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# Advantages of Catheter-Based Adenoviral Delivery of Genes to the Heart for Studies of Cardiac Disease

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## 1. Introduction

With the advent and rapid advancement of genetically engineered methodologies (ie., cDNA, siRNA and transgenics), there has been an unprecedented acceleration in the identification of the underlying maladaptive processes of heart disease. New pharmaceutical and gene therapy strategies targeted at correcting these maladaptions show unprecedented promise. In particular, the transfer of exogenous cDNA or siRNA by viral based vehicles show great promise for both the overexpression or suppression, respectively, of the proteins linked to these maladaptations in heart. Indeed, the translation of these current viral based studies from animal models of cardiac disease to humans are now in clinical trials (Jessup et al., 2011; Hajjar et al., 2008; Jaski et al., 2009; Gwathmey et al., 2011). However, progress is slowed by weak and inefficient techniques for the transport and delivery of exogenous genes to the heart in animal and human.

Much like the transport of consumer goods across the country, the transport of exogenous genes to the heart requires both an efficient route of transport and an efficient delivery vehicle. This chapter briefly compares the efficiency of the various delivery routes to the heart, and we compare two of the more popular vehicles (adenovirus vs adeno-associated virus). We will argue that the adenoviral (Adv) delivery of genes by a catheter-based route provides considerable advantages for molecular based studies of heart disease. Alternatively, the emerging and less invasive adeno-associated vector (AAV) is better suited for therapeutic gene transfer in human. Several groups have demonstrated that simple systemic injection of the AAV-cDNA in rodents enables significant gene transfer and long-term expression of the targeted proteins in heart (see review Wasala et al., 2011). However, in larger animals and humans, the promise of this less invasive approach has not translated well. In larger mammals, gene transfer to the heart still requires an invasive catheter-based surgical procedure (White et al., 2011, Kaye et al., 2007; Hayase et al., 2005). Here, we will discuss some of these gene transfer strategies and their limitations.

We will also describe our surgical advancement to the conventional catheter-based technique for gene delivery to the heart. This approach significantly improves gene transfer

to the heart and eliminates both virus accumulation and gene transfer to non-target organs in rat (O'Donnell et al., 2004, 2005, 2008, 2009). The earlier conventional form of this open-chest catheter-based approach has been used extensively to deliver the cDNA for the $Ca^{2+}$-ATPase, SERCA2a, to heart as a potential therapeutic strategy for the treatment of heart failure (Del Monte et al., 2002). While there are a number of reports that this strategy improves cardiac hemodynamics, function, and survival (Miyamoto et al., 2000; Del Monte et al., 2001), the results via our modified approach challenge this data and the safety of the treatment proposed for humans (O'Donnell et al., 2008). Our finding has recently been supported by a second report in transgenic mice overexpressing SERCA2a subjected to ascending aortic constriction (Pinz et al., 2011). Here, we briefly review this opposing data, and discuss conditions for this discrepancy, which require consideration as a safe and effective treatment strategy continues to evolve for clinical applications. The controversy of this therapeutic strategy, as it relates to the stage and model of the disease, is also discussed in a recent commentary by Sipido and Bangheluwe (Sipido et al., 2010).

## 2. Methods of gene delivery to the heart

The development of efficient techniques for direct in vivo gene transfer is important not only to support basic science studies in animal models, but also for gene therapy of heart disease in human. However, progress to date has been limited by difficulties in the available gene delivery systems (Prasad et al., 2011). Ideally, the gene delivery approach would be minimally invasive and provide a robust and homogenous transfection to the whole heart without an autoimmune or toxic side effect. To this end, several delivery approaches have been developed and examined both in animals and humans as illustrated in Figure 1. These methods include direct injection of the viral vehicle into (a) the pericardial sac, (b) the intramyocardial tissue, (c) the intraventricular chamber, (d) intravenous delivery, or (e) catheter-based delivery of the virus into the aortic root which enables delivery of the virus to the whole heart via the coronary arteries.

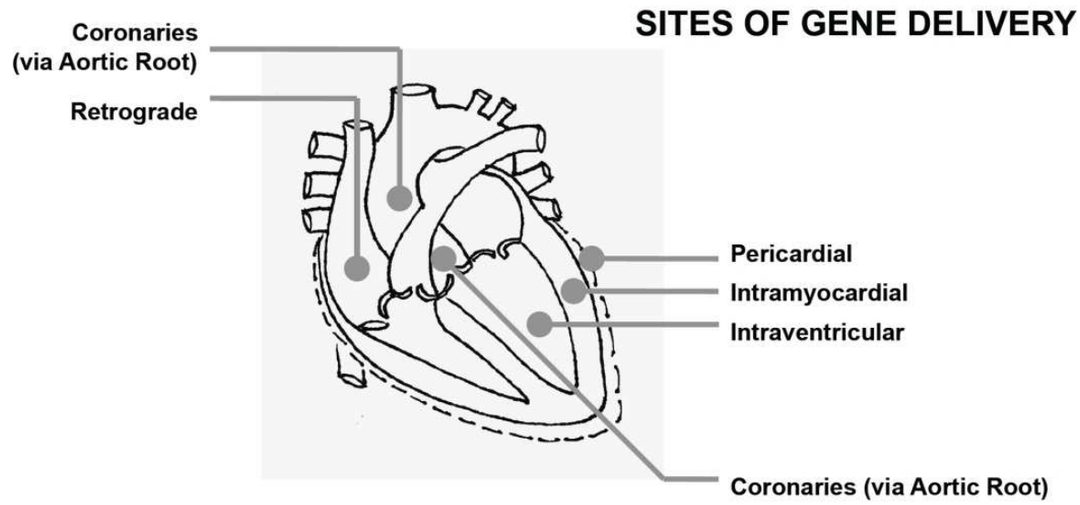


Fig. 1. Sites of gene delivery to the heart via viral_cDNA based methodologies. The highest level of gene transfer has been achieved by coronary perfusion of the heart via catheter-based delivery routes.

### 2.1 Routes of gene delivery

The different routes of viral delivery demonstrate different efficiencies of gene transfer and durations of gene expression in heart (Wasala et al., 2011). The direct injection of the viral-cDNA package into the heart tissue results in a very localized gene transfer at the site of injection (Svensson et al., 1999; Su et al., 2000). When an adenoviral vector is used, the expression peaks at one week and subsequently declines (Guzman et al., 1993). Similarly, intravenous injection of the adenoviral-cDNA package results in peak expression in heart within a week in adult rat and mice (Stratford-Perricaudet et al., 1992). However, unlike the localized injection, the intravenous injection results in widespread infection of peripheral tissue (liver, skeletal muscle, lung). Adenoviral gene transfer also results in an inflammatory response in the heart after 4-5 days, and the expression of the targeted protein is gone after a few weeks (Kass-Eisler et al., 1993). Therefore, studies must be completed within 3 days of gene transfer when the overexpression is maximal and the inflammatory response is minimal.

Unlike the adult mouse or rat model, intraventricular injection of the adenoviral-cDNA package into neonatal mouse hearts leads to the overexpression of exogenous gene for several months (2-12 mths) with no inflammatory response (Stratford-Perricaudet et al., 1992). This chronic overexpression is due to incorporation of the transferred gene into the animal's genome. However, the two month expression window in mice is often not long enough to study chronic models of heart disease, and infection of non-target tissue is still observed (Stratford-Perricaudet et al., 1992).

As an alternate method to intravascular or intraventricular administration, when the adenoviral-cDNA package is injected into the pericardial sac of adult rats, gene transfer is exclusively restricted to the pericardial cell layers (Fromes et al., 1999). However, injecting a mixture of collagenase and hyaluronidase together with the virus, leads to a somewhat larger diffusion (40%) of the transgene through the myocardial wall. The collateral effects of the collagenase / hyaluronidase are unclear.

The transfer of exogenous genes to the whole heart, in vivo, by the catheter-based approach has consistently demonstrated the most promising strategy. The approach was first described by Barr in 1994 (Barr et al., 1994). A catheter was inserted into the right carotid artery and advanced to the coronary ostia before injecting 1 ml of Adv.cmv.lacZ into the coronary artery. A cross section of heart stain for lacZ expression showed myocytes expressing the exogenous gene. However, infection was sparse and efficiency was <1%. An important advancement to the approach was presented by Hajjar's laboratory in 1998 (Hajjar et al., 1998). The viral solution was injected into the aortic root after first cross-clamping the aorta and pulmonary artery. This enabled both a high injection pressure and longer incubation periods prior to releasing the cross-clamp. Subsequent refinements to this basic approach have since yielded efficiencies as high as 40-80% in hamster (Ikeda et al., 2002), mouse (Champion et al., 2003; Iwatate et al., 2003), and rat (Ding et al., 2004). However, the approach still results in (a) infection of non-target tissue (liver, skeletal muscle, lung), (b) an inflammatory response within a week, and (c) some groups report that the level of gene transfer to the heart by the conventional approach is low (Wright et al., 2001; Ding et al., 2004; O'Donnell et al., 2005).

