Delta-Sarcoglycan Gene Therapy Halts Progression of Cardiac Dysfunction, Improves Respiratory Failure and Prolongs Life in Myopathic Hamsters

Running Title: Hoshijima et al: Late-Stage Gene Therapy in Cardiomyopathy

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Abstract

Background—The BIO14.6 hamster provides a useful model of hereditary cardiomyopathies and muscular dystrophy. Previous delta-sarcoglycan (δSG) gene therapy (GT) studies were limited to neonatal and young adult animals, and prevented the development of cardiac and skeletal muscle dysfunction. GT of a pseudo-phosphorylated mutant of phospholamban (S16EPLN) moderately alleviated the progression of cardiomyopathy.

Methods and Results—We treated 4 month-old BIO14.6 hamsters with established cardiac and skeletal muscle diseases intravenously with a serotype-9 adeno-associated viral vector carrying δSG alone or in combination with S16EPLN. Prior to treatment at age 14 weeks, the left ventricular (LV) fractional shortening by echocardiography was 31.3% vs. 45.8% in normal hamsters. In a randomized trial, GT halted progression of LV dilation and LV dysfunction. Also, respiratory function improved. Addition of S16EPLN had no significant additional effects. δSG-GT prevented severe degeneration of the transverse tubular system in cardiomyocytes (electron tomography), and restored distribution of dystrophin and caveolin-3. All placebo-treated hamsters, except animals removed for the hemodynamic study, died with heart failure between 34 and 67 weeks of age. In the GT group, signs of cardiac and respiratory failure did not develop, and animals lived for 92 weeks or longer, an age comparable to that reported in normal hamsters.

Conclusions—GT was highly effective in BIO14.6 hamsters even when given in late stage disease, a finding that may carry implications for the future treatment of hereditary cardiac and muscle diseases in humans.

Key Words: gene therapy, cardiomyopathy, muscles, heart failure, ventilation
Inherited forms of muscular dystrophy including the Duchenne/Becker Muscular Dystrophies (DMD/BMD) (i.e. dystrophinopathies) and Limb-Girdle Muscular Dystrophies (LGMDs) commonly affect both skeletal and cardiac muscles \(^1\). While progressive weakness of neck, trunk, and limb muscles are disabling in such patients, cardiac complications and respiratory failure are major determinants of prognosis \(^2,3\). Among the genes mutated in inherited muscular dystrophies, most encode membrane-cytoskeletal proteins that constitute a macro-molecular complex termed the dystrophin-glycoprotein complex (DGC) \(^1\).

BIO14.6 hamsters have a spontaneous genetic deletion around the first exon of the \(\delta\)-sarcoglycan (\(\delta\)SG) gene \(^4,5\), mutations of which have been linked to human LGMD type 2F \(^1\). Due to the absence of \(\delta\)SG, BIO14.6 hamsters are unable to form the sarcoglycan (SG) protein complex, a subcomponent of the DGC, on the sarcolemma. Although BIO14.6 hamsters appear normal when born, they die early, with life-span of approximately 12 months, primarily due to the development of severe congestive heart failure \(^6\).

Phenotypic consistency has made the BIO14.6 hamster a preferred animal model of muscular dystrophy and hereditary cardiomyopathy used for testing new therapies, including gene replacement using viral vectors \(^7\)\(^{-12}\). However, previous experimental trials of gene therapy (GT) in BIO14.6 hamsters \(^7\)\(^{-12}\) and one trial in mice \(^13\) have focused on the short or long-term effectiveness of disease prevention by treating SG deficiency early in life (neonatal and young adult animals, up to 9 weeks of age), and the effects of GT in late stage disease, when cardiac and respiratory dysfunction are well established, have not been examined.
In the present study, we delivered the δSG gene using an adeno-associated virus serotype 9 (AAV9) vector by intravenous bolus injection, allowing us to test the efficacy of GT in both the cardiac and skeletal muscles in 4 month old BIO14.6 hamsters with well established cardiac and respiratory failure. In addition, since a substantial number of cardiomyocytes are lost by 4 months of age \(^{14,15}\) and surviving cells are under stress, we added an additional group of BIO14.6 hamsters in which GT with a pseudo-phosphorylated mutant (S16E) of phospholamban (PLN) was combined with δSG GT. Based on our previous studies in BIO14.6 hamsters \(^{16}\) and post-infarction rats \(^{17}\), we expected that the positive inotropic effect of S16EPLN treatment could further enhance any benefits from δSG GT alone.
Methods

Additional details are available on-line in “Expanded Methods”.

Animals

Male BIO14.6 hamsters and golden Syrian hamsters were obtained from Bio Breeders Inc (Watertown, MA). All animal-related procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of California San Diego.

