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REVIEW

SERCA2a gene therapy in heart failure: an anti-arrhythmic positive inotrope

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Therapeutic options that directly enhance cardiomyocyte contractility in chronic heart failure (HF) therapy are currently limited and do not improve prognosis. In fact, most positive inotropic agents, such as β-adrenoreceptor agonists and PDE inhibitors, which have been assessed in HF patients, cause increased mortality as a result of arrhythmia and sudden cardiac death. Cardiac sarcoplasmic reticulum Ca^{2+}-ATPase2a (SERCA2a) is a key protein involved in sequestration of Ca^{2+} into the sarcoplasmic reticulum (SR) during diastole. There is a reduction of SERCA2a protein level and function in HF, which has been successfully targeted via viral transfection of the *SERCA2a* gene into cardiac tissue *in vivo*. This has enhanced cardiac contractility and reduced mortality in several preclinical models of HF. Theoretical concerns have been raised regarding the possibility of arrhythmogenic adverse effects of SERCA2a gene therapy due to enhanced SR Ca^{2+} load and induction of SR Ca^{2+} leak as a result. Contrary to these concerns, SERCA2a gene therapy in a wide variety of preclinical models, including acute ischaemia/reperfusion, chronic pressure overload and chronic myocardial infarction, has resulted in a reduction in ventricular arrhythmias. The potential mechanisms for this unexpected beneficial effect, as well as mechanisms of enhancement of cardiac contractile function, are reviewed in this article.

Abbreviations

APD, action potential duration; $[Ca^{2+}]_i$, intracellular (cytoplasmic) Ca^{2+} concentration; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CUPID, Calcium Up-regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease Trials; CICR, Ca^{2+}-induced Ca^{2+} release; DAD, delayed afterdepolarization; (–)dP/dt, maximum rate of left ventricular pressure generation in systole (or reduction in diastole); EAD, early afterdepolarization; ECC, excitation contraction coupling; HF, heart failure; I_{Ca}, L-type Ca^{2+} current; I_f, 'Funny'-current; I_{K1}, inward rectifying potassium channel; I_{TI}, transient inward current; I/R, ischaemia/reperfusion; LTCC, L-type Ca^{2+} channel; LV, left ventricle; LVAD, left ventricular assist device; MI, myocardial infarction; MOI, multiplicity of infection; NCX, Na^+–Ca^{2+} exchanger; NT-proBNP, N-terminal pro–B-type natriuretic peptide; PLB, phospholamban; rAAV, recombinant adeno-associated virus; RyR2, cardiac ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; SUMO, small ubiquitin-like modifier; TAC, transverse aortic constriction; VA, ventricular arrhythmia; VF, ventricular fibrillation; VO_2-max, maximal rate of oxygen consumption; VT, ventricular tachycardia

Introduction

Chronic heart failure (HF) has reached epidemic proportions in Western countries. The Framingham Heart Study measured the lifetime risk for congestive cardiac failure at 20% in both men and women (Lloyd-Jones *et al.*, 2002). Mortality as a result of HF remains high despite the implementation of current evidence-based therapies. Five-year survival is only 32% in patients with systolic dysfunction and little better (35%) in patients with HF and

preserved ejection fraction (Owan *et al.*, 2006; Jhund *et al.*, 2009).

Several pharmacological therapies improve both symptoms and prognosis in patients with HF. These include neurohormonal antagonists, such as β-adrenoceptor antagonists (β-blockers), ACE inhibitors and aldosterone antagonists. Recently, it has also been shown that heart rate reduction by specific inhibition of the 'Funny'-current (I_f) using ivabradine can also improve prognosis and symptoms (Swedberg *et al.*, 2010). On the contrary, the use of positive inotropes to directly target the ventricular dysfunction that characterizes HF has proven to be counterproductive – many traditional agents increase mortality, principally by increasing sudden cardiac death (Packer *et al.*, 1991; O'Connor *et al.*, 1999; Toma and Starling, 2010) and accelerating progression of left ventricular dysfunction (Packer *et al.*, 1984). Cardiac sarcoplasmic reticulum Ca^{2+}-ATPase2a (SERCA2a) gene therapy is one of a number of promising therapies that target specific protein derangements in HF. Others include myosin activators (Teerlink *et al.*, 2011), ryanodine receptor (RyR) stabilizers (Wehrens *et al.*, 2005) and Ca^{2+}/calmodulin kinase (CaMKII) inhibition (Anderson *et al.*, 1998), but a detailed description of these is beyond the scope of this article.

Our group and others have shown that, despite increasing cardiac contractility, SERCA2a gene therapy reduces the risk of potentially fatal arrhythmias in preclinical HF models (Lyon *et al.*, 2011; Cutler *et al.*, 2012b). Promising data have already been reported from a phase 1/2 trial, which demonstrated no pro-arrhythmic effect in HF patients (Jessup *et al.*, 2011) and an ongoing phase 2b trial will assess this in a larger number of patients. If SERCA2a gene therapy makes the transition to the clinic, it may become the first anti-arrhythmic, positively inotropic, agent available in the treatment of HF.

SERCA2a function

The role of SERCA2a in myocyte contraction

The generation of cardiac contractile force is a complex process that has been reviewed elsewhere (Bers, 2001). A highly simplified scheme of the process is shown in Figure 1. The process of excitation contraction coupling (ECC) in the ventricular myocyte begins with the entry of Ca^{2+} via the L-type Ca^{2+} current (I_{Ca}) (Bers, 2002). This relatively small Ca^{2+} influx is amplified by a larger Ca^{2+} release from the sarcoplas-

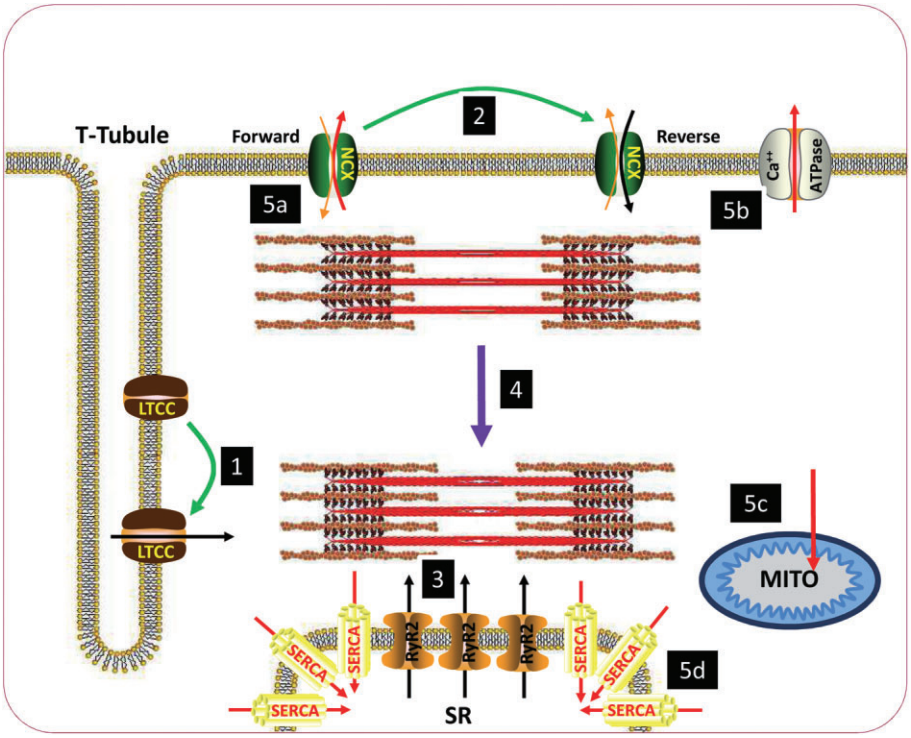

Figure 1

Schematic diagram of excitation-contraction coupling in the ventricular cardiomyocyte. (1) Sarcolemmal depolarization causes L-type Ca^{2+} channels (LTCC) to open, resulting in influx of Ca^{2+} during systole. Ca^{2+} enters initially through the LTCC. (2) During the early stages of the action potential, the Na^+–Ca^{2+} exchanger (NCX) also transitions from functioning in forward mode (Na^+ in Ca^{2+} out) to reverse mode (Ca^{2+} in Na^+ out) to further enhance the rise in $[Ca^{2+}]_i$. (3) The initial rise in $[Ca^{2+}]_i$ induces a larger release of Ca^{2+} from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR2), resulting in a large rise in $[Ca^{2+}]_i$. (4) The large rise in Ca^{2+} causes contraction of the myofilaments. (5) $[Ca^{2+}]$ is subsequently reduced to bring about diastole through movement out of the cytosol (a) via NCX in forward mode and (b) the sarcolemmal Ca^{2+}-ATPase. (c) Ca^{2+} is also buffered by mitochondria. (d) The majority of Ca^{2+} movement in diastole is into the SR via SERCA2a. Green arrows denote transitions brought about by sarcolemmal depolarization. Black arrows denote routes of influx of Ca^{2+} to the cytosol during systole. Purple arrow denotes contraction of myofilaments. Red arrows denote routes of efflux of Ca^{2+} during diastole.