Nevertheless, several groups have demonstrated that the adenoviral delivery of exogenous genes to the heart by the catheter-based approach can enhance cardiac function and provide

a potential therapeutic strategy for the treatment of heart disease. For a detailed discussion of the various trans-genes examined over the past decade, see the recent review by Wasala (Wasala et al., 2011). In brief, Hajjar's group has demonstrated in both rat and sheep models of heart failure that the overexpresson of SERCA2a leads to enhanced cardiac function, extended life span, and reduced arrhythmias (Miyamoto et al., 2000; Byrne et al., 2008). Similarily, the catheter-based delivery of an adenovirus carrying the phospholamban gene resulted in a significant change in left ventricular function (Hajjar et al., 1998). Ross has also demonstrated that coronary delivery of adenovirus carrying the cDNA for δ-sarcoglycan, in a sarcoglycan deficient hamster model of dilated cardiomyopathy, results in enhance left ventricular function (Ideda et al., 2002). Using a modified catheter-based technique, Maurice has shown that the overexpression of a β-adrenergic receptor significantly enhanced heart contractility and hemodynamic performance in rabbits (Maurice et al., 1999).

Cumulatively, these studies demonstrate the therapeutic utility of the catheter-based adenoviral delivery approach for the modulation of heart function, but a common limitation for these studies was that the overexpression of the exogenous gene was low in the heart, the transfer of the exogenous gene to peripheral organs was also observed, and cardiac inflammation was observed by one week. Furthermore, while the results proved promising, it is not completely resolved whether the functional improvement with the treatment strategy was a direct result of the targeted transgene or due to potential peripheral mechanisms.

Our group has since advanced the conventional catheter-based approach to reduced peripheral effects. This advanced approach results in highly efficient and global gene transfer to the whole heart with minimal infection of peripheral tissue. We've since used this catheter-based delivery approach to determine if the overexpression of the Ca2+-ATPase, SERCA1a, can be used as a therapeutic strategy for the treatment of cardiac hypertrophy and ischemia reperfusion. In support of other reports (Chen et al., 2004; Taluker et al., 2007), we found the strategy significantly improved post-ischemic recovery of cardiac function and metabolism in rat (O'Donnell et al., 2009). However, the strategy compromised cardiac function in the hypertrophic heart (O'Donnell et al., 2008). This finding contradicts the earlier reports which used the conventional catheter-based cross-clamp approach to delivery the cDNA for SERCA2a (Miyamoto et al., 2000; Del Monte et al., 2001; Byrne et al., 2008). Whether this discrepancy is related to (a) the higher level of gene transfer achieved by our approach (ie., a dose response), (b) the elimination of gene transfer to peripheral tissue by our approach, (c) the specific isoform of SERCA gene transferred (SERCA1 vs SERCA2), or (d) the stage of the disease model (early vs late stage heart failure) is unclear and discussed under section 4.5.

### 2.2 Vehicles of gene delivery

Equal to the selection of the delivery route is the selection of the viral vehicle used to deliver the exogenous cDNA. Several viral vectors are currently being investigated for gene delivery to provide either transient or permanent transgene expression. These include adenovirus (Adv), adeno-associated virus (AAV), retrovirus, lentivirus, and herpes simplex virus-1 (HSV-1). The Adv can carry a ~30 kb genome, and the expression of the exogenous gene is initially very high, reaching its maximal effect within the first 2-5 days, as discussed. While the adenovirus can efficiently transduce the myocardium, the vector is limited by short-term gene expression (2 weeks) and there is an immune response to viral proteins

after five days (Gilgenkrantz et al., 1995) which can cause significant myocardial inflammation. Therefore, Adv is useful when a high level of expression is required, and studies can be completed before the inflammatory response.

The recombinant adeno-associated viral vectors (AAV) offers an attractive alternative. See the recent review by Reyes-Juarez and Zarain-Herzberg for a detailed discussion (Reyes-Juarez et al., 2011). In brief, the AAV (25 nm) is smaller than the conventional Adv adenovirus which enables the virus to cross the endothelial barrier with greater ease in rat and mouse (Xie et al., 2002; Dhu et al., 2003). Consequently, several groups have shown that simple intravenous or intramuscular injections yield highly efficient gene transfer to the heart or skeletal muscle (Zhu et al., 2005; Blankinship et al., 2004; Kawamoto et al., 2005). This makes delivery both minimally invasive and highly efficient, two important criteria toward controlling gene transfer. In addition, the AAV serotype is not pathogenic, there is little evidence of inflammation with use, and expression is long term (years) (Gregorevic et al., 2004; Aikawa et al., 2002). Unfortunately, as with the conventional adenovirus (Adv), expression of genes delivered via the AAV within non-target cells remains problematic causing deleterious side effects (Aikawa et al., 2002; Su et al., 2004). However, this cross-infection can be reduced by using specific serotypes of the AAV (AAV-1, 2, 5, 6, 7, 8, and 9). These serotypes have an array of tissue tropisms and binding characteristics (Zhu et al., 2005; Du et al., 2004) making the discovery of a cardiac specific AAV a promising strategy.

In rodent models, AAV has been delivered via the catheter-based coronary perfusion approach, direct myocardial injection, and intravenous delivery (Wasala et al., 2011). Svensson and colleagues delivered AAV-2_LacZ to adult mouse heart by coronary perfusion and direct injection (Svensson et al., 1999). While the expression of exogenous genes were minimal at 2 weeks, expression was robust at 8 weeks with 50% of the cardiomyocytes transduced by the trans-coronary perfusion. Similarily, Hoshijima reported the expression of genes persisted for 30 weeks in hamsters with AAV-2 (Hoshijima et al., 2002). Using a far less invasive approach, Gregorevic injected AAV-6 into the tail vein of adult mice and found extensive gene transfer to heart and skeletal muscles (Gregorevic et al., 2004). Xiao and colleagues reported similar high gene transfer to heart and skeletal muscle with intraperitoneal and/or intravascular injection of AAV-2 (Wang et al., 2005). Others have also shown that AAV-9 is 10 fold stronger than AAV-2 and 8 in transducing mouse heart (Bish et al., 2008; Inagaki et al., 2006), though this serotype also displays a strong tropism for gene transfer to off target organs including liver. However, in dividing/regenerative cells such as in liver, the expression of exogenous genes via AAV persist for only 1-2 months, while in non-dividing cells such as in heart, the expression is long-term.

To reduce the expression of the exogenous genes in non-target tissue, French and colleagues recently described an AAV vector system employing the cardiac troponin T promoter (cTnT) (Prasad et al., 2011). AAV-9 mediated gene expression from the cTnT promoter was 640-fold greater in heart compared to liver in one week old mice. However, this transcription targeting with tissue-restricted promoters did not reduce virus accumulation or gene transfer to off-target tissue. Only the expression of the off-target gene was significantly reduced, thus deleterious side effects of viral accumulation in peripheral tissue (liver) could still persist.

These earlier successes in the rodent models provided the impetus for investigators to test the various AAV serotypes in larger animals (canine, swine). Both the minimally invasive systemic delivery approach and the invasive catheter-based coronary perfusion approach were examined. The minimally invasive approach did not yield the high level of gene transfer observed for the rodent models (Wasala et al., 2011). In newborn dogs, Yue delivered AAV via a systemic vein injection and assessed gene transfer in the heart (Yue 2008). The AAV-9 serotype resulted in minimal transduction of the heart yet significant transduction of skeletal muscle. Similarily, Bish delivered AAV-6,8, and 9 to dogs via a relatively non-invasive percutaneous transendocardial delivery approach and observed poor gene transfer to the heart with AAV 8 and 9 (Bish et al., 2008). Alternatively, gene transfer to the heart was significantly improved when the AAV was delivered by the more invasive catheter-based coronary perfusion approach. In swine, Kasper tested direct intracoronary delivery of AAV-2 and observed 8 weeks expression with no inflammatory response (Kasper et al., 2005). Similarily, Raake reported long-term expression via coronary retro-infusion using AAV-6 in heart, though transfection was also detected in liver and lung (Raake et al., 2008). In a swine model of heart failure, Hardri reported that intracoronary delivery of SERCA2a, via AAV-1, significantly improved systolic function and coronary blood flow (Hardri et al., 2010). Thus, while the AAV approach provides for long-term expression of targeted genes to the heart in the large animal models, it still requires the more invasive catheter-based approach for delivery to achieve adequate transgene efficiency. Even with the catheter-based approach, the accumulation of viral particles and transfer of genes to non-target tissue persist if the delivery is not contained within the heart.

