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Neonatal Gene Transfer of Serca2a Delays Onset of Hypertrophic Remodeling and Improves Function in Familial Hypertrophic Cardiomyopathy

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Abstract

Familial Hypertrophic Cardiomyopathy (FHC) is an autosomal dominant genetic disorder linked to numerous mutations in the sarcomeric proteins. The clinical presentation of FHC is highly variable, but it is a major cause of sudden cardiac death in young adults with no specific treatments. We tested the hypothesis that early intervention in Ca²⁺ regulation may prevent pathological hypertrophy and improve cardiac function in a FHC displaying increased myofilament sensitivity to Ca²⁺ and diastolic dysfunction. A transgenic (TG) mouse model of FHC with a mutation in tropomyosin at position 180 was employed. Adenoviral-Serca2a (Ad.Ser) was injected into the left ventricle of one day old non-transgenic (NTG) and TG mice. Ad.LacZ was injected as a control. Serca2a protein expression was significantly increased in NTG and TG hearts injected with Ad.Ser for up to 6 weeks. Compared to TG-Ad.LacZ hearts, the TG-Ad.Ser hearts showed improved whole heart morphology. Moreover, there was a significant decline in ANF and β -MHC expression. Developed force in isolated papillary muscle from 2–3 week old TG-Ad.Ser hearts was higher and the response to isoproterenol (ISO) improved compared to TG-Ad.LacZ muscles. *In situ* hemodynamic measurements showed that by 3 months the TG-Ad.Ser hearts also had a significantly improved response to ISO compared to TG-Ad.LacZ hearts. The present study strongly suggests that Serca2a expression should be considered

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DISCLOSURES

The authors declare that they have no competing financial interests.

as a potential target for gene therapy in FHC. Moreover, our data imply that development of FHC can be successfully delayed if therapies are started shortly after birth.

Keywords

Familial Hypertrophic Cardiomyopathy; Serca2; treatment; gene therapy

INTRODUCTION

Familial Hypertrophic Cardiomyopathy (FHC) is an autosomal dominant genetic disorder largely expressed in genes encoding sarcomeric proteins. The clinical presentation of FHC is highly variable, but is a major cause of sudden cardiac death in young adults [1,2]. Although linkage of FHC to sarcomeric genes is well established, the clinical course is not well defined and therapeutic approaches are lacking. Currently patients with FHC are being treated with conventional drugs for heart failure (HF) at the onset of clinical symptoms [3]. Further understanding of FHC progression and molecular mechanisms may lead to improve treatments or prevention.

Animal models of FHC provide an opportunity not only for studying important human mutations and understanding molecular mechanisms involved in the disease process but also provide important insights into possible prevention and treatment. Semsarian *et al* [4] reported that the Ca^{2+} channel blocker, diltiazem, prevented cardiomyopathy in $\alpha\text{MHC}^{403/+}$ mice. In the same mouse model the HMG-CoA reductase inhibitor, simvastatin, induced regression of cardiac hypertrophy and fibrosis and improved cardiac function [5]. Treatment of another mouse model linked to FHC (TnT-Q92 mice) with losartan, an Angiotensin II blocker, reversed interstitial fibrosis and the expression of collagen 1 α (1) and transforming growth factor- β 1 [6]. We have previously reported that the hypertrophic phenotype in a well-characterized transgenic (TG) mouse model of FHC with a mutation in tropomyosin (Tm) at position 180. (Tm180) (Glu180Gly) with diastolic dysfunction, which displayed increased myofilament Ca^{2+} sensitivity, can be rescued by cross-breeding with a chimeric Tm that exhibits decreased myofilament sensitivity to Ca^{2+} [7]. Moreover, Coutu *et al.* [8] have reported that increased expression of parvalbumin, a soluble Ca^{2+} -binding protein, corrects the slower relaxation in adult cardiac myocytes expressing Tm180.