mic reticulum (SR) – so-called Ca^{2+}-induced Ca^{2+} release (CICR). The resulting increase in intracellular (cytoplasmic) Ca^{2+} concentration ($[Ca^{2+}]_i$) results in conformational changes in myofilament proteins, which ultimately leads to cross-bridge cycling and myocyte contraction. Other than relatively small areas of the terminal cisternae that are closely apposed to the sarcolemma, known as the junctional SR, most of the SR is specialized for Ca^{2+} uptake (the longitudinal SR) such that the vast majority of protein particles in the SR membrane are SERCA2a (Stewart and MacLennan, 1974; Franzini-Armstrong, 1975). SERCA2a transports the Ca^{2+} released during ECC back into the SR lumen against the electrochemical $[Ca^{2+}]$ gradient using chemical energy from ATP. The mechanism of Ca^{2+} pumping follows the E1-E2 model (Figure 2). An initial state in which 2 Ca^{2+} ions can be accepted from the cytosol (E1) transitions, via an intermediate occluded state, to a final state (E2) in which the lumen is accessible by the Ca^{2+} ions concurrent with a sudden reduction in affinity of the protein for Ca^{2+}, thus facilitating its rapid release into the SR (MacLennan and Green, 2000; Wuytack *et al.*, 2002). There are different SERCA isoforms that are functionally adapted to their location: SERCA1a is predominantly expressed in fast-twitch skeletal muscle; SERCA1b is expressed mainly in developing fetal fast-twitch skeletal muscle; SERCA2a is the dominant isoform in cardiomyocytes, but is also expressed in slow-twitch skeletal and smooth muscle; SERCA2b expression occurs in most cell types (the so-called 'house-keeping' isoform), and predominates in vascular smooth muscle; and finally, SERCA3a and 3b are expressed in the reticuloendothelial system (Leberer *et al.*, 1989; Schulte *et al.*, 1993).

SERCA2a is a key regulator of cardiac contractile force. An increase in SERCA2a function augments SR Ca^{2+} content, which increases Ca^{2+} transient amplitude (and thus force generated). This is because SR Ca^{2+} release is steeply dependent on SR Ca^{2+} content (Gyorke and Gyorke, 1998; Bode *et al.*, 2011). The function of SERCA2a within an organism is dynamic, matching cardiac function to physiological demands by increasing during exercise or other forms of stress. Binding of phospholamban (PLB) to SERCA2a inhibits the pump and reduces Ca^{2+} influx into the SR lumen by decreasing its Ca^{2+} affinity two to three times (Tada *et al.*, 1975) and also by reducing energetic efficiency (Frank *et al.*, 2000). At physiologically relevant values of diastolic $[Ca^{2+}]$, the presence of PLB reduces the Ca^{2+} transport rate from about 90 to 30 $\mu M \cdot s^{-1}$ (Bers, 2001). Stimulation of β-adrenoceptors causes a conformational change of PLB secondary to phosphorylation of the Ser^{16} site by PKA (Talosi *et al.*, 1993). This change reduces the affinity of active monomeric PLB for SERCA2a but enhances affinity for other PLB molecules such that detached PLB forms homopentamers, an inactive reservoir of the protein (Cornea *et al.*, 1997; Kimura *et al.*, 1997; Hou *et al.*, 2008). In this way, the affinity of SERCA2a for Ca^{2+} is increased and uptake rate is enhanced.

PLB can also be phosphorylated at Thr^{17} by CaMKII. This site appears to be more important in frequency-dependent responses (Hagemann *et al.*, 2000; Zhao *et al.*, 2004) such as the force–frequency relationship and frequency-dependent acceleration of relaxation, which are often impaired in diseased myocardium (Pieske *et al.*, 1992). Apart from PLB binding, pump function is modulated significantly by the intracellular cytosolic milieu, including ATP, pH, $[Mg^{2+}]$ and $[Ca^{2+}]$ (Dupont, 1977; Bers, 2001), and by post-translational modification, including SUMOylation (see below). Sarcolipin (SLN) is a 31 amino acid structure originally identified in skeletal muscle (MacLennan and Kranias, 2003), but more recent analyses indicate that SLN is also expressed in cardiac tissue, predominantly in the atria (Babu *et al.*, 2007a). Compared with PLB, much less in known about SLN, but they have similar transmembrane sequences, suggesting they share a similar genetic heritage and they may bind to the same regulatory site on SERCA2a. There is evidence that SLN could be an important regulator of atrial Ca^{2+} transport and may be responsible for mediating at least some of the β-adrenoceptor responses in atria (Babu *et al.*, 2007b).

Transgenic models

Transgenic animal models have further highlighted the importance of normal SERCA2a expression and function in Ca^{2+} homeostasis. Germline homozygous SERCA2a knockout is embryonically lethal (Periasamy *et al.*, 1999). Heterozygous SERCA2a knockout reduces protein levels to 55% that of normal and results in increased propensity to HF following transverse aortic constriction (TAC) (Schultz *et al.*, 2004). A model of conditional cardiac-specific SERCA2a knockout in adult mice induces an HF phenotype without any superimposed insult (Andersson *et al.*, 2009; Louch *et al.*, 2010). The importance of SERCA2a as the specific cardiac isoform has also been revealed by transgenic mouse studies.

Cardiac-specific overexpression of the SERCA1a isoform, which has higher kinetic turnover of Ca^{2+}, led to increases in systolic and diastolic cardiac function in the normal mouse heart (Jane Lalli *et al.*, 2001). In contrast, SERCA1a gene transfer into failing rat hearts actually impaired contractile function (O'Donnell *et al.*, 2008). The reason for this unexpected finding may be curtailment of the rise of the Ca^{2+} transient, which has been observed with high-dose SERCA1a gene transfer [multiplicity of infection (MOI) 50], but not low dose (MOI 10) (Teucher *et al.*, 2004). This highlights the role of SERCA proteins as a buffer of cytosolic Ca^{2+}, which can reduce Ca^{2+} transient amplitude when faster kinetics of SERCA are included in computational models (Higgins *et al.*, 2006). Such rapid kinetics would be seen with SERCA1a. Overall, this highlights the importance of using the cardiac-specific isoform SERCA2a in gene therapy strategies.

Abnormalities of Ca^{2+} handling in HF

Abnormal SERCA2a function

One of the hallmarks of the cellular phenotype of HF in animal models and human studies is prolongation of the Ca^{2+} transient, particularly in the decay phase (Gwathmey and Morgan, 1985; Gwathmey *et al.*, 1987). In human preparations, Beuckelmann *et al.* (1992) revealed that this occurred even in the absence of changes in I_{Ca}. Furthermore, even with intracellular $[Na^+]$ controlled and action potential clamped, Ca^{2+} transient decay was slower and diastolic Ca^{2+} levels were higher than in control myocytes (Beuckelmann *et al.*, 1992). These changes in Ca^{2+} handling can, in part, be attributed to reduced SERCA2a gene expression and protein levels, which