Viral vectors

The same δSG and S16EPLN expression module that was prepared in our previous studies was utilized.

Gene transfer procedures

BIO14.6 hamsters (80-100 gram body weight) were anesthetized with ketamine (100 mg/Kg) and xylazine (2.5 mg/kg). Through a small incision in the left inguinal region, the femoral vein was exposed and cannulated with flame-stretched polyethylene tubing (PE-50, Becton-Dickinson, Parsippany, NJ) and AAV9 vectors (8x10^{11} viral genome particles per 100 gram of body weight) were delivered.

Assessment of cardiac function

The methods for transthoracic echocardiographic analysis of left ventricular (LV) function and retrograde LV catheterization using a micro-manometer in anesthetized hamsters have been described. Examiners were blinded to the treatments.
Assessment of respiratory function

A barometric method of plethymography with continuous flow was used to calculate tidal volume and minute ventilation in spontaneously breathing, non-anesthetized hamsters. Hypercapnia-induced ventilation estimated the additional capacity of animals to respond to a ventilatory stimulus.

Electron microscopy

The sample preparation procedures have been described. Electron tomography was obtained in thick sections (500 nm) using an intermediate high-voltage EM system (400 kV). The tomographic reconstruction was carried out as described.

Immuno-fluorescent staining

Snap-frozen heart tissues were cryo-sectioned and unfixed specimens were stained using an indirect immuno-fluorescence strategy as described.

Statistics

Comparison between groups used a mixed-effects linear model or a repeated-measures two-factor ANOVA with post hoc tests. Values are mean±SEM, unless stated differently.
Results

The study design is shown in Figure 1A. We treated 16-week old male BIO14.6 hamsters with intravenous injection of an AAV9 vector carrying a human copy of the δSG gene (δSG/AAV9), δSG/AAV9 in combination with S16EPLN carried by an AAV9 vector (S16EPLN/AAV9), and placebo (saline injection). S16EPLN treatment was shown in our previous study to be moderately effective in reducing the degree of heart failure in BIO14.6 hamsters over 7 months. We chose saline-treatment as control based on a finding that echocardiography detected no functional difference between saline-treated animals and animals treated with an AAV9 vector carrying lacZ β-D-galactosidase (n=5 each group) over 6 months (data not shown).

AAV9 vectors are known to effectively transduce genes in cardiac muscle. Prior to gene transfer, echocardiographic assessment at 14 weeks of age confirmed that left ventricular (LV) function in these BIO14.6 hamsters was substantially depressed compared to age-matched normal golden Syrian hamsters: the % fractional shortening (%FS) was 31.3±4.89% in the BIO14.6 (n=30) vs. 45.8±2.85% in the normal controls (n=6), (P<0.001) (Figure 2A, Pre). Enlargement of the LV chamber was not yet statistically significant at this age (Figure 2B, Pre). However, the LV end-diastolic wall stress estimated by the LV end-diastolic dimension/posterior wall thickness ratio (LVEDd/PWTh) in BIO14.6 hamsters was twice that in normal hamster (7.19±0.15, n=30, vs. 3.78±0.20, normal hamsters, n=6, P<0.001) (Figure 2D). No early mortality or procedure-related health problems were observed after the gene transfer procedure.
The efficiency of δSG replacement was nearly 100% both in ventricular cardiomyocytes (Figure 1C and D) and in fast-twitch skeletal muscles including quadriceps (Figure 1F), anterior tibial, and extensor digitorum longus muscle (data not shown). The efficiency was slightly lower in slow-twitch muscles, such as soleus (Figure 1G). No δSG expression was detectable in non-muscle organs/tissues including liver, lung, kidney, and brain (Figure 1H-K), and the LV coronary vascular system was devoid of staining.

δSG gene transfer halted the development of cardiac failure

After initial echocardiographic studies, the hamsters were randomly separated into subgroups (10 δSG/AAV9-treated animals, 10 δSG/AAV9 and S16EPLN/AAV9 treated, and 10 saline-treated placebo animals) (Figure 1A).

Placebo-treated group: By 25 weeks after placebo treatment, the general health of the BIO14.6 hamster in the placebo was deteriorating, by developing physical weakness including markedly reduced spontaneous activity and poor appetite, and clinical signs of congestive heart failure including dyspnea and anasarca.

One animal died at 18 weeks after the placebo-treatment, and the %FS of the other placebo animals had declined by 25 weeks by an average of 56%, to 13.8±2.13% (n=9) (Figure 2A). The placebo group developed severe LV chamber dilation (Figure 2B). The LV wall thickness remained markedly thinned (Figure 2C), and therefore the estimated diastolic wall stress continuously increased in this group (Figure 2D). Late echocardiographic studies (49 and 65 weeks after treatment) were not obtained for the placebo group. After 5 randomly selected
animals were, as scheduled, removed and used for a hemodynamic study at 28 weeks together with other GT-treated animals, all remaining placebo animals died or were severely morbid by the time of the late echocardiography.