In studies reported here we have tested the hypothesis that early intervention in Ca^{2+} regulation may prevent pathological hypertrophy and improve cardiac function in an FHC that is associated with increased myofilament sensitivity to Ca^{2+} and diastolic dysfunction. We employed a TG mouse model of FHC with a mutation in Tm at position 180 [9–11]. Similar to other FHC mutations, mice expressing the mutant Tm180 show progressive development of cardiac hypertrophy with atria being the most affected, ventricular fibrosis and diastolic dysfunction associated with increased myofilament Ca^{2+} sensitivity [9]. Our approach involved delivery of a single dose of Serca2a using adenoviral vector.

Although transient expression of exogenous Serca2a using adenoviral techniques in different animal models of HF has been previously reported [12,13], to the best of our knowledge no studies have reported neonatal gene transfer of Serca2a to delay or prevent the development of hypertrophy and to improve whole heart function in FHC. Here we report molecular, biochemical and functional data showing that the delivery of a single dose of Serca2a in one day old mice is sufficient to alter hypertrophic gene expression, improve whole heart morphology and the response to β -adrenergic stimulation in the FHC Tm180 mouse model.

METHODS

Recombinant adenoviruses

First-generation type 5 recombinant human adenovirus was used in these studies to deliver β -galactosidase (Ad.LacZ) and rabbit Serca2a (Ad.Ser) as previously described.[12] The Ad.LacZ and Ad.Ser vectors were under control of the cytomegalovirus (CMV) and α -myosin light chain (α -MLC) promoters, respectively [14].

Adenovirus-mediated gene transfer to the postnatal mouse heart *in vivo*

Adenoviral gene transfer to one day old mice was performed as described by Christensen *et al* [15]. TG and NTG mice were initially injected with either Ad.LacZ (NTG-Ad.LacZ, TG-Ad.LacZ), saline (NTG-saline, TG-saline) or not injected (NTG, TG). At the age of 3 months we performed *in situ* measurements and compared hemodynamic parameters. Since we did not see any differences among NTG (NTG-Ad.LacZ, NTG-saline and NTG) or TG (TG-Ad.LacZ, TG-saline and TG) groups, for future experiments we used NTG and TG mice injected with either Ad.Ser or Ad.LacZ. All experiments were conducted and care of animals provided in compliance with animal care policies according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985) and approved by the Institutional Animal Review Board of the University of Illinois at Chicago.

Whole mount X-gal histochemistry of Ad.LacZ (β -gal) hearts

Ad.LacZ infected hearts were dissected out of two day old mice. The hearts were washed 3 $\times$ with ice cold PBS and fixed with 2% formaldehyde and .2 % glutaraldehyde in PBS for one hour at 4°C. The hearts were rinsed 3 $\times$ with PBS and stained overnight at 37°C with X-gal. The hearts were rinsed 3 $\times$ with PBS and visualized for LacZ expression.

Quantitative RT-PCR

To determine the level of expression of the Serca2a, PLB, β -MHC, α -MHC, ANF and GAPDH genes, real-time one-step quantitative RT-PCR with SYBR Green detection (Qiagen, Valencia, CA) was performed using the LightCycler thermocycler (Roche Diagnostics) as previously described [16].

Western blots analysis

The left and right ventricles from 1 week, 3 week, and 3–4 month old mice was obtained after each experiment and immediately frozen in liquid nitrogen. The ventricular homogenates were analyzed by standard Western blotting to compare phospholamban (PLB) and Serca2A levels, and PLB phosphorylation at Ser16 [17,18].

Determination of myofilament protein phosphorylation

Mouse cardiac tissue was flash frozen in liquid nitrogen and stored in a -80°C freezer. Myofibrils were purified from the frozen cardiac tissue as described by Layland [19] with a few modifications. To determine the phosphorylation status of troponin I and myosin binding protein-C SDS-PAGE was performed using a phospho-specific Pro-Q Diamond stain (Invitrogen, Eugene, OR) and total protein stain SYPRO Ruby (Invitrogen, Eugene, OR) [19]. To determine the phosphorylation status of myosin light chain-2, troponin T, and Tm a 4.5–5.5 pH range 2-D DIGE gel was run and analyzed as previously described [20]. The 2-D DIGE gels were run to fully separate the TG from the NTG Tm protein that was unable to be fully separated using 1-D gel approaches.