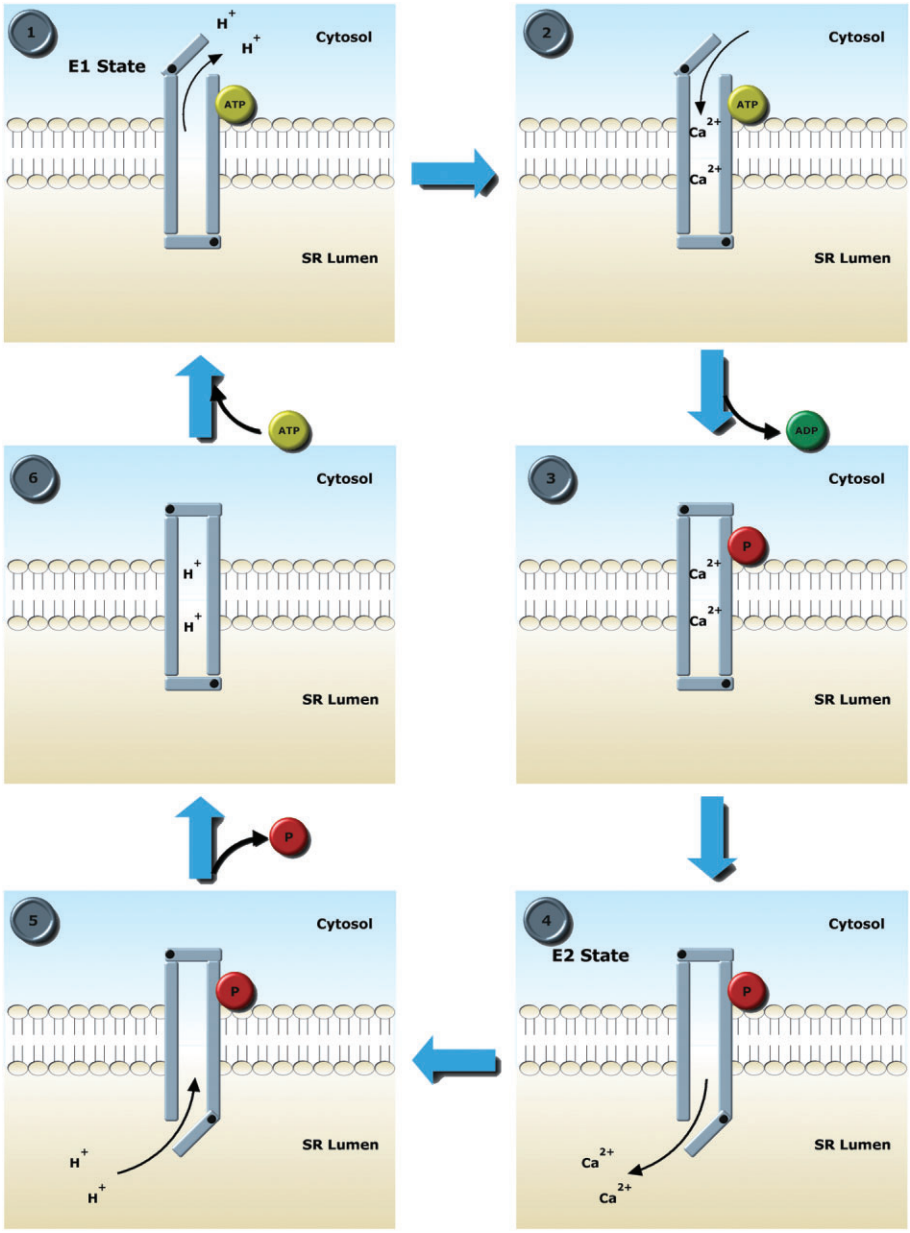

Figure 2

E1-E2 Ca transport scheme via which SERCA2a transports Ca into the sarcoplasmic reticulum (SR) lumen. Stage 1: In the E1 conformation, the high-affinity Ca^{2+} binding sites are accessible by the cytosol. Two protons leave the pump as ATP binds to allow access to the cytosol. Stage 2: 2 Ca^{2+} ions bind with high affinity. Stage 3: Bound ATP is used to phosphorylate Asp^{351}, causing a conformational change which occludes Ca^{2+}. At this point, the pump is in the high-energy unstable phosphointermediate condition. Stage 4: A further conformational change brings about the E2 form of SERCA2a where the SR lumen is accessible to Ca^{2+}. Ca^{2+} ions are released because of a lower affinity in the E2 state. Stage 5: Protons replace Ca^{2+}. Stage 6: Asp^{351} is dephosphorylated, bringing the pump into a low-energy intermediate form, which is unstable and returns to the E1 state.

are reported in many animal models of chronic HF and human failing myocardium (Hasenfuss, 1998). While SERCA2a levels often decrease in HF, most findings indicate that PLB protein levels are unchanged (MacLennan and Kranias, 2003). This implies that the remaining SERCA2a is also more likely to be inhibited by excess monomeric PLB. The phosphorylation state of PLB in HF is complex, with reduced levels of phosphorylation at Ser^{16} found in human tissue (Schwinger *et al.*, 1999; Ai *et al.*, 2005) but enhanced phosphorylation at Thr^{17} (Ai *et al.*, 2005; Mills *et al.*, 2006). Although the net effect of the extent of phosphorylation is unclear, the sum of the changes in PLB : SERCA2a expression and phosphorylation is reduced SERCA2a Ca^{2+} uptake into the SR (del Monte *et al.*, 1995).

Consequences include delayed cytoplasmic Ca^{2+} clearance, resulting in prolonged relaxation times, increased chamber stiffness and a reduction in the rate of systolic pressure generation. The prolongation of Ca^{2+} transient decay and reduction in SERCA2a levels appear to occur in human failing hearts regardless of the aetiology of cardiac dysfunction (del Monte *et al.*, 1995; Schmidt *et al.*, 1998). Therefore, an important advantage of SERCA2a gene therapy is that it is expected to be beneficial in a wide range of HF patients. This is in comparison to the relatively limited patient population that might benefit from the targeting of an individual mutation causing dilated cardiomyopathy.

It is important to note that HF is not one single pathological entity and SERCA2a function may not be uniformly impaired in all cases. However, in the later stages, the phenotype may become more uniform. For example, we have found that, regardless of aetiology, the cellular phenotype of end-stage human HF generally includes impaired SERCA2a function (del Monte *et al.*, 1995). This common finding, despite differences in the underlying primary pathology, may relate to neurohormonal changes that occur as a result of reduced cardiac output, including increased adrenergic hormones, B-type natriuretic peptide and hormones of the renin–angiotensin–aldosterone axis, which have all been shown to reduce SERCA2a expression or function with chronic exposure (Flesch *et al.*, 1997; Linck *et al.*, 1998; Sodi *et al.*, 2008). On the other hand, we acknowledge that end-stage HF may not be representative of the whole range of disease. For example, during early stages of pressure overload, SR Ca^{2+} cycling may be enhanced rather than impaired (Carvalho *et al.*, 2006), with reduced SERCA2a levels ensuing during the transition to cardiac decompensation (Feldman *et al.*, 1993). Even in human end-stage HF, Hasenfuss *et al.* (1999) identified significant differences between myocardium exhibiting both systolic and diastolic dysfunction [reduced SERCA2a levels, normal Na^+–Ca^{2+} exchanger (NCX) levels] and myocardium with systolic dysfunction but preserved diastolic function (preserved SERCA2a levels, enhanced NCX levels). A potential caveat is that SERCA2a activity was not always reported, and reduced SERCA2a function may reflect not only lower protein levels but also post-translational modification of the protein which is expressed. Given these differences dependent on aetiology and cardiac function, we could speculate that therapies to enhance SERCA2a function would be especially helpful in decompensated HF (rather than compensated hypertrophy) in which both systolic and diastolic function are deranged. Dose titration of SERCA2a gene therapy may therefore be important, with higher doses being required with end-stage disease.

Arrhythmogenic Ca^{2+} handling abnormalities in HF

The SR is not only an important store of Ca^{2+} that can be released during contraction, but also the potential origin of spontaneous arrhythmogenic SR Ca^{2+} release. Such SR Ca^{2+} leak manifests either as leak, which is visible by high-speed confocal fluorescence imaging as Ca^{2+} sparks (Cheng *et al.*, 1993) or waves (Bers, 2001), or as more subtle leak, which has been called Ca^{2+} quarks (Lipp and Niggli, 1996; Lipp and Niggli, 1998) or spark-independent Ca^{2+} release (Zima *et al.*, 2010). Spontaneous Ca^{2+} release from the SR can result in triggered cellular activity and subsequent arrhythmia via extrusion of excess cytoplasmic Ca^{2+} via NCX which exchanges 3 Na^+ ions for 1 Ca^{2+} and is thus electrogenic such that clearance of Ca^{2+} results in depolarization of myocytes towards the threshold potential for voltage-gated sodium channels. This NCX-mediated current is the basis of delayed afterdepolarizations (DADs). Cellular triggered activity also comes in the form of early afterdepolarizations (EADs). These are a different entity resulting in a second depolarization prior to the end of the action potential, perhaps as a result of reactivation of L-type Ca^{2+} channels (LTCCs) (Bers, 2001), although more recent evidence also points to an important role for SR Ca^{2+} leak in EAD genesis (Volders *et al.*, 2000; Zhao *et al.*, 2012).

As discussed previously, SERCA2a function and SR Ca^{2+} load are reduced in several models of HF and in human HF cardiomyocytes. All else being equal, therefore, it would follow that spontaneous SR Ca^{2+} leak would also be reduced. On the contrary, many researchers, including our own group (Lyon *et al.*, 2009, 2011), find that leak is enhanced in HF. This forms the basis of a Ca^{2+} paradox in HF (Kass *et al.*, 2008). The reasons for the paradox remain unclear and may involve increased intracellular [Na^+] in HF which could hinder Ca^{2+} efflux via NCX and thus enhance RyR2 opening via interaction with Ca^{2+}-sensitive sites at the cytosolic domain (Sossalla *et al.*, 2010). Another important mechanism might be a reduced threshold for SR Ca^{2+} leak in HF similar to that characterized in catecholaminergic polymorphic ventricular tachycardia (VT) (Jiang *et al.*, 2004). This could enhance leak despite lower SR Ca^{2+} content. The leak threshold may differ in different models and aetiologies of HF (Respress *et al.*, 2012).