**GT Groups:** Both BIO14.6 hamsters treated with δSG/AAV9 alone and those treated with δSG/AAV9 and S16PLN/AAV9 showed vigorous spontaneous activity, maintained good appetite, and displayed no obvious appearance of systemic edema or respiratory distress.

Echocardiography at 25 weeks after GT showed that treatment preserved LV function remarkably well in both treatment groups, demonstrating complete prevention of a decline in the %FS (Figure 2A), suppression of progressive LV dilation (Figure 2B), and a significant increase in diastolic LV wall thickness (Figure 2C) which reduced the estimated LV diastolic wall stress relative to the placebo group (Figure 2D). The rate of decline in %FS and the rate of LV dilation were significantly smaller in the treatment groups than in the placebo (P<0.0001), but there was not a statistically significant difference between the δSG/AAV9-treatment alone and the combined treatment of δSG/AAV9 and S16PLN/AAV9 (%FS, P=0.21; LVEDd, P=0.10). LV wall thickness was significantly greater at 25 weeks in the treatment groups than in placebo (δSG/AAV9 alone, P=0.002; δSG/AAV9 and S16PLN/AAV9, P=0.0125). Estimated diastolic wall stress did not change significantly after GT (at 25 weeks vs. pre: δSG/AAV9 alone, P=0.73; δSG/AAV9 and S16PLN/AAV9, P=0.17).
Late-stage echocardiography (49 and 65 weeks after GT) showed that a favorable effect of the δSG replacement therapy on cardiac function was maintained. Only mild fall in the %FS (Figure 2A) and gradual LV chamber enlargement (Figure 2B) were recognized.

In animal groups treated with δSG/AAV9 alone and with δSG/AAV9 and S16EPLN/AAV9, a beneficial increase in LV wall thickness observed at 25 weeks (see above), might have served to prevent LVEDd/PWTh from increasing significantly at 49 and 65 weeks (Figure 2D).

**Hemodynamic Studies:** To confirm that prevention of the progressive heart failure phenotypes by GT was due, at least in part, to a direct beneficial effect on LV function and myocardial contractility, BIO14.6 hamsters from each treatment group (n=5 per group) were subjected to invasive cardiac hemodynamic examination (catheter tip micromanometry) at 28 weeks after GT (at age 44 weeks) (Figure 3). At rest and with a treatment of increasing doses of dobutamine, the maximum first derivative of LV pressure (max LV dP/dt), an index of LV myocardial contractility, was significantly higher in both of the treated groups compared to the placebo group (Figure 3A). GT also caused marked enhancement of LV relaxation, reflected in an increase in the peak negative first derivative of LV pressure (min LV dP/dt) (Figure 3C) and a shorter time constant of LV pressure decay (tau) (Figure 3D), a relatively load independent measure of relaxation.

**δSG gene transfer halted deterioration of respiratory function**

Most of the placebo-treated BIO14.6 hamsters showed clinical signs of severe respiratory distress before they died. Although lung congestion secondary to heart failure can be the cause
of dyspnea, respiratory muscles are among the most severely affected skeletal muscles in BIO14.6 hamsters\textsuperscript{14, 15}. Therefore, we combined the 84-86 weeks old δSG/AAV9-treated animals with (n=4) and without (n=3) S16EPLN treatment and applied whole body plethysmography in wake animals breathing spontaneously. Because untreated BIO14.6 hamsters do not survive to this age, we studied 42-46 weeks old BIO14.6 hamsters as controls without GT. Golden Syrian hamsters (42-46 weeks old) served as normal controls.

**Untreated group:** Basal respiratory function (tidal volume and minute ventilation) was mildly depressed in the non-treated group compared to normal hamsters (Figure 4 A and B). However, the hypercapnic ventilatory response to 8% CO\textsubscript{2} was severely impaired. The tidal volume increased by only 45%, compared to highly significant increases (81%) in normal hamsters (Figure 4A). Hypocapnia also inefficiently stimulated minute ventilation of non-treated BIO14.6 hamsters (Figure 4B).