Hydroxyproline assay

Hydroxyproline (HOP) content was determined as previously described [21,22] with some minor modifications. Liquid nitrogen frozen mouse cardiac tissue was minced into a pre-weighed Pyrex 9826 screw cap vial to determine the wet weight of the tissue (5–10mg). The tissue was hydrolyzed overnight in 200uL of 6M HCL in an oven set to 11°C. After the hydrolysis 80 µL of isopropanol was added to 5 uL of hydrolysate, and then 40 uL of chloramine-T solution (7% chloramine-T in water) mixed with acetate citrate buffer (0.695M anhydrous C₂H₃O₂Na, 0.174M C₆H₈O₇, 0.435M NaOH, and 38.5% [v/v] 2-propanol) in a 1:4 ratio was added, vortexed, and allowed to incubate and oxidize for 5 min at room temperature. Next 0.5 ml of Ehrlich's reagent (3g of 4-(dimethylamino) benzaldehyde, 10 ml ethanol, 675 µl sulfuric acid) mixed with 2-propanol in a 3:13 ratio was added, vortexed, and incubated for 30 min at 55°C then quenched in an ice bath. The samples were then centrifuged at 5,000×g for 1 min and the optical density of the supernatant was determined at 558 nm. A standard curve of *trans*-4-hydroxy-L-proline (0–500 nM) was included in each assay to determine the nM HOP/mg of tissue.

Isolated papillary muscles

Experiments with papillary muscles were performed as previously described [17,23,24].

In situ measurements

In situ measurements were performed as we previously described [25,26]. In order to analyze the myocardial response to β-adrenergic stimulation, we infused two concentrations of ISO (0.08 and 0.32 ng ISO/g b.w./min) via the femoral vein.

Data computation and statistical analysis

All results were presented as mean ± SE. The significance of difference between the means was evaluated by one-way ANOVA, ANOVA for repeated measurements followed by the Student-Newman-Keuls test. A value of P<0.05 was the criterion for significance.

RESULTS

Gene transfer of Serca2a in one day old neonatal mice results in efficient and long-term expression

We tested the efficiency of neonatal gene transfer using a recombinant adenovirus encoding β-gal. Figure 1A shows representative hearts from two day old mice injected at one day of age with empty adenovirus vector or Ad.LacZ. As previously shown by Christensen *et al* [15] the entire outer layer of the myocardium expressed β-gal. However, when we injected the hearts in neonatal mice older than 1 day the efficiency was reduced (data not shown). Therefore, for all of the future experiments we injected mice with rabbit Ad.Serca2a (Ad.Ser) or Ad.LacZ during the first 24 hours after birth. The expression of exogenous Serca2a was detected by PCR up to 3–4 month of age and resulted in the improvement of the whole heart morphology (Figure 1B, 1C and 1D). The left panels of Figure 1C show representative hearts of 6 week old NTG mice injected with Ad.LacZ or Ad.Ser. The right panels show representative hearts from 6 week old TG mice injected with Ad.LacZ or Ad.Ser. TG hearts injected with Ad.LacZ show typical atrial enlargement, but TG hearts injected with Ad.Ser show lack of atrial enlargement and normal looking ventricles. Figure 1D shows that at 3–4 months of age heart weight to body weight was increased in TG-Ad.LacZ mice compared to other groups. This increased hypertrophy observed in TG-Ad.LacZ mice was also associated with increased fibrosis measured as hydroxyproline content (Figure 1E). Fibrosis was reduced in TG-Ad.Ser compared to TG-Ad.LacZ hearts; however, it was higher when compared to NTG-Ad.Ser group.