There are several modifications to the RyR2 that occur in HF and may reduce the threshold for spontaneous SR Ca^{2+} release. There is evidence that phosphorylation of RyR2, and reduced binding of the channel's stabilizer FK506-binding protein 12.6 as a result, causes an increase in SR Ca^{2+} leak in HF (Marx *et al.*, 2000; Kushnir and Marks, 2010). Other post-translational modifications to RyR2 such as nitrosylation and oxidation may also be important and have been reviewed elsewhere (Aracena *et al.*, 2005; Vassort and Lacampagne, 2005; Zissimopoulos *et al.*, 2007). The leaky SR in HF both predisposes to arrhythmias via the depolarizing influence of the NCX current and reduces contractility due to further depletion of SR Ca^{2+} content. Each occurrence of SR Ca^{2+} leak could also be more arrhythmogenic in HF than in the healthy heart due to an increase in NCX activity which has been seen in some settings, although this is not always associated with reduced SERCA2a activity (Hasenfuss *et al.*, 1999; Pogwizd *et al.*, 2001). Kho *et al.* identified a common regulator for SERCA2a and NCX in the small ubiquitin-like modifier type 1 (SUMO1) protein. This protein both up-regulates SERCA2a function and down-regulates NCX function, and is reduced in a mouse model of HF. These mice showed reduced SERCA2a Ca^{2+} uptake and enhanced NCX function (Kho *et al.*, 2011). In addition, there is down-regulation of inward rectifying potassium channel (I_{K1}) expression (Pogwizd *et al.*, 2001), which normally stabilizes resting membrane potential. The result is that for any given spontaneous Ca^{2+} leak in HF cells, a greater membrane potential (V_m) depolarization occurs via NCX. In the intact organ, mechanical dysfunction

and fibrosis result in a mechanoelectrically heterogeneous ventricular substrate in which triggered arrhythmias can lead to sustained arrhythmias via structural and functional re-entry (Weiss *et al.*, 2005). The transient outward current (I_{to}) density has also been shown to decrease in HF patients (Beuckelmann *et al.*, 1993). I_{to} plays a major role in controlling the duration of the action potential and it is thought that such a decline in current is one of the reasons for APD prolongation in HF. APD prolongation will provide a setting for EADs because the opportunity for Ca^{2+} current reactivation is increased.

Alternative strategies to enhance SERCA2a function in HF

Conventional positive inotropy and arrhythmogenesis in HF

Drugs that have been used conventionally for positive inotropy such as adrenoceptor agonists and PDE inhibitors enhance SERCA2a function via increased PLB phosphorylation. However, the global enhancement of PKA activity phosphorylates numerous other targets and significantly enhances metabolic demand. With respect to Ca^{2+} cycling, phosphorylation of LTCC, PLB and SR Ca^{2+} release channels (such as RyR2) increases SR Ca^{2+} content and causes an increased propensity to diastolic Ca^{2+} leak from the SR to cause DADs. EADs are also exacerbated by conventional positive inotropes due to enhancement of I_{Ca} and reduction in the rate of its inactivation (both Ca^{2+} and voltage dependent) (Zeng and Rudy, 1995), as well as the increase in SR Ca^{2+} leak. Hence, as a result of enhancement of cellular triggered activity, positive inotropes can be arrhythmogenic, even in the absence of underlying cardiac disease (Pogwizd *et al.*, 2001; Cox *et al.*, 2005).

In HF, conventional positive inotropy can thus be seen as a perfect storm; the increased tendency for arrhythmias on an already arrhythmic substrate. This may be why, despite improving cardiac contractility and reducing pulmonary venous pressure, agents such as dobutamine and milrinone have been unsuccessful (Packer *et al.*, 1984, 1991; O'Connor *et al.* 1999; Penson *et al.*, 2007). These agents increase mortality, in large part, due to a rise in ventricular ectopy, arrhythmia and sudden death. SERCA2a gene therapy aims to enhance cardiac contractility but circumvent such problems by targeting derangements of specific molecular pathways in HF.

Specific pharmacological enhancement of SERCA2a function

An alternative to conventional positive inotropy or SERCA2a gene therapy in rescuing impaired SR Ca^{2+} uptake in HF is direct pharmacological activation of SERCA2a or inhibition of PLB binding, although to date most agents purported to have such activity have had insufficient efficacy or specificity to be considered for clinical use in humans. This is despite the initial promise shown by a group of phytochemicals, including the polyphenol ellagic acid, to increase the maximum reaction velocity of SERCA2a (Antipenko *et al.*, 1999). More recently, istaroxime, a new non-glycoside agent that inhibits the Na^+/K^+-ATPase, has been shown to elevate SERCA2a activity in addition to its primary mode of action. *In vivo* studies showed that the incidence of lethal arrhythmias was reduced compared with digoxin at a dose that caused equivalent positive inotropy, probably due to a relative reduction in NCX-mediated transient inward current (I_{TI}) (Rocchetti *et al.*, 2003; 2005). Istaroxime also enhances SERCA2a activity in SR vesicle preparations and increases SR Ca^{2+} content in isolated myocytes treated with the agent (Rocchetti *et al.*, 2005). Istaroxime infusion in a guinea pig TAC HF model restored echocardiographic measures of systolic and diastolic cardiac function to levels comparable with non-failing controls (Micheletti *et al.*, 2007). A recent phase 2 trial of 120 hospitalized patients with HF revealed that the drug was well tolerated with a modest drop in pulmonary capillary wedge pressure (3–5 mmHg) compared with placebo (Gheorghiade *et al.*, 2008). Istaroxime inhibition of the Na^+/K^+-ATPase might lead to similar problems found with digoxin therapy where elevated diastolic Ca^{2+} leads to subsequent impairment of diastolic function and arrhythmia. Its parallel SERCA2a activation may circumvent the potential problem but this will only be resolved with larger trials.

SERCA2a gene therapy aims to rescue the reduction of SERCA2a protein levels in HF and has more extensive safety and efficacy data than any small molecule strategy to enhance SERCA2a function. Even if more specific molecules are discovered, it is currently unclear whether pharmacological stimulation of SERCA2a function will be as effective as gene therapy in HF due to the reduction in levels of the target protein limiting the potential for such an agent to enhance SR Ca^{2+} uptake.

Gene delivery of SERCA2a

It is important to appreciate the substantial technical difficulties that have been overcome to take SERCA2a gene therapy into man. These issues have been reviewed elsewhere (Schmidt and Hajjar, 2001; Huq *et al.*, 2002; Lyon *et al.*, 2008) and will only be explored briefly here. Viral vectors have shown greater transfection efficacy than non-viral methods (Lyon *et al.*, 2008). The choice of viral vector is important. Retroviruses require active division of the host cell for their genetic material to be incorporated into host DNA and therefore have limited utility in mature myocardium. Adenoviruses have been used successfully in animal models of SERCA2a gene therapy and are appealing because they carry double-stranded (ds)DNA, which does not integrate into the host genome, thus limiting side effects as a result of insertional mutagenesis of host DNA. However, targeted adenoviral-mediated delivery is limited by the virus predilection to infect multiple human organs, in addition to its immunogenicity resulting in both immune myocarditis and a limited duration of therapeutic gene expression after the host's immune response is established (Yang *et al.*, 1994; Calabrese and Thiene, 2003).

Focus has therefore shifted to the recombinant adeno-associated viruses (rAAVs), which are currently the viral vector most suited to myocardial gene delivery (Lyon *et al.*, 2008). Like adenoviruses, they can infect non-dividing cells

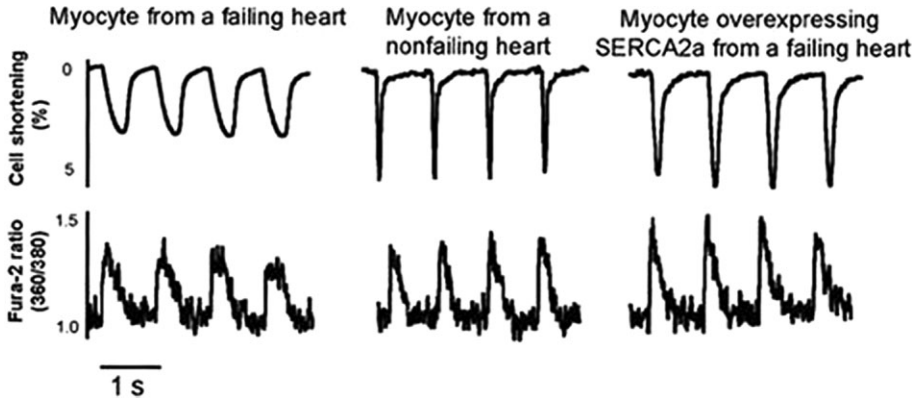

Figure 3

Rescue of human failing ventricular cardiomyocyte contraction and relaxation by *in vitro* Ad.SERCA2a gene transfer. Analysis of % shortening (above) and Fura-2-reported Ca^{2+} transients (below) reveals lower amplitude, slower contractions and Ca^{2+} transients in heart failure cells. This was rescued by SERCA2a gene transfer via an adenoviral vector. Reproduced with permission from Wolters Kluwer Health: Circulation [del Monte *et al.* (1999)]. © 1999.

and have high transduction efficiency without integration into the host genome (Schmidt and Hajjar, 2001). It was hoped that because rAAVs are derived from non-pathogenic parvoviruses, they would be less likely to induce an immune response giving the potential for lifelong gene expression (del Monte and Hajjar, 2003). However, with increasing experience of these vectors, it has been found that the presence of neutralizing anti-AAV antibodies, both pre-existing and generated as a result of therapeutic use, is problematic in up to 50% of patients (Louis *et al.*, 2013). The presence of such antibodies, even at low titres, is associated with reduced or absent therapeutic benefit (Jaski *et al.*, 2009). Strategies to circumvent the immune response via creation of novel, genetically diverse AAV mutants, chemical shielding or pharmacological inhibition of neutralization are reviewed elsewhere (Louis *et al.*, 2013).