**GT group:** The basal tidal volume of GT-treated BIO14.6 hamsters was not significantly different from that in normal hamsters (Figure 4A). In 8% CO\textsubscript{2}, tidal volume increased by 66% and minute ventilation increased by 117%, to a functional level that was not significantly different from that measured in younger normal hamsters (Figure 4 A and B). There was no significant difference between the three animal groups (normal hamsters, BIO14.6 hamsters, and GT-treated BIO14.6 hamsters) with respect to respiratory frequency, both at rest and after receiving hypercapnic stimulation.
Efficiency of δSG replacement in respiratory muscles: GT restored δSG in respiratory muscles (diaphragm and the intercostal muscle: ICM), and efficiency was nearly 100% in ICM (Figure 4D and H), although δSG expression was lower and inhomogeneous in the diaphragm (Figure 4F and J). In addition, αSG and βSG immuno-staining was observed on the sarcolemma in treated BIO14.6 hamsters (data not shown), confirming that the SG complex was restored on the sarcolemma in respiratory muscles.

δSG gene transfer preserved ultrastructure and subcellular components in cardiac muscle

Degeneration of the transverse tubular system (T system) was ameliorated: δSG is known to localize both on the peripheral sarcolemma and along the T system25, 26 (Figure 1C and D). Therefore, using an advanced 3-dimensional (3-D) electron microscopic technology (electron tomography), we characterized T system abnormality, which was previously reported27.

As shown in Figure 5D and F (see also Video 1), high-resolution electron tomography (voxel size: 1.42 nm x 1.42 nm x 1.42 nm) revealed a strikingly deformed cardiac T system in placebo-treated BIO14.6 hamsters. Interestingly, multiple cystic invaginations with diameters of 75-100 nm were observed on T system membranes (Figure 5F). Such T system abnormalities were absent in BIO14.6 hamsters treated with δSG replacement (Figure 5 C, E, and G).

DGC was reconstituted: Despite preceding focal lytic changes in the myocardium, δSG GT effectively recruited and stabilized αSG (Figure 6 C and F), as well as βSG and γSG (data not shown), on the sarcolemma of surviving cardiomyocytes; the SGs were restored on both the
peripheral sarcolemma and the internal sarcolemma of the T system. δSG replacement also favorably affected the entire DGC, including the correction of the abnormal distribution of dystrophin at intercalated discs (Figure 6 I and H), which was reported previously. β-dystroglycan (β-DG) is considered to serve as an intermediate molecule connecting the SG complex and dystrophin; however, the subcellular distribution of β-DG (Figure 6 J-L) did not coincide with dystrophin redistribution (Figure 6 G-I).

δSG GT prevented changes in caveolin-3 (Cav3) distribution: Because the multiple micro cystic invaginations of the T system membranes found on electron tomography resemble caveolae (see Discussion), we analyzed the distribution of Cav3, a key molecule in the formation of caveolae in cardiac muscle using immuno-staining. Unexpectedly, T system-associated Cav3 staining was greatly reduced in untreated BIO14.6 hamsters, while the punctate staining pattern of Cav3 along the peripheral sarcolemma remained (Figure 7 B, E, and H). This abnormal Cav3 distribution was not seen in the δSG/AAV9-treated BIO14.6 hamsters (Figure 7 C, F, and I).

GT prolonged the lifespan of BIO14.6 hamsters

As noted above, due to the development of severe symptoms of cardiac and respiratory failures, none of placebo-treated BIO14.6 hamsters (n=5) survived to undergo late echocardiography. These animals died of the heart failure between 34 and 67 weeks of age. In contrast, there was no animal loss in the GT-treated groups (n=10) until one animal was found dead at 92 weeks of age, followed by two animals at 93 weeks. These animals showed gradual reduction of activity but did not present obvious signs of heart failure or respiratory distress. Kaplan-Meier analysis
with the log-rank test revealed significant improvement in animal survival in the treatment groups (each group, \( P=0.004 \)). The addition of S16EPLN/AAV9 treatment provided no additional benefit (\( P=0.48 \)). It should be noted that the late deaths at 92 and 93 weeks are within the reported lifespan of the normal Syrian hamster, 82±25 weeks in one study \(^{29}\) and 106±26 weeks (mean±SD) in another study \(^{30}\). The remaining 7 animals in the GT group were euthanized at 96 weeks in order to perform tissue studies.
Discussion

While previous gene therapy trials treated young BIO14.6 hamsters and δSG-null mice before the onset or at an early stage of the disease \(^7\text{--}^\text{13}\), we chose 16 weeks of age in the current study as the time for GT in BIO14.6 hamsters when LV dysfunction is moderately severe \(^18\). In the BIO14.6 hamster, the lytic changes in the myocardium and respiratory muscles become evident by 20-40 days of age \(^15\) and subside by 150-160 days to be replaced by fibrosis and calcification \(^14\text{, }15\). The progression of cardiac and skeletal muscle weakness lags somewhat behind histological changes \(^14\text{, }15\). For example, the LV was not significantly dilated and the %FS was only mildly reduced (37-40%) just prior to treatment in our previous δSG GT trial in 7 to 9 week old BIO14.6 hamsters \(^8\). In addition, myopathic changes do not appear until 3 months of age in δSG-null mice \(^31\), which were treated in a previous GT study \(^13\). Accordingly, this study appears to be the first to show that GT at a relatively advanced stage of disease in this form of hereditary cardiac and skeletal myopathy can halt the rapid progression of clinical signs and echocardiographic findings of LV dysfunction, ameliorate many of the structural abnormalities in cardiac muscle, and favorably affect survival.

