCA2a gene was cloned to a recombinant adeno-associated virus type 1 (AAV1) vector and transferred into cardiomyocytes through a percutaneous intracoronary delivery method.

In the phase 1 portion of the CUPID 1 trial (Jaski et al., 2009), 9 CHF patients were divided into 3 cohorts and assigned 3 sequential doses (1.4×10^{11} DNase-resistant particles (DRPs), 6×10^{11} DRPs or 3×10^{12} DRPs). After AAV1/SERCA2a administration via percutaneous intracoronary infusion, patients in the 3 cohorts showed improvements in many biological parameters (Table 1), and no safety concerns at 6- or 12-months follow-up were reported.

The phase 2 portion was a randomized, double-blind, placebo-controlled, dose-ranging study (Jessup et al., 2011). In this study, 39 CHF patients were randomly assigned to four groups, the placebo group, the low-dose group (6×10^{11} DRPs), the mid-dose group (3×10^{12} DRPs) or the high-dose group (1×10^{13} DRPs). The effects of AAV1/SERCA2a administration on the biological parameters applied in the phase 1 portion and time to recurrence and terminal events were observed. Though the low- and mid-dose groups only showed improvements in some parameters, the high-dose group demonstrated significant improvements in several biological parameters and a reduction in the time to

recurrence and terminal events at 6- and 12-months follow-up, while patients from the placebo group showed aggravating conditions. In addition, Zsebo et al. (2014) tracked the impact of AAV1/SERCA2a on recurrent and terminal events for 36 months, finding a significant reduction in the number of recurrent cardiovascular events in the high-dose group and delayed times to recurrence in the low- and mid-dose groups compared with the placebo group. Furthermore, no treatment groups in the CUPID 1 trial showed safety concerns.

Following the positive results of the 2 portions of the CUPID 1 trial, a larger CUPID 2 trial, which was a phase 2b, double-blind, placebo-controlled, multinational, multicenter, randomized, event-driven clinical trial, was conducted in 2012 (Greenberg et al., 2016b). This study comprised 250 advanced HF patients, and 123 received intracoronary AAV1/SERCA2a infusion at a dose of 1×10^{13} DRPs, while the rest received placebo. Though there was no safety signal observed during the study, the treatment group did not show improvements in time to recurrence or terminal events versus the placebo group.

There were three other clinical trials associated with AAV1/SERCA2a. AGENT-HF (NCT01966887), which was designed to study whether AAV1/SERCA2a could influence ventricular remodeling using multimodality cardiac imaging technology, began in 2013. SERCA-

Table 1. Clinical trials associated with SERCA2a gene therapy in CHF.

Trial (year)	Phase	Vector	Delivery	Primary endpoints	Results/Status
CUPID1 (2007-2009)	I	AAV-1	Intracoronary	Biological parameters: NYHA, 6MWT, VO ₂ max, NT-proBNP, QOL, echocardiographic function (6 months, 12months)	Met the primary endpoints
CUPID1 (2009-2012)	II	AAV-1	Intracoronary	Biological parameters, time to recurrent and terminal events (6 months, 12 months, 36months)	High-dose group met all the primary endpoints
CUPID2 (2012-2016)	II	AAV-1	Intracoronary	Time to recurrent cardiovascular events (range 1.8-29.4 months, median of 17.5 months)	No improvements in primary outcomes
AGENT-HF (2013)	II	AAV-1	Intracoronary	Changes in left ventricular end-systolic volume (6 months)	Recruitment was terminated
SERCA-LVAD (2014)	II	AAV-1	Intracoronary	Safety and feasibility (6 months)	Recruitment was terminated
NCT0234642 (2015)	I	AAV-1	Intracoronary	Safety and efficacy (24 months)	Recruitment was terminated

6MWT, 6 min walk test; NT-pro BNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; QOL, quality of life; VO₂ max, maximal oxygen consumption.

Table 2. Characteristics of patients enrolled in CUPID trials.

Trial	Phase	NYHA class	LVEF	Nabs titer	NT-proBNP	Other conditions
CUPID1	I	III/IV	≤30%	1:2or <1:2	Not included in the inclusion criteria	with stable optimal outpatient therapy and implantable defibrillators
CUPID1	II	III/IV	≤35%	<1:2	Not included in the inclusion criteria	with stable optimal outpatient therapy and implantable defibrillators
CUPID2	II	II-IV	≤35%	<1:2	>1,200 pg/ml, or >1,600 pg/ml if atrial fibrillation is present	recurrent heart failure hospitalizations within 6 months

LVEF, left ventricular ejection fraction.

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LVAD (NCT00534703) was conducted in 2014, aiming to investigate the safety and feasibility of AAV1/SERCA2a. All patients enrolled in this trial were fitted with a left ventricular assist device (LVAD), with a purpose of obtaining myocardial tissue samples to test the quantity of successfully transferred vector DNA. In the third clinical trial (NCT02346422), researchers prepared to test the safety and efficacy of SERCA2a gene transfer by using AAV1 at a dose of 2.5×10^{13} DRPs. However, these three studies were terminated because of the neutral results of CUPID 2. Therefore, it is necessary to analyze the neutral results of CUPID 2 to provide a basis for future studies.