In large animals, the highest efficiency of cardiac-specific gene transfer by viral delivery has been achieved using a closed-loop coronary recirculation strategy. White et al reported a novel surgical procedure (MCARD) that allowed for closed recirculation of AAV vectors in the cardiac circulation using cardiopulmonary bypass in sheep (White et al., 2011). This approach resulted in highly efficient, cardiac specific gene transfer using the scAAV6 vector. Similarily, Kaye and colleagues have reported a recirculation approach (V-Focus) with moderate results (Kaye et al., 2009). Coronary venous blood containing the viral vectors was recaptured from the coronary sinus with the use of a percutaneously positioned occlusive balloon recovery catheter. The captured blood, containing the viral vectors, was re-oxygenated, and returned directly to the left coronary territory via a non-occlusive catheter placed percutaneously in the left main coronary artery. At the conclusion of the recirculation period, blood continued to be removed from the coronary sinus for a short time interval to capture any remove and unsequestered virus, thereby minimizing gene transfer to non-target tissue.

### 2.3 The pro's and con's of the adenovirus versus adeno-associated virus

Below, we provide a summary of some of the key advantages and disadvantages of the adenovirus vs adeno-associated vector used to deliver exogenous genes to the whole heart, in vivo, via the different delivery approaches.

- While the catheter-based delivery of adenovirus requires a surgically invasive approach in rodent, compared to the non-invasive delivery of AAV (ie., delivery by simple tail vein injection), the catheter-based approach can be cardiac specific. That is, after the Adv virus is delivered to the heart via the catheter-based cross-clamp approach,

unsequestered virus can be flushed from the heart before releasing the cross-clamp. Co-infection of peripheral tissue is therefore minimal. With tail vein delivery of AAV there is still co-infection of peripheral organs (liver, skeletal muscle).

- While the delivery of AAV via the tail vein in rodent models is minimally invasive, delivery of AAV in larger animals and humans still currently requires an invasive catheter-based closed-loop recirculated strategy. This is required to maximize the concentration of the virus delivered to the heart, while reducing viral accumulation and gene transfer to non-target tissue.
- An important distinction between the Adv and AAV approach relates to both how quickly and how long the delivered gene is overexpressed. The Adv_cDNA delivery provides maximum overexpresson of the protein within one week of gene transfer, compared to a gradual (1-3 mths) increase in expression via AAV_cDNA delivery. While the overexpression via Adv is relatively immediate, the overexpression is short term (2 weeks). Alternatively, protein overexpression is sustained for 3-12 months following AAV-cDNA delivery. Thus, the AAV-cDNA package provides chronic overexpression of targeted proteins, and Adv-cDNA provides acute overexpression.
- This important distinction between an acute or chronic expression predisposes the two viral packages for very different experimental applications. The immediate response of the Adv is ideal for experimental studies designed to examine the direct effects of overexpressing the targeted protein on specific biochemical, molecular, or functional responses. With AAV, any changes in biochemical, molecular, or cardiac function is in response to the gradual and long term overexpression of the targeted protein. It is difficult to define whether the observed changes with AAV_cDNA delivery are a direct response to the change in targeted protein, or if they are a secondary response due to whole organ or whole body adaptations.
- The adenovirus, Adv, is physically a much larger virus compared to the AAV. This is both good and bad. While the smaller size of the AAV enables the virus to cross the endothelial barrier with greater ease compared to the Adv, the smaller physical size of the AAV limits the length of the genome that can be packaged into the capsid. That insert capacity for the recombinant AAV is <5.2 kbases, whereas the insert capacity for the Adv is ~10 to 30 kb in length. The genome must include the inserting gene, a promoter, and a polyadenylation linker. The promoter can be as large as 1.5 kb and the polyA linker 1kb, leaving ~3.4 kb for inserting the gene in the AAV, and ~6.5 kb in the Adv. Thus, while the smaller physical size of the AAV is advantageous in terms of crossing the endothelial barrier, the larger Adv can deliver much larger genes. To circumvent the difficulty of crossing the barrier with the Adv, the use of vascular permeabilizing agents such as histamine, papaverine, substance P, low Ca2+ cardioplegia, and/or whole body cooling have been employed to relax the barrier and improve viral delivery.
- The Adv can be used to transfer exogenous genes to the heart multiple times. The AAV can only be used once due to an autoimmune response to the virus.
- The synthesis of the adenovirus-cDNA package is much easier than the AAV-cDNA package. The insertion of the cDNA into the Adv vector and the subsequent amplification of the virus is now routine, available thru multiple vendors and core facilities, and can be amplified in standard laboratories. Amplification via cell-to-cell transmission in HEK 293 cells with the Adv enables large-scale concentrations ($10^{13}$

vp/ml) and volumes. On the other hand, AAV production requires vector quantities which are not easily produced in laboratory or most research-grade vector core facilities. The most established methods for producing AAV use adherent HEK 293 cells chemically co-transfected with plasmids encoding the necessary virus proteins (Kotin, 2011). However, the absence of cell-to-cell transmission limits AAV production to cells initially transfected with plasmid DNA, thereby limiting the production titer and volume ($10^{10}$ vp/ml at best).

- The Adv induces an inflammatory response in heart after 4-5 days. The inflammatory response is minimal with AAV with the first treatment.
- While some of the serotypes of AAV do show specificity for heart, infection of non-target tissue presists. To improve cardiac specific expression of the protein, cardiac promoters are used instead of the ubiquitously strong CMV promoter (AAV9 with cardiac troponin T promoter cTnT; Prasad et al., 2011). While this does reduce the expression of the gene in non-target tissue, expression in the heart is also attenuated without the strong CMV promoter. Furthermore, while the expression of the gene in non-target tissue is reduced by using the cardiac-specific promoters, the accumulation of viral particles in non-target tissue (liver) is unchanged.

## 3. Two catheter-based methods for adenoviral gene delivery to rat heart, in vivo

### 3.1 The conventional catheter-based, cross-clamp method

The conventional open-chest cross-clamp approach has been described extensively in previous reports (Miyamoto et al., 2000; Del Monte et al., 2001, 2002). In brief, the chest was entered by a median sternotomy and a 22-gauge catheter containing 100 ml of adenoviral solution was advanced from the apex of the left ventricle to the aortic root. The aorta and pulmonary arteries were clamped distal to the site of the catheter and the viral solution (~$2x10^{12}$ vp/ml) was injected (high pressure). The clamp was maintained for 30 seconds. Only those hearts demonstrating the hallmark 'blanching', or whitening, during viral injection were maintained post-operatively for assessment of infection.

### 3.2 The catheter-based, retrograde perfusion method

Our advancement enables a completely isolated heart to be continuously retrograde perfused, in vivo, thereby (a) providing a blood-free pretreatment period with endothelial barrier relaxation agents (ie., calcium free Tyrode solution) prior to the delivery of the viral solution, and (b) enabling unsequestered virus to be flushed from the heart prior to releasing the cross-clamp, thereby eliminating gene transfer and viral accumulation in non-target organs (O'Donnell et al., 2005, 2006).

Figure 2 illustrates the scheme of the catheter-based approach for the retrograde perfusion of the isolated heart, in vivo. In brief, adult male Sprague–Dawley rats (350 g) were anesthetized (isoflurane), intubated, and placed on an ice pad to cool the core body temperature to 30°C. While the temperature lowered, the chest was opened from the right side at the second or third intercostal space. The pericardium was opened and 4–0 silk was sutured at the apex of the heart (see Figure 2). The suture was used to control handling / positioning of the heart. A 20-gauge catheter was inserted through the apex into the left

ventricle. The tip of the catheter was advanced to the aortic root. Placement of the tip was verified by observing the tip through the wall of the ascending aorta. The catheter was connected to tubing for delivery of perfusate solution and adenovirus via a peristaltic pump. The catheter was tied to the 4–0 suture that was placed at the apex of the heart. This kept the catheter from sliding out of position during the perfusion protocol.