We consider that the failure of the S16EPLN treatment to provide an additional effect in the current study is primarily due to the overwhelming efficacy of δSG GT. In addition, the efficacy of S16EPLN therapy is sensitive to the stoichiometry of sarcoplasmic reticulum calcium-ATPase and native PLN molecules \(^16\), which might not be optimal in the current study, where recipient BIO14.6 hamsters were much later into treatment than in our earlier study \(^16\).
We believe that this study provides the initial demonstration of a successful in vivo treatment of respiratory dysfunction by GT in a muscular dystrophy model. Since cardiac function and lung function are highly interrelated, we speculate that the improvement of respiratory function found in the current study is probably due to both a direct effect of GT on respiratory muscles and a secondary effect from improved cardiac function.

The study also applied electron tomography and revealed an enormous T-system dilation and multiple small cystic invaginations associated with the T-system in placebo-treated BIO14.6 hamsters (Figure 5 D and F). From their size and shape, we initially speculated that these cystic membrane structures represented caveolae. Caveolae are frequently observed on the peripheral sarcolemma of cardiac myocytes and at the tips of elongating cardiac T-tubules in young mammals. In the past, caveolae were thought to be the precursor of T-tubules. However, this is unlikely in this case, since Cav3, which is prerequisite for caveola formation in striated muscles, was found to be dissociated from the T system in BIO14.6 hamsters (Figure 7 E and H). The cause of Cav3 dislocation is not clear, as Cav3 may not be an integral component of the DGC. Nonetheless, it remains possible that the translocation of Cav3 might be a factor leading to disorganization of the cardiac T system in BIO14.6 hamsters, as suggested in studies in humans with LGMD.

There is an increasing awareness of the clinical importance of cardiac involvement in muscular dystrophy. Currently, cardiac and skeletal muscles are often significantly damaged when patients are first diagnosed, despite increasing use of newborn screening. The δSG-deficient BIO14.6 hamster used in the current study develops myolysis and progressive weakness in both
cardiac and skeletal muscles resembling the clinical manifestation of DMD/BMD. Also, plasma membrane fragility has been suggested as the central disease mechanism of dystroglycanopathies and genetic SG defects, as recently reviewed\textsuperscript{1}. Importantly, recent improvements in health care practices have extended the lifespan of DMD/BMD patients and, as a result, nearly 90% of DMD/BMD patients now die from cardiac or muscular respiratory failure\textsuperscript{2}. Accelerated cardiac damage found in dystrophin-deficient mice that were selectively treated for skeletal muscle myopathy further supports the need for cardiac therapy in muscular dystrophy\textsuperscript{42}. In addition, approximately 25% of all so-called idiopathic cardiomyopathies in humans are genetic, and these individuals might not be identified at an early disease stage by family studies and/or genetic screening\textsuperscript{43}. Therefore, we consider that the therapeutic efficacy of relatively late gene replacement demonstrated in the present study carries significant clinical implications for the future of GT in humans, even when cardiac and respiratory dysfunction are well established.
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Disclosures

None.
References


Figure Legends

Figure 1. Systemic induction of δSG in BIO14.6 hamsters using an AAV9 vector

A shows the time course of the study. The human copy of δSG was systemically transferred intravenously to BIO14.6 hamsters at the age of 16 weeks, with or without a pseudophosphorylated mutant PLN (S16EPLN), both carried by an AAV9 vector. GT, gene transfer. B and E show negative δSG immuno-staining in left ventricular and quadriceps muscles in placebo (saline)-treated BIO14.6 hamsters. C, D, and F-K are representative δSG immuno-staining in various tissues collected from δSG/AAV9-treated BIO14.6 hamsters at 28 weeks (wks) after gene transfer. D is a high magnification view of a subarea of C (dashed line square). δSG induction was confirmed on both the peripheral sarcolemma and the T system (D). Bars, 10 μm in B-F, 100 μm in G-K.