Neonatal gene transfer of Serca2a delays the expression of atrial natriuretic factor (ANF) and β -myosin heavy chain (β MHC) and alters expression of phospholamban (PLB) and endogenous Serca2a

To assess whether the improvement of the whole heart morphology of TG hearts injected with Ad.Ser is associated with any significant changes in expression of hypertrophic gene markers, we performed quantitative RT-PCR at different time points in NTG and TG hearts injected with Ad.Ser or Ad.LacZ. Figure 2A shows that from 3 weeks of age expression of ANF was increased in TG hearts compared to NTG hearts. However, Ad.Ser injection significantly reduced expression of ANF up to 6 weeks of age in TG hearts. At 1 week, expression of β -MHC was significantly increased in TG-Ad. LacZ hearts compared to TG-Ad.Ser and NTG-Ad.Ser hearts (Figure 2B). At 3–4 month the expression of β -MHC was significantly increased in both groups of TG hearts compared to NTG hearts. Next we examined the expression of PLB and endogenous Serca2a at different time points (Figure 2 C–D). At 1 week of age expression of PLB was reduced in NTG and TG hearts injected with Ad.Ser compared to NTG and TG hearts injected with Ad.LacZ. At 3 week of age expression of PLB was increased in NTG-Ad. LacZ hearts compared to other groups. Both in NTG and TG hearts injection of Ad.Ser resulted in reduced expression of endogenous Serca2a compared to hearts injected with Ad.LacZ at 1 and 3 week of age (Figure 2 D).

Next we examined whether the reduced expression of endogenous Serca2a and expression of exogenous Serca2a resulted in altered total expression of Serca2a at the protein level at different time points. Figure 3A shows a representative Western blot of Serca2a in NTG and TG hearts injected with either Ad.Ser or Ad.LacZ at 1 week. Figure 3B–D shows the average expression of Serca2a at the protein level in NTG and TG hearts injected with either Ad.Ser or Ad.LacZ at 1 week (panel B), 3 weeks (panel C) and 3–4 months (panel D). At 1 and 3 weeks of age expression of total Serca2a at the protein level was significantly increased in Ad.Ser hearts compared to Ad.LacZ hearts. At 3–4 months of age there was no difference of total Serca2a expression among the groups (Figure 3D). Since expression of PLB gene was altered in mice injected with Ad.Ser (Figure 2C) we also tested the expression of PLB at protein level. No statistically significant changes in expression of PLB at total protein level or its phosphorylation at Ser16 were found among the groups ($n=4-8$ per group). Figure 3 (panels E and F) shows a representative Western blots of PLB and phosphor-PLB at 3 weeks (panel E) and 3–4 months of age (panel D).

Contractile parameters and the response to isoproterenol (ISO) are improved in papillary muscles isolated from 2–3 week old TG hearts injected with Ad.Ser

Since in hypertrophy and heart failure the response to β -adrenergic stimulation is normally blunted, we determined whether this is true in our model of FHC and whether injection with Ad.Ser is able to improve the response to β -adrenergic agonist ISO in papillary muscles isolated from 2–3 week old mice. Figure 4A shows that the response to ISO was blunted in papillary muscles from TG-LacZ mice compared to other groups. However, papillary muscles from TG-Ad.Ser mice showed a normal response to ISO. At baseline and all concentrations of ISO (Figure 4B) time to 90% relaxation (RT_{90}) normalized to developed force was prolonged in TG-LacZ muscles compared to other groups.

Effect of Ad.Ser injection on baseline hemodynamic parameters and the response to ISO in 3–4 month old mice

To further determine the beneficial effect of exogenous expression of Serca2a in TG Tm180 mice on cardiac function, we measured left ventricular (LV) pressure *in situ* in NTG-Ad.LacZ, NTG-Ad.Ser, TG-Ad.LacZ and TG-Ad.Ser hearts at baseline and during β -adrenergic stimulation. In the basal state there were no differences in developed pressure (DP) among the groups, however the heart rate (HR) was slightly decreased in TG groups (Figure 5A and 5B).