The heart was externalized from the rib cage and all vessels leading to and from the heart (superior/inferior vena cava, pulmonary artery/vein, and ascending aorta) were occluded simultaneously with a single cross-clamp. The clamp was distal to the catheter positioned in the aortic root. A 24-gauge catheter was inserted into the right ventricle. This catheter provided a path for perfusate efflux from the heart. Next, the heart was retrograde perfused, in vivo, with well oxygenated calcium-free tyrode solution for 7.5 min via the catheter positioned in the aortic root. The perfusate flow rate was 3–30 ml/min, and the efflux was discarded. Following this permeability treatment phase with calcium free buffer, the perfusion was stopped and the adenovirus was injected thru the perfusion catheter (0.2 ml of AdV.cmv.SERCA1 in PBS; $10^{12}$ viral particles/ml). Excess solution dripped from the efflux catheter. This allowed the adenovirus to circulate down the coronaries. Next, the efflux catheter positioned in the right ventricle was removed, and an additional 0.5 ml/kg of adenovirus (~0.2 ml) was delivered to the aortic root (1 sec) at a peak pressure of 300 ± 100 mmHg. After 90 s, catheters were positioned in the right and left ventricles, and unsequestered virus was flushed from the heart with well oxygenated Krebs buffer containing calcium delivered through the perfusion line. This washout period with buffer containing $Ca^{2+}$ (1.5 mM) aided in contractile recovery. The chest was then closed, air was evacuated, and the body was warmed to 37°C. Once the animal could breath independently, the rat was moved to an oxygen chamber until it recovered from the anesthesia (1 h). After 2-3 days, hearts were excised for the analysis of SERCA expression (Western blots), functional measurements (in vivo and ex vivo), and metabolic measurement ($^{31}P$ and $^{13}C$ NMR experiments).

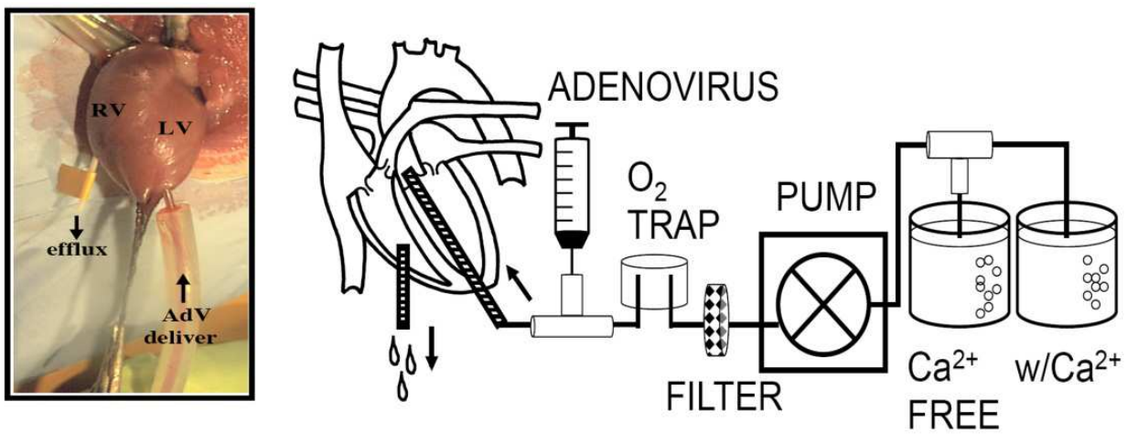


Fig. 2. Photo of a rat heart instrumented for catheter-based delivery of adenovirus into the aortic root. The hearts were completely isolated in vivo and retrograde perfused with calcium free Tyrode solution for 7.5 minutes before a single bolus of Adv.cmv.LacZ ( $10^{12}$ vp/ml in 400 ml PBS) was delivered into the aortic root. A catheter positioned in the aortic root provided the route for Adv delivery. A second catheter in the right ventricle provided a route for perfusate efflux during the retrograde perfusion period. (Reprinted from O'Donnell et al., 2006)

We noted several key methodological nuances that influenced the level of infection in the heart. First, during viral injection, no tension could be put on the heart. If the heart was stretched or compressed during injection of the virus, infection was focus to the atria and right ventricle, suggesting compromised coronary perfusion. In addition, the heart did not perfuse properly if the left ventricle filled with perfusate during the isolation. If the left ventricle appeared to hyperextend during perfusion, a second catheter was inserted into the left ventricle to reduce intraventricular pressure. We also noted an important limitation with the preparation of the virus. Viral amplifications taken out past five passages yielded the very high titers required; however, the expression of LacZ was reduced in the infected heart. This is consistent with the known rearrangement of the Ad5.LacZ vector at the sixth passage. For this reason, we always started with first-generation virus and stopped the amplification at the fifth passage.

*Survival rates.* The survival rate following this invasive procedure is on the order of 80-100% in healthy animals. It depends largely on the skill of the surgeon. This is clearly a non-trivial procedure. We find it takes 1-3 months of weekly practice for a previously untrained technician to master the skills required for any type of open-chest survival surgery (ie., aortic banding or catheter based viral_cDNA delivery). In addition, there is a risk that a novice surgeon can damage the aortic valve when the catheter is inserted through the apex of the heart and advance to the aortic root (as detected by echocardiograms). Survival rates for rats in early stages of decompensated left ventricular heart failure (10-12 weeks post banding) is >70% following the surgical procedure. Rats do not survive this procedure, or any open-chest surgical procedure, if performed at end-stage heart failure (>20 weeks post-banding).

### 3.3 Reporter gene considerations

Reporter genes are used to assess the efficiency of gene delivery by the various delivery approaches and delivery vectors. Two of the more common reporter genes include GFP and LacZ (β-Gal). GFP has not always been favored as a reporter gene for studies of gene transfer to the heart, in part because it can be difficult to distinguish authentic GFP fluorescence above endogenous background autofluorescene (Prasad et al., 2011). This is illustrated in Figure 3. This figure shows high regions of fluorescence which could be interpreted as areas of GFP expression. However, this is actually background autofluorescene from a section of untreated heart which had not been uniformly sliced and mounted on the slide.

The reporter gene, LacZ, is an attractive alternative. Following vector delivery of cDNA for LacZ, the efficiency of LacZ gene transfer is assessed after X-Gal staining of the whole heart or individual slices of the heart cross-section. Positive cells for Lac Z expression turn blue with X-Gal staining. In the cross-sectional preparations, the efficiency of gene transfer is calculated as the number of blue cells relative to the total number of cells. In whole heart staining, the heart is first fixed, stained, and digested (collagenase) by retrograde perfusion (O'Donnell et al., 2005, 2006). Then, the efficiency of gene transfer is measured in the isolated cell preparation as the fraction of blue cells expressing LacZ.

The LacZ approach does require careful interpretation of the results. First, some organs do have a low level of endogenous LacZ expression. It is not always clear if this endogenous

signal increases in response to the disease model or the surgical intervention of gene transfer. Therefore, it is important to confirm the relative change in endogenous signal in sham-operated animals and disease models without viral gene transfer. In the case of the aortic-banded pressure-overloaded rat model of cardiac hypertrophy, the change in endogenous LacZ signal is minimal in the heart (O'Donnell et al., 2008). Secondly, the expression of LacZ can be assessed by a colorimetric assay for the increase in blue signal from tissue samples. However, the colorimetric approach is not a direct measure of the efficiency of gene transfer. While it is a useful tool to measure a relative change in the level of LacZ expression, the actual RLU reading (relative light units) provides no insight for the number, or efficiency, of cells infected. Finally, care must be taken in reporting the efficiency of LacZ gene transfer by photographic illustration. As illustrated in Figure 3, an image which shows only a small region of heart for LacZ expression is not representative of the whole heart, and it does not properly reflect the efficiency of transfer. Nevertheless, when properly performed, the LacZ reporter gene is an excellent and widely used tool to assess and compare the efficiency of gene transfer by various delivery techniques.

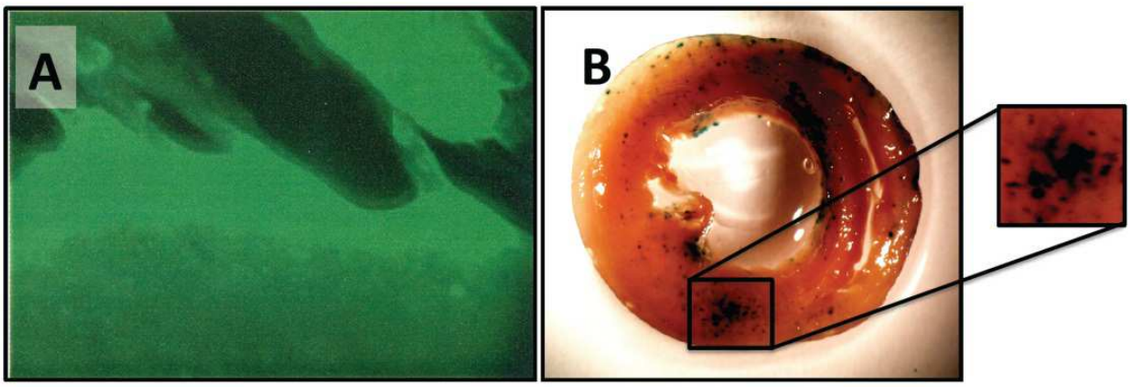


Fig. 3. Adenoviral gene transfer for the reporter gene (A) GFP and (B) LacZ in heart by the conventional cross-clamp technique. Interpretation of reporter gene data can often be misleading. While there is significant green fluorescence visible in a section of heart on the left, this figure is actually background fluorescence. The heart was not infected with Adv.cmv.GFP. This section of heart was not evenly sliced and mounted on the slide, thereby accounting for regions of greater GFP expression. The heart on the right was indeed infected with Adv.cmv.LacZ via the conventional cross-clamp approach. While the full cross-section indicates that the efficiency of transfer was low (ie., dark blue cells), the selected inset shown on the right inappropriately suggests that the transfection was quite good.