An important advantage of the use of rAAVs is their tropism for specific tissues allowing targeting of the diseased organ, in this case the myocardium. Of the 11 serotypes currently identified, AAV1, 6, 8 and 9 have higher myocardial tropism than any of the alternative candidate myocardial vectors (Pacak *et al.*, 2006; Palomeque *et al.*, 2007). However, as there may still be significant infection of other organs (Cheng and Smith, 2013), delivery of the vector has until now usually been achieved in a targeted manner via various methods, including coronary infusions, retrograde venous infusion via the coronary sinus or injection into the pericardial sac (del Monte and Hajjar, 2003). A hope for the future is that more highly cardiotropic rAAVs may be developed that circumvent the need for such invasive techniques by allowing peripheral venous administration (Pacak *et al.*, 2006).

One potential concern in any gene delivery system is that transfection will result in patchy expression of the gene within the myocardium. This could potentially enhance arrhythmias as any electrophysiological heterogeneities caused could induce wavebreak and re-entry. Mechanisms designed to enhance delivery, such as coronary infusions – rather than direct myocardial injection – and high viral tropism may result in global rather than patchy uptake. These approaches appear to have been successful thus far, with the beneficial effects on arrhythmia in preclinical and clinical settings (below) suggesting that enhanced electrophysiological heterogeneity of the myocardium is not a major problem with current gene delivery techniques.

Rescuing EC coupling in HF models with SERCA2a gene therapy

Rescuing contractile impairment in HF

The first cell model used to study SERCA2a gene therapy was neonatal rat cardiomyocytes. This is relevant to HF because the disease process is characterized by a regression to a less mature phenotype. These studies revealed that increased SERCA2a expression increased amplitude of Ca^{2+} transients, enhanced relaxation kinetics and reduced diastolic Ca^{2+} (Hajjar *et al.*, 1997). This was only achieved in the absence of elevated PLB – that is, the SERCA2a : PLB ratio was of paramount importance (Giordano *et al.*, 1997; Meyer *et al.*, 1999). Subsequently, it was demonstrated that *in vitro* transfer and expression of the *SERCA2a* gene in failing human cardiomyocytes from hearts explanted from transplant recipients could rescue deranged contraction and relaxation parameters, and this was mirrored by improved kinetics of intracellular Ca^{2+} transients and normalization of diastolic [Ca^{2+}] (Figure 3) (del Monte *et al.*, 1999). The next step in translation to human HF therapy was to assess whether gene transfer could rescue established HF in animal models.

Initial *in vivo* studies of acquired HF focused on the TAC model in the rat (Miyamoto *et al.*, 2000). Adenoviral SERCA2a gene therapy delivered via coronary infusion restored reduced SERCA2a expression to levels comparable with non-failing sham-operated rats. Cardiac contractile parameters, specifically the maximum rate of pressure generation (dP/dt) in the left ventricle (LV) and maximum rate of relaxation (–dP/dt), were also returned to sham-operated levels. In the same model, SERCA2a gene therapy reduced

mortality compared with untreated TAC animals. In contrast, TAC animals treated with dobutamine had accelerated mortality, analogous to the clinical experience using this positive inotrope (del Monte *et al.*, 2001). Large animal studies followed, with advancements in vector technology leading to the use of rAAVs in these preclinical experiments. In a porcine model of chronic volume overload-induced HF due to mitral regurgitation, AAV1.SERCA2a restored SERCA2a levels to normal and rescued cardiac contractile function (Kawase *et al.*, 2008). Similar results have been obtained in a variety of large animal models, including pacing-induced HF in sheep (Byrne *et al.*, 2008) and dog (Mi *et al.*, 2009).

These effects may not only be of importance in enhancing impaired contractile function, but also of value in reducing the formation of arrhythmias. Contractile impairment in HF is closely coupled with arrhythmias through mechanoelectrical coupling. Heterogeneity of contractility is common in HF, particularly where there is an ischaemic aetiology. Hence, there are regions in the heart in which acute stretch during systole can lead to activation of depolarizing stretch-activated ion channels to bring about ventricular premature beats (Janse *et al.*, 2003). Rapid relaxation of these segments can also bring about triggered activity via rapid release of Ca^{2+} from myofilaments, which can induce Ca^{2+}release from the SR and subsequent Ca^{2+} waves and DADs (ter Keurs, 2011). On a cellular level, it has been found that acute stretch of isolated cardiomyocytes can lead to enhancement of spontaneous SR Ca^{2+} release via increased production of reactive oxygen species (Byrne *et al.*, 2008; Iribe *et al.*, 2009; Prosser *et al.*, 2011). Chronic stretch of cardiomyocytes, as seen in dilated cardiomyopathies, can also contribute to arrhythmia via altered transcription of ion channels and cellular Ca^{2+} overload (Janse *et al.*, 2003).

Indirect benefits of SERCA2a gene therapy

In addition to the effects on contractility, reduced SERCA2a levels can have less direct but equally damaging effects that contribute to the pathophysiology of HF. Mitochondrial saturation by the elevated resting Ca^{2+} levels impairs mitochondrial energetics, increasing cardiomyocyte oxidative stress (Duchen *et al.*, 2008). Myocardial oxidative stress damages numerous effector systems in the failing cardiomyocyte, including central proteins in Ca^{2+} cycling (Cutler *et al.*, 2012a), leading to a positive feedback cycle between rising calcium levels, oxidative stress, mechanical dysfunction, electrical instability and ultimately cell death. Furthermore, increased diastolic [Ca^{2+}] drives several pathological signalling pathways in ventricular cardiomyocytes, including calcineurin-NFAT, CaMKII-histone deacetylase and stromal interaction molecule 1, which can lead to hypertrophy and adverse cardiac remodelling (Kho *et al.*, 2012).

Abnormalities of both cardiac metabolism (measured by the ratio of myocardial phosphocreatine : ATP using NMR spectroscopy) and the energetic cost of contractility were restored in SERCA2a-treated animals with HF induced by TAC or pacing (del Monte *et al.*, 2001; Sakata *et al.*, 2007a; Byrne *et al.*, 2008; Kawase *et al.*, 2008). Similar findings were reported by Sakata *et al*. in a rat model of diabetic cardiomyopathy (Sakata *et al.*, 2006) and by our group in a myocardial infarction (MI) rat HF model (Lyon *et al.*, 2011). Enhanced myocardial energetics may be explained, at least partially, by the alteration of balance of cytoplasmic Ca^{2+} efflux from SERCA2a to NCX in HF and back towards SERCA2a in SERCA2a gene therapy. The stoichiometry of ATP molecules consumed to Ca^{2+} ions transported is 1:2 (ATP : Ca^{2+}) for SERCA2a and 1:1 for NCX (indirectly via the Na^+/K^+-ATPase), hence contributing to the reduction in efficiency seen with reduced SERCA2a levels (Sakata *et al.*, 2006). The improvement in oxygen cost of contractility contrasts with the neutral (or negative at higher doses) effects on this parameter by dobutamine and levosimendan (Muller *et al.*, 2010).

In addition, SERCA2a appears to have some effects on myocardial perfusion. Coronary blood flow was shown to increase following its administration in a model of diabetic cardiomyopathy (Sakata *et al.*, 2007b), although it was not clear whether this was related to improved myocardial relaxation facilitating diastolic coronary blood flow or a direct vascular mechanism. More recently, evidence has emerged that SERCA2a is expressed in vascular smooth muscle of treated HF animals and that this is related to restoration of normal levels of endothelial NOS and thus reduced vasoconstriction (Hadri *et al.*, 2010). SERCA2a gene therapy has also been shown to result in reduced smooth muscle proliferation and neointimal thickening in response to injury in rat carotid and human internal mammary artery (Lipskaia *et al.*, 2005; 2013).

Modulation of arrhythmia with SERCA2a gene therapy

Possible detrimental effects of SERCA2a overexpression on failing hearts

SERCA2a, as a key regulator of SR Ca^{2+} content, is important in dictating Ca^{2+} available for CICR and thus the cardiac contractile force subsequently generated. This can be understood in the context of the fundamental SR Ca^{2+} release event: the Ca^{2+} spark. When summated in their thousands, sparks form the large cytosolic rise in Ca^{2+} (the Ca^{2+} transient) that causes contraction (Cannell *et al.*, 1995; Lopez-Lopez *et al.*, 1995). There is a large amount of gain in the system, provided both by the higher Ca^{2+} flux carried by RyRs compared with LTCCs and by the persistence of Ca^{2+} release beyond the initial I_{Ca} trigger. The quantity of Ca^{2+} leaving the SR during both individual spark events and their summated transient is steeply dependent on SR Ca^{2+} content (Gyorke and Gyorke, 1998; Trafford *et al.*, 2000; Bode *et al.*, 2011). Thus, increasing SERCA2a activity increases the amplitude of each spark and the summated systolic Ca^{2+} transient (Jane Lalli *et al.*, 2001). However, because Ca^{2+} sparks are also the fundamental building blocks of Ca^{2+} waves (Cheng *et al.*, 1996), which result in pro-arrhythmic DADs, the beneficial effects on contractility could theoretically result in an increased risk of arrhythmia as seen with conventional positive inotropes. This is particularly concerning in the context of the 'leaky' SR in HF. Seemingly supporting the concept of arrhythmogenesis with SERCA2a overexpression, accelerating SR-cytoplasmic Ca^{2+} cycling above normal levels is deleterious with pro-arrhythmic sequelae in a number of transgenic mouse models (Chopra *et al.*, 2007; Yuan *et al.*, 2007). In addition, in humans with a homozygous PLB null mutation, a fatal dilated cardiomyopa-

thy phenotype results (Haghighi *et al.*, 2003). Also, sudden arrhythmic death was transiently increased in a transgenic SERCA2a overexpressing rat following acute coronary ischaemia and reperfusion (Chen *et al.*, 2004), although surviving animals then had reduced post-infarction LV impairment compared with wild-type controls. This enhanced arrhythmia is in contrast to the majority of studies that have found beneficial effects.