The max rate of contraction measured as the positive dP/dt (dP/dt_{max}) was increased and the rate of relaxation measured as negative dP/dt (dP/dt_{min}) was decreased in TG group compared to NTG (Figure 5C and 5D). The left ventricular diastolic pressure (LVEDP) was not different among groups. LVEDP was 4.8 ± 0.4 mmHg in NTG-Ad.LacZ group, 4.7 ± 0.3 mmHg in NTG-Ad.Ser group, 3.6 ± 0.3 mmHg in TG-Ad.LacZ group and 3.6 ± 0.5 mmHg in TG-Ad.Ser group.

Next we compared the responses of these four groups of animals to β -adrenergic stimulation. Figure 5E shows the response (% change) to 0.08 ng/g b.w./min of ISO and Figure 5F to a maximal dose of ISO (0.32 ng/g b.w./min). We have previously shown that the maximum effect was obtained with this dose of ISO [25, 26]. HR increased significantly in all groups of animals at both doses of ISO, but did not differ among groups. At the lower dose of ISO the % change in dP/dt_{max} and dP/dt_{min} was significantly greater in TG mice injected with Ad.Ser compared to TG-Ad.LacZ animals (Figure 5E). At the higher dose of ISO (0.32 ng/g b.w./min) the percent change of dP/dt_{min} was also significantly greater in TG-Ad.Ser compared to TG-Ad.LacZ hearts (Figures 5F).

Adenoviral expression of Serca2a does not alter isoform expression or phosphorylation levels of myofilament proteins

The activity of the myofilament can be influenced by isoform switching and/or alteration in levels of protein phosphorylation. To determine the protein phosphorylation levels NTG and TG samples were randomly labeled with Cy3 and Cy5 separately then equally mixed and separated on a 2D-DIGE gel. Figure 6A shows a representative 2D-DIGE image of ventricular myofibril homogenates from NTG-LacZ and TG-LacZ 3 week old mice. The phosphorylation levels of TnI, myosin binding protein-C, and TnT did not significantly change in either 3 week or 3–4 month old animals (data not shown). However, in the 3 week age group phosphorylation level of mutated Tm in TG-Ad. Serca2a and TG-Ad.LacZ groups were lower compared to the WT Tm in the transgenic animals (Figure 6B). At 3–4 months of age the phosphorylation levels of mutated and WT Tm were not different among all 4 groups of animals (Figure 6C). Moreover, we found that the phosphorylation level of MLC_2 was reduced in TG mice compared to NTG both at 3 week and 3–4 months of age (Figure 6D and 6E). Despite the altered level of gene expression of β MHC (Figure 2B) the protein expression level did not significantly change (data no shown). In 3 week old animals, replacement of the WT Tm with mutated Tm at the myofibril level was 71.0 ± 1.1 % in TG-Ad.Ser mice and 71.7 ± 2.5 in TG-Ad.LacZ mice. In the 3–4 month old animals, replacement of the WT Tm with mutated Tm at the myofibril level was 66.0 ± 2.9 % in TG-Ad.Ser mice and 64.6 ± 2.5 in TG-Ad.LacZ mice.

DISCUSSION

Experiments reported here are the first to show that a single dose gene transfer of Serca2a in neonatal mice can delay the development of the hypertrophic phenotype and improve cardiac function in an FHC mouse model that is associated with increased myofilament sensitivity to Ca^{2+} and diastolic dysfunction. We found that this one time gene delivery resulted in a significantly increased expression of total cardiac Serca2a protein up to a few weeks of age and was sufficient to improve morphology and response to β -adrenergic stimulation up to 3–4 months of age.