Figure 2. δSG replacement with S16EPLN therapy prevented the progression of cardiac dysfunction and chamber dilation in BIO14.6 hamsters

Normal, placebo-treated, and BIO14.6 hamsters treated with the δSG/AAV9 alone or in combination with S16EPLN/AAV9 (denoted as BIO δSG and BIO δSG+S16E, respectively) were followed by serial echocardiograms. A shows preservation of %FS with treatment, as an index of LV systolic function. B indicates prevention of LV dilation with treatment. C demonstrates changes in diastolic LV wall thickness. In D, estimated diastolic wall stress (LVEDd/PWTh) is significantly reduced by the treatment (relative to the placebo). See the text for details. The blue dashed lines are pair-wise comparisons between animal groups tested by a
mixed-effects linear model (*P<0.05 or “n.s.”). Depressed cardiac function of BIO14.6 hamsters prior to the treatment was shown (#P<0.05, vs. normal at pre-GT). Error bars are means±SEM. Pre, pre-GT; n.s., no significance.

Figure 3. Chronically improved cardiac contractility and relaxation by δSG replacement therapy in BIO14.6 hamsters

BIO14.6 hamsters treated with δSG/AAV9 alone or in combination with S16EPLN/AAV9 (BIO δSG and BIO δSG+S16E, respectively) for 28 weeks were subjected to hemodynamic analyses. At rest (dobutamine, 0 μg/Kg/min) measures of LV contractility (max LV dP/dt) shown in A and relaxation (min LV dP/dt and tau) shown in C were significantly higher in treated animals than in placebo-treated controls. Since min LV dP/dt is sensitive to changes in peak LVP, tau (a relatively load-independent index of relaxation) was also evaluated (D) and improved at rest in the GT groups. Dobutamine enhanced LV systolic and diastolic functions dose-dependently in both placebo and GT groups. The blue dashed lines are pair-wise comparisons between animal groups (*P<0.05 or “n.s.”). #P<0.05, vs. placebo at rest. Error bars are means±SEM. n.s., no significance.

Figure 4. δSG replacement improved respiratory function and restored the SG complex on the respiratory muscle sarcolemma

A and B show ventilatory function in BIO14.6 hamsters (84-86 weeks old, n=7) treated with δSG/AAV9 at 16 weeks of age, with and without additional S16EPLN treatment, compared to
that of untreated younger BIO14.6 hamsters and normal golden hamsters (42-46 weeks old, n=6 in each group). In both tidal volume (A) and minute ventilation (B) measurements, beneficial therapeutic effects are shown at rest (Basal) and after exposure to hypercapnia (8% CO₂). The mean values of measurements in treated hamsters are statistically indistinguishable from those measured in younger normal hamsters. Unadjusted p-values of pair-wise comparisons are shown in A and B. # p-value is smaller than the critical level of significance. * p-value is smaller than the critical level of significance, vs. basal. The Error bars are means±SEM.

Representative immuno-staining of restored δSG is shown in the diaphragm (F and J) and intercostal muscles (ICM, D and H). C, G, E and I show absence of SG staining in placebo-treated BIO14.6 hamsters. G-J show δSG staining alone. In C-F, sarcolemmal staining with WGA (green) and nuclear staining with DAPI (nuc, blue) are co-visualized. Bars, 100 μm.

**Figure 5. Degeneration of the cardiac T system in BIO14.6 hamsters demonstrated by electron tomography**

A-C, 70-nm thin-section electron micrographs. T, T-tubule. L, lipid droplet. In B, arrows indicate dilated T-tubules in BIO14.6 cardiomyocytes treated with placebo for 28 weeks, compared to normal T-tubules found in golden Syrian hamsters (A) and in BIO14.6 cardiomyocytes treated with δSG/AAV9 alone (C). D-G are composite images of 3-D surface-rendered mesh models of T system (green) and dyadic junctions (red) with 2-dimensional slices, generated from reconstructed electron tomograms. F and G are high magnification views of outlined volumes in D and F (white dashed lines), respectively. Arrows in F indicate multiple
“caveola”-like cystic invaginations of the T system membrane. The size of tomograms is 5.8 x 5.4 x 0.36 μm³ (placebo) and 5.8 x 5.5 x 0.27 μm³ (δSG treated), respectively. See Video 1.

Figure 6. Reconstitution of the DGC by δSG GT in cardiomyocytes
LV tissues were collected from BIO14.6 hamsters after 28-weeks of treatment with δSG/AAV9 alone. The effect of δSG/AAV9 treatment (C, F, I, and L) in BIO14.6 hamsters was referenced to placebo treatment (B, E, H, and K) and normal golden Syrian hamsters (A, D, G, and J). D-F show αSG staining (red) alone. A-C show combination of αSG staining (red) with membrane staining with WGA (green), which stains both the peripheral sarcolemma and T system, and nuclear staining (nuc, blue) with DAPI. G-I shows that dystrophin is expressed on both the peripheral sarcolemma and T system, and aberrantly expressed in intercalated discs only in placebo-treated BIO14.6 cardiomyocytes (H, arrows). The dystrophin expression in intercalated discs was not accompanied by change in β-DG distribution (K). Bars, 10 μm.