### 3.4 Efficiency of gene transfer in heart

Figure 4 shows the extent of LacZ expression, following X-Gal staining, through multiple cross-sections of a heart 72 h after Ad.cmv.LacZ delivery to the heart, in vivo. The heart was isolated *in vivo* and retrograde perfused for 7.5min with calcium-free Tyrode solution before delivery of a single bolus of PBS containing adenovirus (Ad.cmv.LacZ, 400 ml, $10^{12}$ pfu/ml in PBS). Transfected hearts were also digested (collagenase) after X-Gal staining, and the efficiency of gene transfer was assessed by blue cell counts. Importantly, this strategy resulted in significant and global gene transfer to the whole heart. The efficiency of gene transfer was 58 ± 11%.

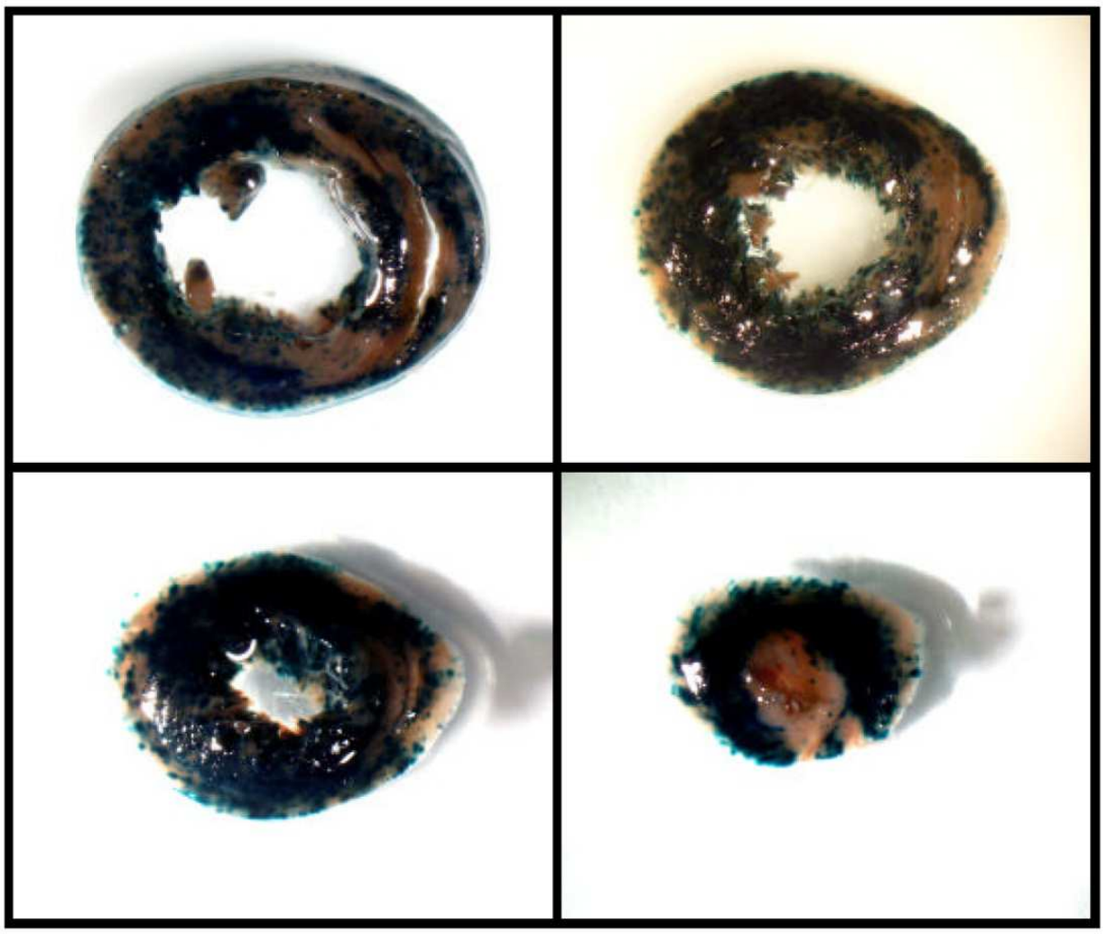

Fig. 4. Cross sections of heart stained (X-Gal) for LacZ expression following gene transfer *in vivo* by the isolated heart perfusion approach. The heart was completed isolated *in vivo* and retrograde perfused with calcium free Tyrode solution for 7.5 minutes before a single bolus of Adv.cmv.LacZ ( $10^{12}$ vp/ml in 400 ml PBS) was delivered into the aortic root. The blue regions demonstrate cells expressing the transferred gene. The efficiency of gene transfer to the heart was 58 ± 11 %. (Reprinted from O'Donnell et al., 2005)

### 3.5 Gene transfer to non-target organs

We assessed the level of Ad.cmv.LacZ infection in heart, liver, lung, and skeletal muscle based on a colorimetric RLU assay quantifying β-Gal activity. The intent was to determine if flushing unsequesterd virus from the heart, prior to removing the cross-clamp, reduced infection of peripheral tissue compared to the conventional cross-clamp approach. The results are shown in Figure 5. In liver, β-Gal activity was dramatically reduced compared to the conventional cross-clamp approach. Infection of lung and muscle was nearly undetectable. Conversely, the level of β-Gal activity in the heart (ie., post gene transfer) was 4–10 times greater by the isolated, retrograde perfusion approach compared to the conventional cross-clamp approach. The reduction in the infection of peripheral organs is consistent with having flushed all unsequestered virus from the heart prior to releasing the cross-clamp.

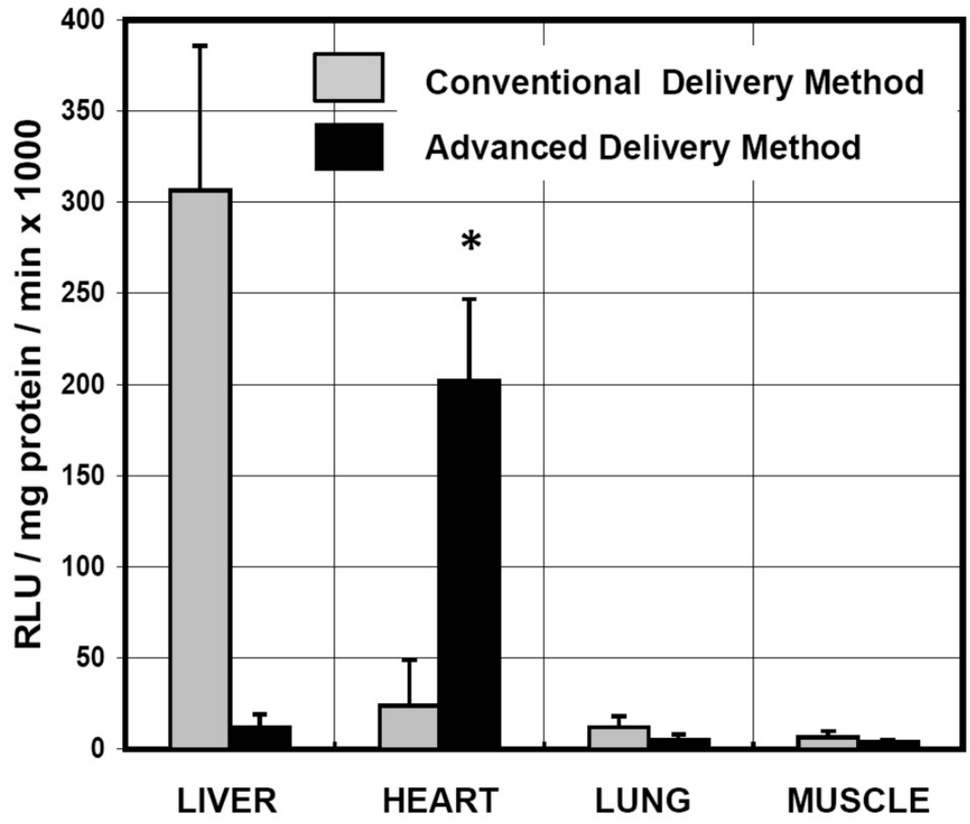


Fig. 5. The solid gray bars illustrate the level of in vivo gene transfer by a conventional aorta/pulmonary artery cross-clamp technique. Infection by our isolated, retrograde perfusion approach is shown in black. The conventional method results in significant infection of the liver and peripheral tissue. Our scheme results in significantly greater gene transfer to the heart while reducing liver infection dramatically. (Reprinted from O'Donnell et al., 2005)