The long lasting or permanent changes in myofilament properties that result from mutations causing FHC often result in maladaptive growth and remodeling. Examples of these FHC-causing mutations at the myofilament level are missense mutations, deletions and truncations of sarcomeric proteins linked to FHC (for reviews see [3,27,28]). It is not known what signaling pathways are involved in the development of pathological phenotypes in hearts expressing these defective myofilaments, but interestingly most of these FHC-causing mutations in thin

filament proteins are characterized by increased myofilament sensitivity to Ca^{2+} and diastolic dysfunction, which may lead to activation of stress-sensitive hypertrophic pathways. Therefore, interventions to improve relaxation are of particular interest. Major changes in relaxation may occur by altering myofilament sensitivity to Ca^{2+} or the rate of Ca^{2+} uptake by SR. Although reducing myofilament sensitivity to Ca^{2+} in FHC seems to be the most straight forward approach for pharmacological treatment there are no approved drugs so far available. In this study we have chosen to improve relaxation by altering SR Ca^{2+} uptake through a transient increase in Serca2a expression using an adenoviral-based technique.

The SR proteins, Serca2a and PLB play a critical role in regulation of SR Ca^{2+} uptake and therefore cardiac relaxation [24,29]. The rate of Ca^{2+} removal by SR depends on the level of expression of Serca2a and PLB and its level of phosphorylation, which are often altered in different disease states [30]. For example in HF the ratio of Serca2a to PLB is decreased, reducing the activity of the SR Ca^{2+} pump [31–34]. Therefore, targeting expression of either Serca2a or PLB in HF is of particular interest [35]. In failing human myocytes PLB had a positive effect on contractility when its expression was decreased using adenoviral vectors expressing antisense PLB [36]. Ablation of the PLB gene by gene targeting also rescued a dilated cardiomyopathic phenotype in a muscle lim protein KO mouse [37]. Moreover *in vivo* gene delivery of pseudophosphorylated mutant PLB using an adeno-associated virus resulted in chronic suppression of HF progression in BIO14.6 CM hamsters [38] and improved function in a sheep pacing model of HF [39]. However, ablation of PLB was not sufficient to rescue *in vivo* cardiac function or hypertrophy in mouse models of overexpressing Gαq or a mutant myosin binding protein C linked to human HCM [40]. It has been recently shown that increasing Serca2a expression using adenoviral gene transfer [12,41] or transgenesis [42,43] can improve survival, ventricular function and cardiac metabolism in a rat model of HF, and it also improves cellular function of human cardiac myocytes isolated from failing hearts [36]. In contrast, reduction of Serca2a levels accelerates the onset of HF during pressure overload [44]. These studies clearly show that reduced Serca2a expression in failing hearts is a key factor responsible for their depressed contractility and relaxation and may prove to be a good target for gene therapy. The first gene therapy trials using Serca2a for HF are in progress in the USA and England (<http://www.clinicaltrials.gov>) using adeno-associated vectors [45]. In fact the safety of SERCA2a gene transfer in humans along with specific biological activities have been demonstrated in phase 1 of the CUPID trial (Calcium Up-Regulation by Percutaneous Administration of Gene Therapy in Cardiac Disease), which has been recently reported [46]. Thus, we hypothesized that increased expression of Serca2a may also alleviate symptoms of diastolic HF in a model of compensated hypertrophy in TG Tm180 mice.