Figure 7. Aberrant distribution of Cav3 in BIO14.6 hamster cardiomyocytes, which was restored by δSG replacement.
A-C show Cav3 immno-staining (red), together with sarcolemmal staining with WGA (green) and nuclear staining with DAPI (nuc, blue). D-I are Cav3 immno-staining only (red). G-I represent high magnification of D-F, respectively (dashed line square). T system-associated distribution of Cav3 in normal hamsters (arrow heads in G) is largely lost in cardiomyocytes in 28-week placebo-treated BIO4.6 hamsters (E and H). Punctate Cav3 staining on the peripheral
sarcolemma (*arrows* in G-I) is, on the other hand, preserved (H). Abnormal Cav3 distribution was restored at 28-weeks after δSG replacement (F and I). Bars, 10 μm.
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SUPPLEMENTAL MATERIAL

Delta-sarcoglycan gene therapy halts progression of cardiac dysfunction, improves respiratory failure and prolongs life in myopathic hamsters (Hoshijima M et al.)

Expanded Methods

Viral vectors

A muscle-specific promoter (C5-12) \(^1\) was used for the muscle-tropic expression of human δSG. C5-12 is a randomly assembled synthesis of muscle promoters and enhancers, which was successfully used in our previous neonatal-juvenile hamster \(^2\) and adult mouse \(^3\) studies. The ubiquitously active human cytomegalovirus immediate-early promoter was used to drive the expression of a pseudo-phosphorylated mutant of the phospholamban gene (S16EPLN). S16EPLN selectively affects cardiomyocytes \(^4\), where sarcoplasmic reticulum ATPase and phospholamban are expressed stoichiometrically.

The gene expression constructs were cross-packaged to make recombinant AAV9 vectors using a triple-transfection procedure \(^5\). The vectors were purified using a cesium chloride sedimentation method and genome copy titers were determined \(^6\), performed by the Penn Vector Core of University of Pennsylvania (Dr. Julie Johnson).

Echocardiographic assessment of LV function

The method for analyzing cardiac function in hamsters by echocardiography has been described \(^7\). Hamsters were anesthetized with intra-peritoneal administration of pentobarbital (65 mg/kg of body weight). The anterior chest wall was shaved and small needle electrodes for simultaneous electrocardiogram were inserted into one upper limb and two lower limbs. Transthoracic
echocardiography was performed using a Philips Sonos 5500 ultrasound system with an L15-
6MHz transducer. Echocardiograms were performed prior to gene therapy (GT) and in the
placebo and GT groups at 5, 13, and 25 weeks after GT, along with studies at 49 and 65 weeks
after GT in the GT groups.

**Hemodynamic measurements**

For hemodynamic measurements at 28 weeks after GT, BIO14.6 hamsters were anesthetized with
intra-peritoneal administration of pentobarbital (65 mg/kg of body weight), intubated, and
ventilated; a bilateral vagotomy was performed, and a 1.4 Fr high-fidelity catheter-tip micro-
manometer (Millar Instruments, Houston, TX) was advanced into the left ventricular (LV)
chamber through the right carotid artery. Peak LV pressure, maximum and minimum LV
dP/dt, and tau were calculated from LV pressure recording, as described.

**Assessment of respiratory function**

On the day of the experiment, animals were placed individually, unrestrained, in a Plexiglas
chamber (4.65 liters), and ventilation was measured using the barometric method of
plethysmography with modification for the employment of continuous flow. Humidity and
temperature inside the chamber were monitored. An electronic gas mixing flow meter was used
to ensure precise control of inspired gas concentrations and high input impedance, and chamber
gas concentrations were measured using a mass spectrometer (MGA 1000, Perkin-Elmer).
Tidal volume was calculated from the ventilation-induced pressure changes as described.
Calibration was carried out using a gas-tight syringe and injection of air pulses (0.1-0.25 ml) in
the chamber at rate similar to the respiration rate of tested animals. The hamsters were
acclimated for ~30-60 min in the chamber environment before recording. After basal ventilation
function was recorded, the chamber air was abruptly changed to 8% CO₂, 21% O₂, and 71% N₂.
for 10 minutes to evaluate hypercapnia-induced ventilation, as in a previous study that reported the age-dependent development of respiratory dysfunction in BIO14.6 hamsters."