## 4. SERCA gene therapy for heart failure: Good or bad?

The changes in intracellular Ca2+ handling reported for the failing myocardium have been linked to the sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) (Gwathmey et al., 1987). Calcium uptake into the sarcoplasmic reticulum (SR) by SERCA determines the rate of calcium removal for relaxation, the SR calcium content, and calcium released for contractions (Periasamy et al., 2001). In the failing heart, it has been proposed that SERCA contributes to reduced contractility and relaxation as the activity and number of these calcium transporters are reduced and calcium transients are blunted and slow to decay (Gwathmey et al., 1987; Chang et al., 1996; Miyamoto et al., 2000).

There is overwhelming evidence that the overexpression of SERCA2a in the failing myocardium from both animal models and humans leads to enhance cardiac function and improved survival (review Gwathmey et al., 2011). Surprisingly, we found the overexpression of the SERCA1 isoform actually compromised cardiac performance, in vivo (O'Donnell et al., 2008). Our finding was recently supported by a second report (heart failure model in SERCA2a transgenic mice) (Pinz et al., 2011). These opposing results raise concerns regarding the safety of the current translational clinical studies in human (Hajjar et

al., 2008; Jaski et al., 2009; Gwathmey et al., 2011). Here, we will briefly highlight the progression of the earlier studies in whole heart and isolated cardiomyocytes which support the therapeutic strategy. We will discuss the opposing results from our study and the recent transgenic mouse study, and we will provide some considerations which may account for the discrepany between reports. The controversy of this therapeutic strategy as it relates to the stage and model of the disease is also discussed in a recent commentary by Sipido and Bangheluwe (Sipido et al., 2010).

### 4.1 Positive outcome with SERCA in isolated cardiomyocytes from human

Indeed, a number of groups have reported that the overexpression of either the isoform for SERCA2a or SERCA1 in isolated cardiomyocytes lead to enhanced cell shortening, and accelerated uptake of calcium by the sarcoplasmic reticulum. In non-treated myocytes isolated from patients with end-stage heart failure, the calcium transient is blunted and the sequestration of calcium slowed (Gwathmey et al., 1987). If this is truly a maladaptive alternation, and not compensatory, then it is reasonable to hypothesized that increasing the rate of calcium sequestration may enhance the relaxation rate. Indeed, Del Monte et al demonstrated that the transfer of SERCA2a cDNA into isolated cardiomyocytes from failing human hearts restored contractile function (Del Monte et al., 1999). Similarily, Weisser-Thomas provided further evidence that the overexpression of the SERCA1 isoform in isolated cardiomyocytes from patients in heart failure also had beneficial effects (Weisser-Thomas et al., 2005).

### 4.2 Positive outcome with SERCA2a in whole heart models of heart failure

The encouraging results from the isolated cardiomyocyte studies provided the impetus to examine the effects in animal models of heart failure. Hajjar subsequently developed the catheter-based cross-clamp approach for the delivery of genes to rat heart in vivo (Del Monte et al., 2002). His group demonstrated that the overexpression of the SERCA2a isoform in the aortic-banded rat model of left-ventricular function improved cardiac function and survival (Miyamoto et al., 2000; Del Monte et al., 2001). The strategy was then performed in a larger animal model (sheep) with heart failure induced by fast pacing (Byrne et al., 2008). Results were similarly encouraging. These early studies in animals models of cardiac disease have now been translated to clinical trials, and results from recent early phase clinical trials in humans find fewer episodic events and improvement in multiple end-points (such as left ventricular end-systolic volume, walking tests, oxygen consumption) with no adverse events, following delivery of SERCA2a via AAV-1 via a catheter based approach (Hajjar et al., 2008; Jaski et al., 2009; Gwathmey et al., 2011). However, LV ejection fraction did not increase appreciably with any of the three AAV_SERCA2a doses examined (Jessup et al., 2011). Two other clinical trials targeting SERCA2a are currently enrolling patients. The trials are being conducted in the United Kingdom and the Institut of Cardiology Pitié-Salpêtrière, Paris, France, with the primary objective to investigate the impact of AAV6- CMV-SERCA2a on cardiac remodeling parameters in patients with severe heart failure.

### 4.3 Negative outcome with SERCA1a in whole heart models of heart failure

In our study, Ad.cmv.LacZ, Ad.cmv.SERCA1, or PBS was delivered, in vivo, to aortic banded (10-12 wks) and sham operated Sprague Dawley rats by our catheter-based perfusion technique described above. Compared to the cardiac isoform (SERCA2a) SERCA1 is not

regulated by phospholamban, and has a higher activity with a two-fold greater calcium uptake relative to SERCA2a. SERCA1 is also more resistant to oxidative stress and acidosis.

The subset of banded and sham rats receiving Adv.cmv.LacZ ($10^{12}$ viral particles/ml), were used here to confirm the efficiency of gene transfer in the disease model. X-Gal staining for LacZ gene transfer revealed significant and heterogeneously expression throughout both the sham and banded hearts 48-72 hrs following Ad.cmv.LacZ delivery, similar to the level of overexpression illustrated in figure 4. Importantly, there were no "false positives" for endogenous LacZ expression in hearts as a consequence of microinfarctions formed during the intracoronary infusion as previously speculated by Wright et al (Wright et al., 1998).

Western blot analysis for SERCA2a, SERCA1, and calsequestrin are shown in Figure 6 for sham and banded hearts receiving Ad.cmv.SERCA1. In the 10-12 week banded and sham groups receiving the PBS vehicle without adenovirus, SERCA2a expression was similar between groups. SERCA2a levels are not expected to drop until late-stage failure in the rat model (Arai et al., 1996). Importantly, in the banded and sham groups receiving the Adv.cmv.SERCA1, SERCA1 protein was significantly overexpressed. Densitometry analysis of Coomassie blue stained gels indicated that the total SERCA content increased by 34 ± 15% in both banded and sham hearts, and endogenous SERCA2a expression levels were unaffected. Indeed, this level of expression is lower than the level reported for the SERCA1 transgenic mouse model and our own earlier cardiomyocyte data (Cavagna et al., 2000; Loukianov et al., 1998). In transgenic mice expressing SERCA1, total SERCA levels increased by 2.5-fold and endogenous SERCA2a expression were reduced by 50%(Huke et al., 2002; Lalli et al., 2001; Loukianov et al., 1998). In cardiomyocytes, total SERCA levels increased by 3 fold and SERCA2a dropped by 50%. The higher level of gene transfer in isolated cardiomyocytes is consistent with a much higher multiplicity of infection (MOI). In cardiomyocyte the MOI is typically 2 to 10 vp/cell (O'Donnell et al., 2001). In the intact heart, the MOI is typically <0.5 at best (ie., 50% efficiency) (O'Donnell et al., 2008). Nevertheless, a contractile response was still elicited both in vivo and ex vivo following the catheter-based delivery of SERCA1 to whole hearts.