We have previously shown that prolonged relaxation in TG mice expressing the skeletal isoform of troponin I and normal levels of Serca2a protein can be corrected by disinhibiting SR Ca^{2+} uptake via knockout of PLB [24]. These data strongly indicate that slower cardiac relaxation caused by increased myofilament Ca^{2+} sensitivity in ssTnI mice can be corrected by an intervention that enhances SR Ca^{2+} uptake. It has also been demonstrated in various models, including the mouse that increased expression of Serca2a by Ad.Ser [47] or transgenic approaches [48] improves SR Ca^{2+} uptake and relaxation. We therefore took advantage of an adenovirus with a long-lasting expression [15,49] and injected Ad.Ser into one- day old mice in order to increase Serca2a expression for a few weeks after birth. Because expression of Serca2a is low after birth [50], we expected that even a small increase in total Serca2 expression should have a significant effect on the impaired cardiac relaxation seen in Tm180 mice. Our studies clearly demonstrated that increased expression of Serca2a in Tm180 mice, above the normal level seen in NTG mice was able to improve cardiac relaxation, reduce hypertrophy, reduce HOP content and improve the response to β -adrenergic stimulation up to 3–4 months of age. At this time point expression of ANF and β -MHC were higher in TG-Ad.Ser compared to TG-Ad.LAcZ hearts. It has been reported in other animal models of hypertrophy, such as

isoproterenol-induced or aortic-banded model, that expression of hypertrophic markers such as ANF and β -MHC have a biphasic pattern of induction, specifically there is an initial increase in the level of these markers followed by a decrease to a plateau level [51,52]. It is therefore possible, and we can only speculate, that at 3–4 months of age in TG-Ad.LacZ mice the levels of ANF and β -MHC are already in the decline phase, whereas TG-Ad.Ser are still in the initial increase phase. This fits well with our data showing a delay in the development of hypertrophy following Ad.Ser injection.

The mechanisms by which Serca2a improves cardiac function in TG Tm180 hearts are not known and are excellent topic for future studies, but it is clear that positive effects of Ad.Ser treatment are not due to changes in phosphorylation levels of thin and thick myofilament proteins. Although the level of LC₂ phosphorylation is decreased in Tm180 mice both at 3 week and 3–4 months of age, this occurred independently of whether Tm180 mice were injected with Ad.Ser or Ad.Lac. These data suggest that the beneficial effect of Serca2 is not related to altered phosphorylation level of LC₂. Interestingly we did not find any changes in phosphorylation of TnI, a protein with well documented PKA phosphorylation sites that induce myofilament desensitization and increased lusitropy [25,53,54].

We do not know what stress/strain activated pathways are being promoted in Tm180 and probably suppressed by Serca2a expression. However, it is likely that altered relaxation seen in Tm180 hearts is sensed by the protein network present in the Z-disk, costameres, the M-band, or the giant protein titin (for reviews see [55,56]. The data obtained in mechanically stressed cultured cells strongly indicate that extracellular matrix-integrin-costamere protein complexes is a mechanosensor, but there are no data as to how altered relaxation is sensed by this complex (for review see [57]). Answers to this question are important, but it requires development and application of several new technical approaches and is beyond the scope of this paper.

In summary the present studies strongly suggest that Serca2a should be considered as a potential target for gene therapy not only in patients with late stages of HF, but also in patients with FHC. Moreover, our data imply that development of FHC can be successfully delayed if therapies are started shortly after birth. Since FHC is a genetic disorder, neonatal screening and family history can provide early FHC detection. We believe that this study demonstrates the promise of early neonatal intervention in the prevention of FHC.

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Figure 1.

A. Representative hearts from a mouse injected with saline (left heart) or Ad.LacZ one day after birth and stained for lacZ. **B.** Expression of exogenous Serca2a detected by PCR in samples from 1 week, 3 week and 6 week old mice. **C.** Representative hearts from (non transgenic) NTG and transgenic Tm180 (TG) mice injected with Ad.LacZ or Ad.Ser at 6 week of age. **D.** Heart weight to body weight ratio in NTG and TG mice injected with Ad.LacZ or Ad.Ser at 3 weeks and 3–4 months of age. Data are presented as mean $\pm$ SEM, n=8–9 per group. **E.** Hydroxyproline content in ventricles from NTG and TG mice injected with Ad.LacZ or Ad.Ser at 3–4 months of age. Data are presented as mean $\pm$ SEM, n=3–4 per group.

Figure 2.