Beneficial effects on SR Ca^{2+} leak

Contrary to these concerns, we reported that SERCA2a therapy reduced arrhythmia in an established chronic MI model of HF in the rat (Lyon *et al.*, 2011). The HF model was characterized by a high frequency of spontaneous ventricular arrhythmia (VA) with over 4000 spontaneous ventricular ectopic beats per day (corresponding to approximately 1% of all beats). This was significantly reduced in SERCA2a-treated animals. We also assessed the incidence of isoprenaline-induced arrhythmia, a technique used to stress the importance of SR leak : load imbalance in HF. In the untreated HF group, 75% of animals had sustained arrhythmia in the form of sustained VT or ventricular fibrillation (VF), whereas no animals in the HF + SERCA2a gene therapy group had sustained (>4 beats) VT or VF (Figure 4).

We proceeded to study the cellular mechanisms underlying this anti-arrhythmic effect. We found that HF cells had a typical 'leaky' SR phenotype, in part, due to increased frequency of spontaneous Ca^{2+} sparks. As a consequence, SR Ca^{2+} content was reduced in HF cells. Following SERCA2a gene therapy, Ca^{2+} spark frequency remained high, but spark morphology changed substantially with a reduction of spark mass to normal levels. This resulted in improvements to spark-mediated Ca^{2+} leak (calculated by spark mass × frequency) to a level not statistically different from that of age-matched control animals. The reduction in mass had compensated for the higher frequency. Another measure of SR Ca^{2+} leak is total leak, which includes spark and non-spark mediated fluxes and is measured through application of tetracaine (Shannon *et al.*, 2002). This was also reduced to control levels following SERCA2a gene therapy. SR Ca^{2+} content also returned to normal following SERCA2a treatment, presumably in part due to reduced SR Ca^{2+} leak and in part due to rescued SERCA2a levels and SR Ca^{2+} uptake. Isoprenaline-induced triggered cellular activity was also reduced in cardiomyocytes from SERCA2a-treated animals, consistent with the anti-arrhythmic effect observed following *in vivo* isoprenaline challenge.

Clearly, such changes are not predictable from the increase in SR Ca^{2+} content induced by SERCA2a gene

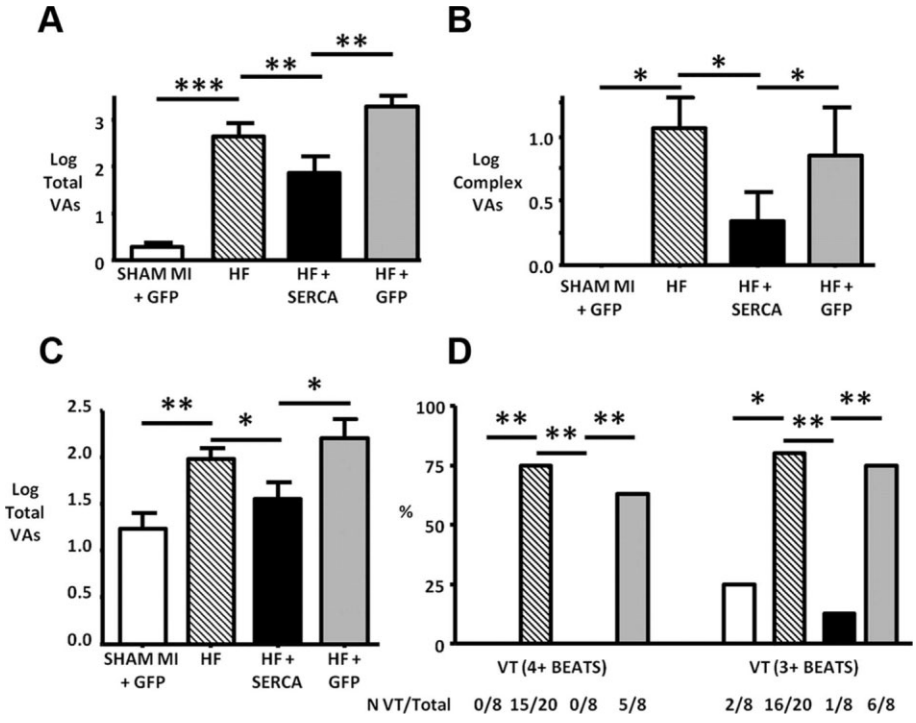

Figure 4

SERCA2a gene therapy rescues pro-arrhythmic phenotype of myocardial infarction (MI)-induced heart failure (HF) in the rat. (A) Simple spontaneous ventricular arrhythmias (VAs) [i.e. ventricular ectopic (VE) beat incidence] during a 24 h study period were significantly increased in HF versus sham animals and rescued in SERCA2a-treated animals. Control virus (HF + GFP) group showed no improvement over HF group. (B) Complex spontaneous VAs [i.e. multiple sequential VEs or ventricular tachycardia (VT)] showed a similar pattern. (C) Total isoprenaline-induced VAs during the 60 min recording period after injection were reduced by SERCA2a gene transfer. (D) The effect on isoprenaline-induced VT was even more marked with 15/20 HF animals suffering isoprenaline-induced VT (4 + beats), but none of the SERCA2a-treated HF animals. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. N VT/Total – number of rats that suffered VT versus total rats assessed with isoprenaline challenge. Reproduced with permission from Wolters Kluwer Health: Circulation Arrhythmia & Electrophysiology [Lyon *et al.* (2011)]. © 2011.

therapy. There must be additional effects of therapy that compensate for the presumed increase in leak simply induced by elevation of SR Ca^{2+} load *per se*. Several possibilities may explain why this might happen. One possibility is direct modification of Ca^{2+} spark morphology due to the buffering ability of increased SERCA2a. Indeed, pharmacological inhibition of SERCA2a has been shown to prolong sparks (Gomez *et al.*, 1996). If the opposite occurs following therapy, curtailed sparks may result.

Secondly, phosphorylation of RyR2 at Ser^{2815} was also reduced by SERCA2a gene transfer (Lyon *et al.*, 2011). Phosphorylation at this site is induced by the activity of CaMKII (Yang *et al.*, 2007). As SERCA2a enhances Ca^{2+} uptake, it could potentially reduce $[Ca^{2+}]_i$ in the vicinity of CaMKII, thus reducing its activation via the Ca^{2+}-calmodulin complex. The lower amount of phosphorylation would be expected to increase the threshold for SR Ca^{2+} release towards normal levels, thus 'resetting' the leak threshold and reducing spontaneous Ca^{2+} release at a given load. We believe a virtuous cycle whereby reduced CaMKII activity is initiated by enhanced SERCA2a activity and reinforced by reduced SR Ca^{2+} leak may ensue. The effects of SERCA2a therapy on other modifications of RyR2 such as oxidation and nitrosylation have not been specifically assessed but may also be significant, particularly given the importance of diastolic $[Ca^{2+}]_i$ in the regulation of mitochondrial function and oxidative stress (Zima and Blatter, 2006; Duchen *et al.*, 2008).

Although not yet studied in detail, it is also possible that SERCA2a therapy is beneficial not only in terms of Ca^{2+} leak reduction but also in terms of reducing adverse consequences of Ca^{2+} leak. It is interesting to speculate on what effect SERCA2a therapy would have on Ca^{2+} waves. On the one hand, the enhanced buffering of cytosolic Ca^{2+} may mean that it is more difficult for waves to initiate and propagate. On the other hand, there is recent evidence that Ca^{2+} uptake by SERCA2a at the wavefront might produce luminal sensitization that is necessary for efficient wave propagation (Keller *et al.*, 2007; Maxwell and Blatter, 2012), and could enhance waves in the presence of SERCA2a up-regulation. The consequences of a wave might also be altered since the amplitude of the ensuing DAD could be reduced by the increase in SERCA2a : NCX ratio, or enhanced as a result of increased SR Ca^{2+} content. These aspects will be the subject of further study.