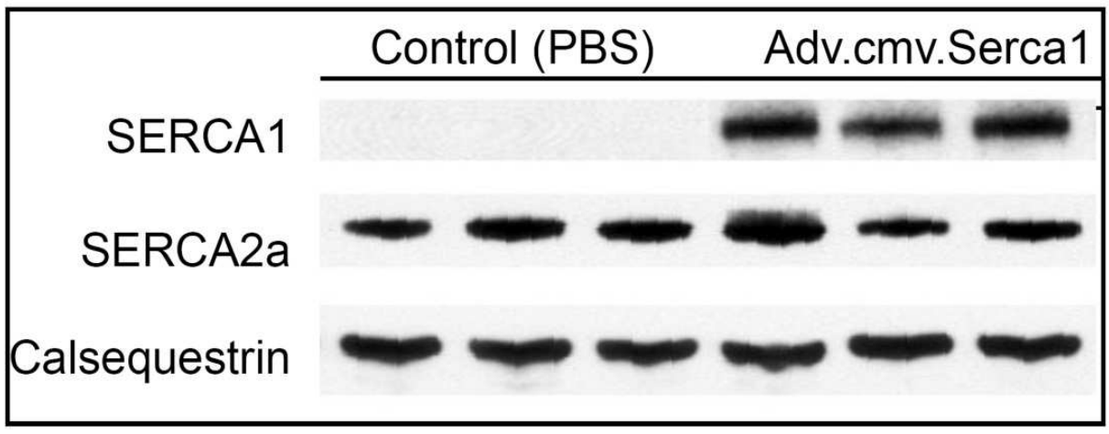


Fig. 6. Western blot data for SERCA1, SERCA2a, and calsequestrin expression following Adv.cmv.SERCA1 gene transfer or PBS (control) in rat heart. Densitometry analysis of Coomassie blue stained gels indicated that the total SERCA content increased by 34 ± 15% with adenoviral transfer of SERCA1 cDNA. Endogenous SERCA2a expression levels were unaffected. (Reprinted from O'Donnell et al., 2009)

*Hemodynmic consequences of SERCA1 overexpression.* The echocardiographic measurements made 48–72 h after PBS delivery or adenoviral gene delivery of SERCA1 cDNA in both sham and banded LVH rat hearts are listed in Table 1. There were no significant differences between sham groups receiving either catheter-based delivery of PBS or Ad.cmv.SERCA1. LV dimensions were similar, as were LV wall thickness and fractional shortening. After 10 wk of aortic banding, LVH_PBS animals showed echocardiographic signs of LVH relative to the SHM_PBS group, as expected, including a significant increase in the LV systolic dimension and a decrease in fractional shortening. Importantly, these parameters were not reverse with SERCA1 treatment. LV diastolic and systolic dimensions actually increased 10–15%, whereas fractional shortening was reduced by an additional 17%. This change indicates further LV remodeling with SERCA1 expression in failing hearts in this study. A decrease in the LV posterior wall systole dimension (LVPWS) in the SERCA1 treated LVH group (LVH_SR1) was also measured, and is consistent with further dilation. Importantly, this data for the overexpression of SERCA1 challenges the existing hemodynamic / functional data for the overexpression of SERCA2a in this rat model of left ventricular hypertrophy (Miyamoto et al., 2000; Del Monte et al., 2001). Whether this is linked to the specific isoform of SERCA is unclear, and it raises a cautionary flag regarding the current clinical trails.

| | Heart/body (g/kg) | Heart rate (bpm) | LVDd (mm) | LVSd (mm) | FS (%) | Vcf (circ/s) | LVPWD (mm) | LVPWS (mm) |
|---|---|---|---|---|---|---|---|---|
| SHM_PBS (n=4) | 6.20 | 335 ± 25 | 8.2 ± 0.9 | 4.8 ± 0.4 | 40.6 ± 5.7 | 0.027 ± 0.005 | 1.3 ± 0.4 | 2.4 ± 0.3 |
| SHM_SR1 (n=7) | 6.19 | 334 ± 36 | 8.5 ± 0.8 | 5.2 ± 0.9 | 38.9 ± 5.6 | 0.026 ± 0.003 | 1.2 ± 0.2 | 2.2 ± 0.3 |
| LVH_PBS (n=6) | 9.22* | 332 ± 20 | 8.5 ± 0.1 | 5.9 ± 0.1* | 30.5 ± 4.2* | 0.025 ± 0.003 | 1.4 ± 0.1 | 2.3 ± 0.1 |
| LVH_SR1 (n=7) | 9.25† | 320 ± 27 | 9.1 ± 0.1† | 6.8 ± 0.1†§ | 25.3 ± 5.3† | 0.020 ± 0.004§ | 1.3 ± 0.1 | 2.0 ± 0.1 |

Table 1. LVDd indicates LV diastole dimension; LVSd, LV systole dimension; FS, fractional shortening; Vcf, velocity of circumferential shortening; LVPWD, LV posterior wall diastole dimension; LVPWS, LV posterior wall systole dimension. *P<0.05 LVH_PBS vs SHM_PBS or SHM_SR1; †P<0.05 LVH_SR1 vs SHM_PBS or SHM_SR1; §P<0.05 LVH_SR1 vs LVH_PBS. (Reprinted from O'Donnell et al., 2008)

Following the in vivo echocardiography measurements, hearts were excised, retrograde perfused, and both cardiac function and metabolic activity were assessed. The ex vivo data revealed a very different functional response to SERCA1 overexpression compared to in vivo data. In excised SHM hearts overexpressing SERCA1, there was a modest increase in RPP yet a profound positive impact on contractility rates (±dP/d*t*). HR was unaffected. The accelerated relaxation (-dP/d*t*) is consistent with a faster removal of calcium from the cytosol as the number of SERCA pumps are overexpressed. These results compared favorably with functional data reported for isolated, work-performing hearts from SERCA1 transgenic mice (Huke et al., 2002). Huke and colleagues also reported a 20% increase in LVDP, whereas □±dP/d*t* nearly doubled, and HR was unchanged. However, much like our own in vivo data, they did not find dramatic changes in vivo (Huke et al., 2002). They suggested that the differences between *ex vivo* and *in vivo* data reflect the presence of *in vivo* compensatory mechanisms that "normalize" cardiac function in transgenic mice.

For the excised LVH hearts overexpressing SERCA1, RPP recovered to normal levels at baseline. However, the relaxation rate (-dP/d*t*) and LVDP did not improve with treatment. Instead, the improvement in the RPP was linked to a 20% increase in HR. These hearts were not paced. This finding was unexpected and suggests a physiological link between SERCA function and HR regulation. It is unclear to what extent SERCA influences the electrical excitability and repolarization of cardiomyocytes or potentially the neurons that innervate the heart. It was also unexpected that the overexpression of SERCA1 in the LVH group did not lead to enhanced LVDP or relaxation rates in isolated hearts. We originally hypothesized that the overexpression of SERCA1 would lead to a faster calcium uptake by the SR, thereby accelerating relaxation and potentiating LVDP as seen in SERCA1 overexpressing shams. However, our finding is more consistent with the hypothesis that calcium sequestration by the SR may not be the limiting factor affecting LVDP and relaxation in the failing heart.

*Experimental summary.* Although previous cardiomyocyte data and non- hypertrophic transgenic mouse models have revealed important and positive functional and metabolic responses to SERCA1 isoform overexpression, this study extended those earlier findings to include the intact functioning hypertrophic heart expressing SERCA1 under both in vivo and ex vivo conditions. In support of previous work, we found that the overexpression of SERCA1 in isolated heart preparations resulted in enhanced function and sustained energy potentials. At higher workloads, SERCA1 expression influenced energy metabolism by increasing glucose oxidation, thereby potentially making overall energy production more efficient. However, under the fully loaded in vivo condition, function was not affected in healthy hearts overexpressing SERCA1 at basal workloads, and hypertrophic hearts revealed depressed function with SERCA1 expression. Further investigation is required to determine if this is a consequence of whole body compensatory mechanisms or a limitation in the competitive handling of calcium between the SR versus myofilaments.

### 4.4 Negative outcome with SERCA2a transgenic mice in heart failure

Pinz examined the effects of SERCA2a overexpresion on cardiac performance and energetic costs in left ventricular hypertrophy transgenic mouse model (Pinz et al., 2011). Mouse hearts were isolated and perfused from wild-type and transgenic mice overexpressing the cardiac isoform of SERCA2a, 8 weeks after ascending aortic-banding (left ventricular hypertrophy). SERCA2a mRNA and protein levels were decreased more than 50% in banded wild-type hearts compared to wild-type shams. The expression of SERCA2a mRNA and protein levels in the transgenic hypertrophic group was normalized compare to the wild-type hearts. They found that overexpressing SERCA2a enhanced myocardial contraction and relaxation in normal transgenic mouse hearts during inotropic stimulation with isoproterenol, and energy comsumption was proportionate to contractile function. However, the increased amount of SERCA2a in hypertrophied hearts was not sufficient to support or increase contractile function of these hearts above the level achieved by hypertrophied wild-type hearts. These results indicated that the positive effect of overexpressing SERCA2a on myocardial contractiliy was not maintined in hypertrophic hearts. Furthermore, despite the finding of greater energy efficency with higher levels of SERCA2a in normal transgenic hearts, they did not find a beneficial energetic effect in hypertrophied hearts overexpressing SERCA2a. Instead, they observed a downward shift in the relationship between contractile force and free energy available from ATP hydrolysis in hypertrophied transgenic hearts when

compared with sham transgenics, suggesting that energy supply may be a limiting factor for the benefit of SERCA2a overexpression in hypertrophied hearts. Taken together, they concluded that the strategy of increasing SERCA activity may not be effective in all models and/or stages of cardiac dysfunction (Pinz et al., 2011).