Analysis of the CUPID trials

Differences in the designs of CUPID 1 and CUPID 2

The causes for the different results seen in CUPID 1 and CUPID 2 are unclear. The patient characteristics (Table 2) of CUPID 2 participants revealed no obvious differences with the CUPID 1 cohort, except for an amendment of the inclusion criteria after the enrolment of 101 patients, which required that patients have raised B-type natriuretic peptide or CHF-related hospital admission within 6 months. Moreover, the protocol amendment seemed to have no impact on the results (Greenberg et al., 2016b). However, patient numbers in CUPID 1 were too small and the condition of patients randomly assigned to the placebo group in CUPID 1 was more serious than in CUPID 2, which could indicate that benefits of the high-dose group in CUPID 1 might be due to chance, despite the significant P-values reported in the study (Jessup et al., 2011).

Different primary endpoints applied in CUPID 1 and CUPID 2 could also have led to discrepancies in the trial results (Table 1). CUPID 1 used multiple end points, including evaluations of efficacy associated with symptoms, functions, biomarkers, as well as remodeling and clinical outcomes, while the primary endpoint of CUPID 2 was time to recurrent cardiovascular events. Unified and effective primary endpoints are needed in future studies.

Insufficient vector delivery and uptake in CUPID 2

Another possible cause for the neutral results in CUPID 2 was insufficient vector delivery and uptake to the myocardium, which would block efficient transgene expression. After receiving tissue samples from 3 patients in CUPID 1 and 23 patients in CUPID 2 who died or received cardiac transplantation and a mechanical circulatory support device, the investigators tested the quantity of vector DNA, finding that the average amount of vector DNA was 43 copies per μg (range <10–192) in CUPID 2, which was much lower than CUPID 1 (range >20–561) (Zsebo et al., 2014, Greenberg et al., 2016b). Though the number of tissue samples was small, the results indicated that vector

delivery and uptake might have been insufficient in CUPID 2. Therefore, limitations of AAV1 and intracoronary delivery methods may have contributed to the low efficiency of SERCA2a gene transfer.

Limitations of AAV1

To date, various vector systems have been developed to bring clinical gene therapy into human beings. The vectors available for cardiovascular gene therapy include plasmids, lentiviral vectors (LV), Ad, AAV and other newer methodologies. Each vector system has its own advantages and disadvantages; however, AAV was selected by most researchers for use in SERCA2a gene therapy clinical trials because of its safety profile, persistent transgene expression and low immune response. Tilemann et al. (2012) reviewed the cardiovascular vector systems before conducting the CUPID 2 trials. Here, we only analyze limitations of AAV1 that may have contributed to the neutral results of CUPID 2.

The presence of neutralizing antibodies (NABs) against AAV1 is one of its disadvantages. The duration of the NAb response was short, usually lasting only days to weeks after uptake of the AAV1 vectors. Hence the presence of NABs was not expected to impact SERCA2a gene transduction in the CUPID trials. However, the outcomes of the CUPID 1 trial provided new information (Greenberg et al., 2016a). In the phase 1 portion of CUPID 1, two patients in the treatment group had NAB titers of 1:2 and showed no improvement in biological activity compared with the patients who had undetectable titers (<1:2).

Tissue obtained from these NAB positive patients was measured, resulting in undetectable vector sequences. Similarly, in phase 2 of the CUPID 1 trial, one patient in the high-dose group seroconverted to detectable NAb titers during screening, and had the worst individual efficacy score compared with the other patients that received high-dose AAV1/SERCA2a. Accordingly, it should be noted that many AAV1/SERCA2a-treated patients in the CUPID 2 trial developed NABs during 12-month follow-up, while no evidence of NABs was found in patients given placebo (Greenberg et al., 2016b). Therefore, the presence of NABs may be at least partially responsible for the failure of the CUPID 2 trial.

Another drawback of AAV is the prevalence of NABs in CHF patients. In CUPID 2, up to 60% of patients were excluded due to the detection of AAV1 NABs in their serum (Greenberg et al., 2016a). Therefore, the widespread application of AAV1/SERCA2a could be limited by the high prevalence of AAV1 NABs, and efforts have been made to find ways to solve the NAB problem. For example, Ishikawa et al. (2014) created an AAV variant by reengineering the AAV surface capsid (BNP116), and this modified reagent was used to transfer cardiac inhibitor-1 into a swine CHF model. They reported that BNP116 had a

unique antigenic advantage compared with AAV. In a sheep HF model, a molecular cardiac surgery with recirculating delivery (MCARD) method was reported to remove AAV1 NABs from cardiac circulation (Fargnoli et al., 2013). Further studies are needed to develop NAB-resistant AAV delivery systems. Furthermore, immunosuppression and plasmapheresis are possible alternatives for reducing the effects of NABs.