Beneficial effects on Ca^{2+} and repolarization alternans

A further mechanism whereby SERCA2a gene therapy can be anti-arrhythmic has been explored extensively by Rosenbaum and co-workers. Beat-to-beat variations in Ca^{2+} fluxes and action potential duration (APD) within myocytes, known as Ca^{2+} and electrical alternans, respectively, are known to be associated with arrhythmia susceptibility (Cutler *et al.*, 2009). Cellular alternans occur when the heart rate exceeds the capability of the cardiomyocytes to cycle Ca^{2+}. Hence, particularly in HF, a reduction in SERCA2a can predispose to alternans and arrhythmia. Cutler *et al.* showed, first in normal myocytes (Cutler *et al.*, 2009) and later in myocytes from a pressure-overload induced HF model in the guinea pig (Cutler *et al.*, 2012b), that enhanced SERCA2a levels reduced the propensity for both Ca^{2+} and APD alternans. Even under constant action potential clamp conditions, this difference persisted, providing further evidence that the effect was due to SR-dependent effects. As a result, pacing-induced VAs were suppressed in hearts with higher SERCA2a expression, with the lowest SERCA2a levels (as seen in HF) being associated with reduced threshold for alternans. Comparatively higher SERCA2a expression in ascending order was seen in (a) control, (b) SERCA2a-treated HF and (c) SERCA2a-treated controls. VF/VT inducibility was reduced predictably as SERCA2a levels increased (Figure 5). Consistent with our study, these experiments also showed that SERCA2a-treated HF also reduced SR Ca^{2+} leak in the whole heart, with reduced Ser^{2814} (the CaMKII site in this species) phosphorylation. Xie *et al.* have also shown that adenoviral SERCA2a gene transfer

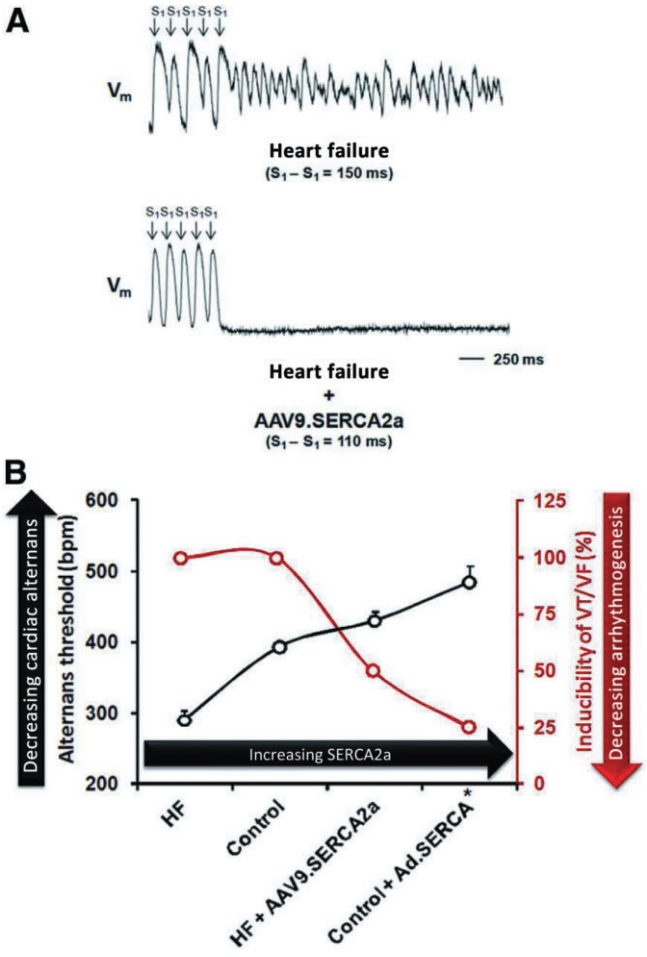

Figure 5

Modulation of alternans-mediated arrhythmia by SERCA2a gene transfer. (A) Optical mapping of a Langendorff-perfused heart failure (HF) heart reveals marked action potential duration (APD) alternans with subsequent induction of ventricular fibrillation (VF). In a HF heart treated with SERCA2a gene therapy (HF + AAV9.SERCA2a), such alternans does not occur despite an even shorter basic cycle length. (B) Graph of increasing threshold for alternans and decreasing inducibility of VT/VF in the presence of higher SERCA2a expression from lowest (HF) to highest (SERCA2a-treated controls – control + Ad.SERCA). Reproduced with permission from Wolters Kluwer Health: Circulation [Cutler *et al.* (2012b)]. © 2012.

in cultured rabbit cardiomyocytes suppresses Ca^{2+} alternans and that the SERCA2a inhibitor thapsigargin increases susceptibility to alternans (Xie *et al.*, 2008). It is important to note that enhancement of SR Ca^{2+} leak alone can result in cellular Ca^{2+} and voltage alternans (Weiss *et al.*, 2011). Thus, the extent to which alternans is a primary event or an epiphenomenon indicating the enhanced SR Ca^{2+} leak in this setting is unclear. Nevertheless, it is likely to be an important mechanistic connection between spontaneous SR Ca^{2+} release and possible VT or VF at the level of the organ.

Other anti-arrhythmic mechanisms

APD prolongation in HF is thought to be an important cause of arrhythmia, both through enhancing dispersion of repolarization and favouring the development of EADs (Kaab *et al.*, 1996). Terracciano *et al.* (2002) showed that SERCA2a gene transfer in cultured rabbit cardiomyocytes reduced APD duration. Consistent with these findings, Davia *et al.* (2001) showed that SERCA2a gene therapy also reduced the incidence of aftercontractions in the same model. Together, these studies suggest that a reduction in EADs, as well as DADs, may play a role in arrhythmia reduction. APD prolongation can lead to increased QT interval on the ECG of the intact animal. Hence, our finding that QT interval is prolonged in HF and rescued in SERCA2a-treated rats (Lyon *et al.*, 2011) is consistent with these data. The reduction in APD induced by SERCA2a probably has its basis in enhanced Ca^{2+}-dependent inactivation of LTCC that is due to the larger SR Ca^{2+} release.

Abnormal automaticity is another potentially important mechanism of arrhythmogenicity in HF (Vermeulen, 1998) and may be independent of SR Ca^{2+} leak (Nuss *et al.*, 1999). This may be enhanced in ventricular cells in HF due to reductions in I_{K1} and enhanced I_f. Given recent insights into the role of SR Ca^{2+} release in normal automaticity, and particularly how it is synergistic with I_f in bringing the membrane potential to threshold (Maltsev and Lakatta, 2009), it is possible that reduction of abnormal automaticity may also play a role in the anti-arrhythmic effect of SERCA2a in HF.

Modulation of ischaemia–reperfusion (I/R)-induced injury may be another mechanism that confers protection against arrhythmias *in vivo*. In a rat I/R rat model, SERCA2a gene therapy 2–6 days prior to I/R reduced episodes of VT/VF (del Monte *et al.*, 2004). While the initial ischaemic area of the heart did not differ between treated and control animals in this study, the final MI area was significantly reduced in SERCA2a-treated animals. This suggests enhanced survival of SERCA2a-treated cardiomyocytes in the presence of ischaemia, potentially due to enhanced buffering of the large rise in cytosolic Ca^{2+}, which can be induced by I/R. The reduction in infarct size would be expected to reduce conduction heterogeneity in the ventricle and thus mediate the decline in the number of arrhythmias observed. These findings were later reproduced in a porcine I/R model in which arrhythmias were reduced following I/R, but not in a permanent coronary ligation model in the same species (Prunier *et al.*, 2008).

More general reverse remodelling of the failing heart as a result of improved cardiac mechanics may also play a significant role in the reduction of arrhythmias. We have shown an example of this at the level of the isolated cardiomyocytes whereby the disruptions in surface structure and t-tubule morphology that occur in the rat chronic MI model of HF (Lyon *et al.*, 2009) are rescued in cardiomyocytes isolated from SERCA2a-treated HF rats (Lyon *et al.*, 2012). Such restoration of ultrastructure could improve arrhythmia via a reduction of 'orphaned' RyRs (those remote from t-tubules), which have been hypothesized to be particularly important in the pro-arrhythmic consequences of HF (Song *et al.*, 2005; 2006; Keller *et al.*, 2007). Enhancing SERCA2a function via transfection of pseudophosphorylated PLB has also been shown to result in significant reduction of macroscopic tissue heterogeneity via reduction of fibrosis in a cardiomyopathic hamster model (Hoshijima, 2005). These structural improvements could reduce the enhanced electrophysiological heterogeneity and thus decrease the likelihood of re-entry in HF. They also emphasize that SERCA2a gene therapy can result in a broader reversal of the pathophysiology of HF than isolated effects on SR Ca^{2+} cycling could confer. A summary of the multifaceted mechanism of action of SERCA2a gene therapy in reducing arrhythmia is shown in Figure 6.

Clinical SERCA2a gene therapy trials

Regardless of the beneficial effects of SERCA2a gene therapy in preclinical models, whether the therapy is destined to form part of the management of HF is dependent on the results of ongoing clinical trials. A completed phase 1/2 trial [Calcium Up-regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease (CUPID)] and the current phase 2B trial (CUPID2) among others will help decide whether SERCA2a will be beneficial in the human HF population.