### 4.5 Potential mechanisms for the discrepancies between reports

#### 4.5.1 Dose-response effects

Teucher and colleaques examined the effects of different levels of SERCA1a expression on contractility and $Ca^{2+}$ cycling in isolated cardiomyocytes in heart of rabbit transfected at different multiplicities of infection (Teucher et al., 2004). They examined whether increased SERCA1a expression levels enhanced myocyte contractility in a gene-dose-dependent manner. At a MOI 10 vp/cell, myocytes expressing SERCA1a (versus Ad-LacZ controls) revealed enhanced SR $Ca^{2+}$ uptake, relaxation rates, SR $Ca^{2+}$ content, isotonic shortening, and $Ca^{2+}$ transient amplitude. At higher SERCA expression levels (MOI 50), myocytes exhibited further increases in SR $Ca^{2+}$ uptake, relaxation rate, and SR $Ca^{2+}$ content but showed depressed contraction amplitude and no Ca2+ transient enhancement versus control. They concluded that high SERCA activity causes a paradoxical decrease in contractile activation because of greater Ca2+ removal from the cytosol, and that the use of SERCA1a for gene therapy in heart failure requires careful control of transfection efficiency and induced expression levels.

#### 4.5.2 Maladaptive myofilament effects

In our study described above, it was unexpected that the overexpression of SERCA1 in the LVH group did not lead to enhanced LVDP or relaxation rates in isolated hearts. We originally hypothesized that the overexpression of SERCA1 would lead to a faster calcium uptake by the SR, thereby accelerating relaxation and potentiating LVDP. Our finding is more consistent with the hypothesis that calcium sequestration by the SR may not be the limiting factor affecting LVDP and relaxation in the failing heart (Janssen et al., 2002; Perez et al., 1999). In brief, if reuptake of Ca2 becomes too fast, the cytoplasmic calcium concentration near the myofilaments will decline rapidly, preventing the appropriate activation of myofilaments and hindering adequate force development (Janssen et al., 2002; Teucher et al., 2004). Thus, the SR competes with troponin C for calcium binding (Hiranandani et al., 2007; Janssen et al., 2002; Loukianov et al., 1998; Teucher et al., 2004). In heart failure, the calcium sensitivity of myofilaments is altered in both human and rodent models (Marston et al., 2008). A potential adverse effect of increasing calcium sensitivity is slowed relaxation and diastolic dysfunction (MacGowan, 2005). If myofilament properties are limiting the relaxation rate and force development in heart failure, the overexpression of SERCA would be competitive and deleterious.

In addition, the majority of studies that concluded that calcium sequestration limited the relaxation rate were performed in isolated cardiomyocytes. Under such unloaded conditions, cross-bridges cycle much faster than loaded cross-bridges, and the sequestration rate by the SR may indeed limit cytosolic calcium decline (Janssen et al., 2007). However, under loaded conditions, relaxation of the myocardium may be more closely linked to myofilment properties (Janssen et al., 2002, 2007). Indeed, Teucher and colleagues (2004)

also reported that moderate SERCA1 gene transfer and expression improved contractility and $Ca^{2+}$ cycling in cardiomyocytes. However, higher SERCA1 expression levels impaired myocyte shortening because of higher SERCA activity and $Ca^{2+}$ buffering. Vangheluwe et al. (2006) also demonstrated that replacement of the SERCA2a isoform with SERCA2b, an isoform with increased $Ca^{2+}$ affinity, led to severe cardiac hypertrophy, stress intolerance, and a reduced life span in transgenic mice.

### 4.5.3 Animal model effects (ie., early vs late stage heart failure)

The discrepancy between the results of our study and the early rat studies may be related to the stage of heart failure model examined. In the earlier work, the effects of SERCA2a overexpression were examined in the aortic-banded rat model at a late stage of heart failure (Miyamoto et al., 2000; Del Monte et al., 2001). At this late stage of decompensated heart failure, endogenous SERCA2a content is significantly reduced in untreated hearts, and the overexpression treatment normalized SERCA2a content and reportedly had beneficial effects on cardiac function. Alternatively, we examined the consequences of SERCA overexpression at an earlier deompensated stage (10-12 weeks post-banding) of the disease in rat (O'Donnell et al., 2008). At this stage, endogenous SERCA2a expression was not reduced, and the overexpression of SERCA was deleterious. However, this argument is not supported by the SERCA2a transgenic mouse model of heart failure (Pinz et al., 2011). The transgenic mouse study was also performed at a stage of disease progression where SERCA2a mRNA and protein levels were reduced by 50%, consistent with the earlier rat study. Unlike the rat studies, normalization of protein levels in the transgenic heart failure group did not improve cardiac performance. Therefore, the opposing results of the earlier SERCA2a studies in the rat compared to our study (and transgenic mouse) study remains unresolved.

### 4.5.4 Problems selecting a proper control gene

There is no ideal vector to use as a control group. The control groups could receive an empty adenovirus (which is not actually empty), a bolus of PBS, or adenovirus carrying cDNA for GFP, LacZ, or scrambled cDNA. With any of the adenoviral packages, there is foreign DNA inserted into the cells, and the cells will need to deal with this foreign nucleotide and subsequent protein expressed. Our group, and others (Weisser-Thomas et al., 2005) have noted that these adenoviral "controls" do affect function relative to non-treated tissue (ie, PBS treatment). Therefore, it is not always clear if the gene of interest (ie., SERCA2a) altered baseline cardiac function, or if the control group (ie., GFP, scrambled cDNA, etc) altered baseline function. The earlier studies used βGal cDNA as the control gene in their studies (Del Monte et al., 2001), and our group used a virus free PBS control group.

## 5. Conclusions

There were an overwhelming number of laboratories examining the new and exciting adenoviral gene therapy strategies in the 1990's. With any new breakthrough, a number of hurdles emerged and the immediate reality of the approach did not live up to the promise. Most groups moved onto the next great thing (ie., stem cells). Some groups persevered with the viral approach, and focused their efforts on resolving the limitations. Today, we have a

far greater understanding of this approach, and we are closer to the promise of this application in human. The current state-of-art approach for human application includes the use of AAV vectors delivered to the heart in a catheter-based, isolated, recirculation path. The AAV vector provides for long-term protein overexpression with minimal inflammatory or toxic effects. A number of different AAV serotypes and gene promoters are now being examined to improve targeting the gene delivery to the heart with the hope of one day simply injecting the viral package into the circulatory system. Until then, the catheter-based delivery of the viral package to the heart is required to optimize gene transfer to the heart, while reducing the accumulation of viral particles and gene transfer to non-target organs in human.

Not only is the development of this novel technology important for treatment of heart disease in humans, it is also a powerful tool for elucidating the molecular basis of cardiovascular diseases. Indeed, the results of these basic science studies in rat and mouse steer us toward the proper gene selection for disease treatments in humans. For these studies, the delivery of the exogenous genes to a completely isolated heart, in vivo, via a catheter-based approach, is required to maximize gene transfer in heart and eliminate viral accumulation and gene transfer to non-target tissue. Unlike the human treatment strategies with the AAV, we argue that the adenoviral vector Adv still provides considerable advantages for molecular based studies. The Adv vector is easy to synthesize in any lab, it provides very robust gene transfer compared to AAV, large genes can be packaged in the virus, and the overexpression of the transfer gene is maximal within 3-4 days (prior to the inflammatory response). As opposed to the chronic AAV and transgenic models, this acute response enables the scientist to directly assess the affects of protein overexpression (or silencing) on molecular processes, cardiac function, and survival.

## 6. Acknowledgement

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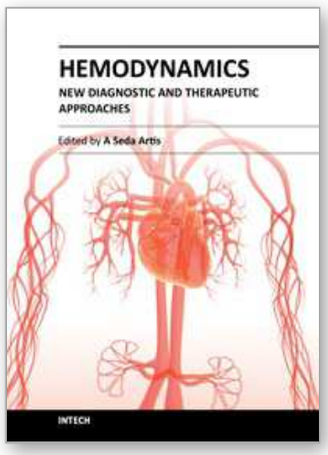


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Hemodynamics is study of the mechanical and physiologic properties controlling blood pressure and flow through the body. The factors influencing hemodynamics are complex and extensive. In addition to systemic hemodynamic alterations, microvascular alterations are frequently observed in critically ill patients. The book "Hemodynamics: New Diagnostic and Therapeuric Approaches" is formed to present the up-to-date research under the scope of hemodynamics by scientists from different backgrounds.

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