Low proportion of empty viral capsids used in CUPID 2

Another possible factor for the controversial outcomes of the CUPID trials might be the different proportion of empty viral capsids used in CUPID 1 (75%) versus CUPID 2 (25%) (Greenberg et al., 2014). Mingozzi et al. (2013) studied the effects of empty capsids on AAV-mediated coagulation factor IX transduction to hepatocytes *in vitro* and *in vivo*, finding that the addition of empty capsids to AAV increased vector transduction by overcoming the inhibitory effects of NABs. Moreover, the benefits of empty capsids increased with dose. Therefore, it is possible that the efficiency of SERCA2a transduction in CUPID 2 was influenced by the application of fewer empty viral capsids. Furthermore, as the anti-AAV antibody effects of empty viral capsids increased with vector dose, it is feasible to increase the vector dose and the proportion of empty capsids to overcome the inhibitory effects of NABs in future studies. However, further investigations are needed to evaluate the safety of increasing vector doses and empty viral capsids.

Drawbacks of the intracoronary delivery method

In addition to the vectors used, delivery methods also play an important role in successful gene therapy. Tilemann et al. (2012) have previously detailed the various delivery methods used in cardiovascular gene transfer. Briefly, the current delivery methods used in cardiovascular gene therapy include direct intramyocardial injection, intracoronary perfusion, retrograde venous infusion and pericardial injection. Compared with other methods, catheter-based intracoronary perfusion was chosen as the delivery method in the CUPID trials not only because it is a safe, simple and practical approach, but also due to its ability to allow gene transduction across the myocardium and homogeneous distribution of vectors. However, relatively low vector transfection efficiency is a major drawback of this technique.

Approaches to increase the efficiency of intracoronary infusion have been developed, including treatments with agents that increase vascular permeability. For example, Karakikes et al. (2012) tested the effects of nitroglycerin (NTG) on AAV1/SERCA2a myocardial transduction using a porcine model and observed a significant increase in SERCA2a expression after NTG and AAV1/SERCA2a were co-administered

by intravenous infusion. Based on this evidence, all patients enrolled in the CUPID 2 trial were designed to receive an intravenous NTG infusion for 15–25 min before AAV1/SERCA2a infusion and continuing throughout the intracoronary administration of AAV1/SERCA2a (Greenberg et al., 2014), in the hope of improving vectors uptake. However, this strategy was only partially useful, because except for the increase in vascular permeability, reported variables associated with this delivery method included virus concentration, contact time, temperature, coronary flow rate, and perfusion pressure (Donahue, 2016). Future studies are needed to create a more controlled environment by confirming these variables.

In addition to intracoronary infusion, other delivery methods should be developed. As previously mentioned, MCARD administration of AAV1/SERCA2a was reported to remove NABs, which is of great significance to AAV-mediated gene transfer (Fargnoli et al., 2013). In fact, it resulted in efficient gene transfer, as well as global and homogeneous vector distribution; however, a major drawback of MCARD is the requirement of open-heart surgery.

Catheter-based trans-endocardial injection is another potential delivery method. To study cell-based therapies for non-ischemic cardiomyopathy (NICM), Mushtaq et al. (2014) used a catheter-based trans-endocardial injection to transfer stem cells under the guidance of a NOGA-XP Cardiac Navigation System. They found that the trans-endocardial delivery method showed highly efficient cardiac specificity compared to the intracoronary delivery method. Furthermore, this method allows direct targeting of the injection area without open-heart surgery; however, the risks associated with myocardial perforation should be noted.

Conclusion

In this review, we discussed SERCA2a gene therapy for CHF from the following perspectives: 1) CHF is a widespread disease that negatively impacts health around the world, and gene therapy has been recently recognized as a potential therapeutic alternative for CHF treatment. 2) SERCA2a regulation is complex; normal Ca^{2+} handling is necessary for cardiac function, and abnormal Ca^{2+} cycling can be mostly attributed to decreased SERCA2a activity in CHF. 3) SERCA2a overexpression has been confirmed *in vivo* and *in vitro* to improve cardiac contraction and energetics and to decrease myocardial fibrosis and ventricular arrhythmias. 4) Clinical trials investigating SERCA2a gene therapy for CHF have been conducted; however, their results are inconsistent. An analysis of the possible causes for these inconsistent results could lead to improved methodologies.

Gene therapy has never been a methodology that could easily succeed in clinical applications, even for monogenic diseases, let alone complex, chronic or systemic diseases such as CHF. Nevertheless, gene

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therapy remains a promising novel alternative for treating CHF. The CUPID trials demonstrated that SERCA2a gene therapy is safe, and further research into approaches to overcome the inhibitory effects of NABs, developing more efficient and safe delivery methods, as well as creating a controllable environment by confirming variants that influence gene transfer will help increase successful gene transfer treatments for CHF patients. Furthermore, because CHF is a multifactorial disease with complex pathophysiological mechanisms and SERCA2a expression and activity are regulated by a multitude of factors, SERCA2a overexpression might be insufficient to positively impact the cardiac processes in some CHF patients. Therefore, future work should also focus on the mechanisms that regulate SERCA2a expression and activity.

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