The CUPID trial programme

The phase 1/2 CUPID trial was launched in 2007 (Hajjar *et al.*, 2008). In the initial open-label phase 1 part of this study, safety was established through dose escalation in 12 patients (Jaski *et al.*, 2009). In the second stage, 39 patients with advanced HF were randomized to receive one of three doses of intracoronary AAV1.SERCA2a or placebo saline infusion (Jessup *et al.*, 2011). Dosing protocol for the treatment groups was low dose (6×10^{11} DNase-resistant particles), medium dose (3×10^{12} DNase-resistant particles) or high dose (1×10^{13} DNase-resistant particles). Virus or saline placebo was administered via antegrade coronary infusion over 10 min. All patients were required to have an implantable defibrillator in response to concerns that SERCA2a gene therapy may increase the rate of VAs. Eight patients received low-dose AAV1.SERCA2a, eight were randomized to a mid-level dose and nine to high dose; 14 patients were randomized to placebo with saline infusion.

The primary endpoint in terms of efficacy was evaluated by examining concordant trends in the following endpoints: Patient's symptom questionnaires, functional status [6 min walk test and VO_2-max (maximal rate of oxygen consumption)], N-terminal pro–B-type natriuretic peptide (NT-proBNP) levels and echocardiographic measures. The AAV1.SERCA2a high-dose group met the pre-specified criteria for efficacy. Patients in this treatment group demonstrated improvement or stabilization in symptoms, functional class, NT-proBNP levels, and LV end-systolic volumes. There was also a significant increase in time to cardiovascular events

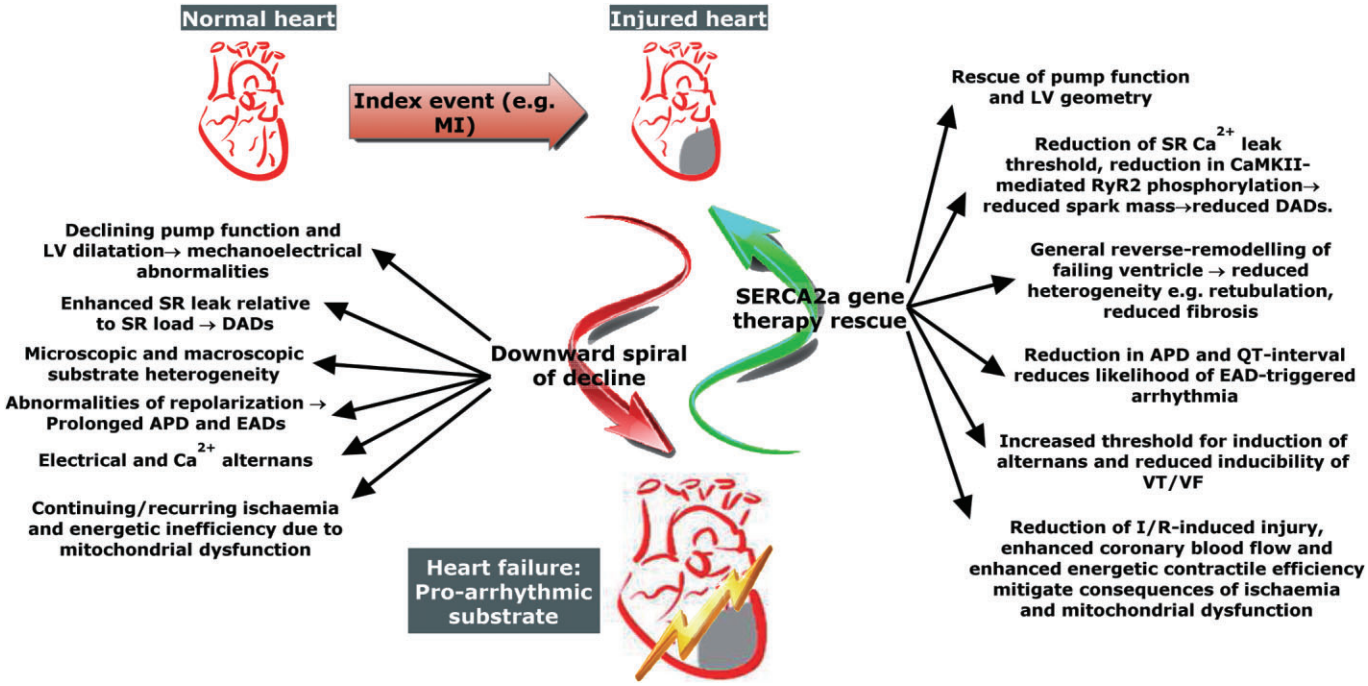

Figure 6

Multiple mechanisms lead to pro-arrhythmic heart failure phenotype and are rescued by SERCA2a gene therapy. An index event causes a degree of cardiac damage and leads to a downward spiral of events which result in impaired pump function and a pro-arrhythmic substrate. Pathological changes leading to arrhythmia, and how they are rescued by SERCA2a gene therapy, are highlighted. APD, action potential duration; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DAD, delayed afterdepolarization; EAD, early afterdepolarization; I/R, ischaemia/reperfusion; LV, left ventricle; MI, myocardial infarction; RyR2, cardiac ryanodine receptor; SR, sarcoplasmic reticulum; VF, ventricular fibrillation; VT, ventricular tachycardia.

and a decreased frequency of cardiovascular events per patient in all patients receiving AAV1.SERCA2a. Importantly, patients in the treatment arm showed no increase in adverse events, disease-related events or arrhythmias, correlating well with the preclinical studies described earlier, and contrary to the perceived concerns. In this trial, a potentially problematic issue of AAV auto-antibodies was identified. Patients with even the lowest titres of AAV auto-antibodies (>1:2) appeared not to receive any therapeutic benefit. This highlights the importance of developing novel vectors that are not neutralized by pre-existing antibodies.

CUPID 2

The CUPID 2 trial is a phase 2b, double-blind, placebo-controlled, multinational, randomized study evaluating the safety and efficacy of intracoronary administration of AAV1.SERCA2a in subjects with HF. Two hundred patients will be recruited with ischaemic or dilated cardiomyopathy with New York Heart Association (NYHA) III/IV class symptoms despite optimal HF treatment. In contrast to CUPID, in this trial patients are not required to have implantable defibrillators, reflecting the balance of evidence thus far in both preclinical and clinical studies regarding arrhythmia risk. Patients will be screened for neutralizing antibodies to AAV1 and must be negative (titre <1:2) to be enrolled. Eligible patients will be randomized in parallel in a 1:1 ratio to receive either AAV1.SERCA2a or placebo. Vector delivery is in the form of a 10 min intracoronary infusion of 1×10^{13} DNase-resistant particles of AAV1.SERCA2a (the high dose in the CUPID trial) with concomitant glyceryl trinitrate to improve vector uptake and transfection efficiency via vasodilatation. To allow sufficient vector delivery, participants must have at least one coronary vessel with angiographically normal flow.

The primary efficacy endpoint is 'time to recurrent HF hospitalizations in the presence of a terminal event [all-cause death, heart transplantation, left ventricular assist device (LVAD) implantation]'. The secondary endpoint is 'time to a terminal event (all-cause death, heart transplantation, LVAD implantation)'. Both primary and secondary endpoints will be analysed using the joint frailty model, a method of statistical analysis of endpoints which, in contrast to standard survival analysis, takes into account all events and not just the first (Huang and Liu, 2007). Exploratory assessments include changes from baseline in the following: NYHA class, 6 min walk test and symptom questionnaire.

Safety will be assessed based on the incidence of adverse events, all-cause mortality and laboratory evaluations. Patients have already been treated in the USA, Denmark and at our centre in the UK. An appropriately powered separate trial in a high-risk arrhythmia population (e.g. HF patients with implantable cardioverter-defibrillators) would be necessary to specifically assess anti-arrhythmic efficacy. Further trials may also be necessary to assess whether the dose would

need to be adjusted depending on the differences in SERCA2a deficiencies seen in different models of HF.

Conclusion

Myocardial SERCA2a gene therapy is the first of several novel molecular-targeted strategies aimed at improving mechanical function of the failing heart without the detrimental side effects of accelerating energetic inefficiencies, apoptosis and arrhythmogenicity associated with classical positive inotropes which increase PKA activity. Ca^{2+} has long been identified as a central regulatory and signalling messenger for numerous intracellular pathways in cardiomyocytes, as the fields of excitation-contraction coupling, excitation-energetic coupling and excitation-transcription coupling all testify. As a result, targeting abnormal SR Ca^{2+} cycling in HF directly confers several simultaneous benefits, including normalized Ca^{2+} transient kinetics, enhanced energetic efficiency and increased cell survival. Evidence of additional beneficial effects, via reduced SR Ca^{2+} leak, increased alternans threshold, reduced propensity for injury during I/R and reverse remodelling of the heart suggest that SERCA2a gene therapy has the potential to become the first anti-arrhythmic positively inotropic treatment strategy in HF. This could improve both quality of life and survival in this group of patients.

Acknowledgements

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Conflict of interest

Dr. Lyon is the UK national lead investigator for the CUPID2 trial and has a consultancy contract with Celladon Corporation for this role.

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