**Assessment of animal health conditions**

The general health condition of BIO14.6 hamsters treated with placebo or therapeutic viral vectors was regularly assessed by a researcher, who was blinded to the difference in treatments.

**Sample preparation for conventional EM and electron tomography**

Hamsters were euthanized and perfusion-fixed through the left ventricle with 2% (weight/volume) paraformaldehyde and 2% glutaraldehyde in 0.15 M sodium cacodylate buffer (pH7.4) at a hydraulic pressure of 90 cm for 5-10 min. The subsequent steps of EM sample preparation were essentially the same as described previously. Thin sections (70nm) were viewed and pictured on a JEOL1200 system operated at 80-100 kV. Thick sections (500 nm) were prepared and a single tilt series of projected images were obtained from –60° to +60° at 2° intervals in a JEOL 4000EX operated at 400 kV as described. The images were recorded at x 10,000 magnification using a 4K x 4K charge-coupled device camera (16 bits per pixel, Spectral Instruments Inc., Tucson, AZ). Volume rendering, segmentation of T system and dyadic junctions, and generation of surface meshes used IMOD (Boulder Laboratory for 3-D Electron Microscopy of Cells, University of Colorado, Boulder, CO), as described. 3-D demonstration of model structures also used Amira (Visage Imaging Inc., San Diego, CA).

**Immunofluorescent staining**

Heart tissues were snap-frozen in isopentane precooled with liquid nitrogen. 10-µm thick unfixed cryo-sections were prepared, immediately blocked in 3% goat serum, 1% bovine serum albumin, 0.5% cold fish gelatin, and phosphate buffered saline at room temperature for 1 hr, and stained using an indirect immuno-fluorescence strategy as described. Monoclonal antibodies against α-
SG (1:50), β-SG (1:100), γ-SG (1:100), δ-SG (1:100), β-DG (1:200), dystrophin (clone Dy8/6C5, 1:100, Vector Laboratories Inc, Burlingame, CA), and Cav3 (1:100, BD Transduction Laboratories) were used. An Alexa Fluor 568 dye-conjugated goat anti-mouse antibody (1:300, Invitrogen) was used for secondary detection. In some sections, wheat germ agglutinin (WGA)-conjugated with Alexa Fluor 488 dye (1:500, Invitrogen) was included to visualize the sarcolemma. Finally, sections were coverslipped with ProLong Gold anti-fade reagent with 4′-6-diamidino-2-phenylindole (DAPI) nucleic acid stain (Invitrogen) and observed on a FluoView FV1000 laser-scanning confocal microscope (Olympus) equipped with an x60 PLAPON objective (oil, NA=1.42).

Statistics
A mixed-effects linear model was applied to assess the effect of GT on repeated-echocardiographic measurements (PROC mixed, SAS Version 9.2, SAS Institute Inc.). The model sets treatment groups and the treatment by time after gene transfer interaction as fixed factors, and mouse identification number and the time after gene transfer as random effects. AR(1) was chosen as the repeated covariance structure. When significant differences between treatment groups were observed, pair-wise multiple comparisons were carried out with the level of significance adjusted by the Tukey strategy.

A mixed-effects linear model (PROC mixed, SAS Version 9.2, SAS Institute Inc.) was used to assess hemodynamic responses to dobutamine infusions (a linear random effect), differences between the treatment groups in response to dobutamine (a fixed treatment by dobutamine interaction) and difference in responses between treatment groups (a fixed effect). When significant differences were observed, pair-wise multiple comparisons were carried out with the Tukey correction to adjust the level of significance.
The repeated-measures two-way ANOVA (SigmaPlot 11, Sistat Software Inc.) was also applied to analyze respiratory functions. Both gas treatments and animal groups were categorical factors. The interaction of 2 factors (gas x animal groups) was included in the model. Factorial difference was tested using the Games-Howell method. When significant differences were observed, pair-wise multiple comparisons were carried out with the Holm-Sidak correction to adjust the level of significance (overall significance level = 0.05).

The Kaplan-Meier algorithm produced estimated survival fractions and the log-rank test (Mantel-Cox) examined statistical significance of differences among treatment groups (SPSS 18.0, Advanced Statistics, IBM). Pair-wise multiple comparisons used the Bonferroni-correction to adjust the level of significance (overall significance level = 0.05).

**Supplemental References for Expanded Methods**


**Legend for the Video file**

Video 1: Micro-degeneration of cardiac T system, which was newly revealed by electron tomography, in placebo-treated BIO14.6 hamsters. 3-D surface-rendered mesh models of T system (green) and dyadic junctions (red) are shown with an ultra-thin 2-dimensional slice of the tomogram. Dilated T-tubules and “caveola”-like multiple cystic invaginations of the T system membrane are demonstrated.