Message levels of atrial natriuretic factor (ANF), β -myosin heavy chain (β MHC), phospholamban (PLB) and endogenous Serca2a at different time points in NTG and TG hearts injected with Ad.LacZ or Ad.Ser. **A.** Expression of ANF. **B.** Expression of β MHC. **C.** Expression of PLB. **D.** Expression of endogenous Serca2a. Expression was normalized to GAPDH and presented as mean $\pm$ SEM. n=5–10 per group

Figure 3.

Expression of total Serca2a, PLB and phosphorylated PLB (phos-PLB) at Ser16 in NTG and TG mice injected with Ad.LacZ or Ad.Ser at different time points. **A.** A representative Western blot for total Serca2a expression at 1 week of age. **B.** The average expression of total Serca2a at 1 week of age. **C.** The average expression of total Serca 2a at 3 week of age. **D.** The average expression of total Serca 2a at 3–4 month of age. **E.** A representative Western blot for PLB and phospho-PLB (Ser 16) at 3 weeks of age. **F.** A representative Western blot for PLB and phospho-PLB (Ser 16) at 3–4 months of age. Data are presented as mean $\pm$ SEM. n=4–8 per group.

Figure 4.

The effect of isoproterenol (ISO) on developed force and relaxation time in papillary muscles isolated from NTG and TG mice injected with Ad.LacZ or Ad.Ser. **A.** Developed force at baseline and during ISO stimulation. **B.** Time to 90% of relaxation normalized to developed force at baseline and during ISO stimulation. Data are presented as mean $\pm$ SEM, n=5–11 per group.

Figure 5.

Hemodynamic parameters during basal state and isoproterenol (ISO) stimulation in NTG and TG mice injected with Ad.LacZ or Ad.Ser. **A.** Developed pressure (DP). **B.** Heart rate (HR). **C.** Rate of contraction (dP/dt_{max}) and **D.** Rate of relaxation (dP/dt_{min}) at baseline. **E.** Percentage change of DP, HR, dP/dt_{max} and dP/dt_{min} during low dose of ISO stimulation (0.08 ng/g b.w./min). **F.** Percentage change of DP, HR, dP/dt_{max} and dP/dt_{min} during max dose of ISO stimulation (0.32 ng/g b.w./min). Data are presented as mean $\pm$ SEM. n=5–10 per group.

Figure 6.

Quantification of myofilament protein phosphorylation of NTG and TG mice injected with Ad.LacZ and Ad.Ser. TnT₃ or 4, troponin T (isoform 3 or 4); p, phosphorylated; MLC₂, regulatory myosin light-chain; U, un-phosphorylated; Tm, tropomyosin; Note the TG animals express both the TG (Tm180) and wild-type (WT) form of Tm. **A.** Representative 2D-DIGE gel showing region of interest from 3 week old mice injected with Ad.LacZ. Myofibrillar fractions were labeled separately and equally mixed. The red signal corresponds to NTG-Ad.LacZ and the green signal corresponds to TG-Ad.LacZ. The legend to the right identifies the labels on the x-axis of the bar graphs (B–E). **B.** Representative 2D-DIGE gel region of Tm from 3 week old TG-Ad.LacZ and TG-Ad.Ser mice. The bar graph shows the 3 week old mice

Tm phosphorylation levels. **C.** Representative 2D-DIGE gel region of Tm from 3–4 month old TG-Ad.LacZ and TGAd. Ser mice. The bar graph shows the 3–4 month old mice Tm phosphorylation levels. **D.** Representative 2D-DIGE gel images showing region of MLC₂ from 3 week old transgenic and wild type mice treated with Ad.LacZ. The bar graph to the right shows the 3 week old mice MLC₂ phosphorylation levels. **E.** Representative 2D-DIGE gel images showing region of MLC₂ from 3–4 month old transgenic and wild type mice injected with Serca2a. The bar graph to the right shows the 3–4 month old mice MLC₂ phosphorylation levels. Values are means±SEM. n=4 per group.