Stable Myocardial-Specific AAV6-S100A1 Gene Therapy Results in Chronic Functional Heart Failure Rescue

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Background—The incidence of heart failure is ever-growing, and it is urgent to develop improved treatments. An attractive approach is gene therapy; however, the clinical barrier has yet to be broken because of several issues, including the lack of an ideal vector supporting safe and long-term myocardial transgene expression.

Methods and Results—Here, we show that the use of a recombinant adeno-associated viral (rAAV6) vector containing a novel cardiac-selective enhancer/promoter element can direct stable cardiac expression of a therapeutic transgene, the calcium (Ca^{2+})-sensing S100A1, in a rat model of heart failure. The chronic heart failure–rescuing properties of myocardial S100A1 expression, the result of improved sarcoplasmic reticulum Ca^{2+} handling, included improved contractile function and left ventricular remodeling. Adding to the clinical relevance, long-term S100A1 therapy had unique and additive beneficial effects over β-adrenergic receptor blockade, a current pharmacological heart failure treatment.

Conclusions—These findings demonstrate that stable increased expression of S100A1 in the failing heart can be used for long-term reversal of LV dysfunction and remodeling. Thus, long-term, cardiac-targeted rAAV6-S100A1 gene therapy may be of potential clinical utility in human heart failure. **(*Circulation*. 2007;115:2506-2515.)**

Key Words: gene therapy ■ heart failure ■ long-term care ■ S100A1 protein

Cardiovascular disease remains a leading cause of mortality worldwide.[1,2] In particular, chronic heart failure (HF) continues to represent an enormous clinical challenge because HF mortality and incidence continue to rise.[2,3] Although therapy has considerably improved HF care over the last 2 decades, existing treatments are not ideal because they often fail to support myocardium and increase global cardiac function.[1–3] Therefore, novel therapeutic approaches to target the underlying molecular defects of ventricular dysfunction in HF are needed. One hallmark molecular defect in failing myocardium is dysfunctional intracellular calcium (Ca^{2+}) handling, and several Ca^{2+}-cycling proteins have been identified as potential targets for reversing failing myocyte function.[4,5]

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S100A1 is a Ca^{2+}-sensing protein of the EF-hand type and a positive inotropic regulator of myocardial function in vitro and in vivo.[6–10c] Consistent with S100A1 being a key player in cardiac contractile function, data generated from S100A1 knockout mice demonstrate that the loss of S100A1 expression leads to an inability of the heart to adapt to acute or chronic hemodynamic stress in vivo.[11,12] Importantly, S100A1-mediated effects on cardiac contractile function do not interfere with basic regulatory mechanisms of myocardial contractility[9] and have been found to be independent of β-adrenergic receptor (βAR) signaling.[6–8,11] Functional properties of S100A1 in cardiomyocytes are caused mainly by increased sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) activity, diminished diastolic SR Ca^{2+} leak, and augmented systolic open probability of the ryanodine receptor, causing an overall gain in SR Ca^{2+} cycling.[12–14] This demonstrates a potential distinct mechanism of action for S100A1 altering Ca^{2+} handling in both phases of SR function.

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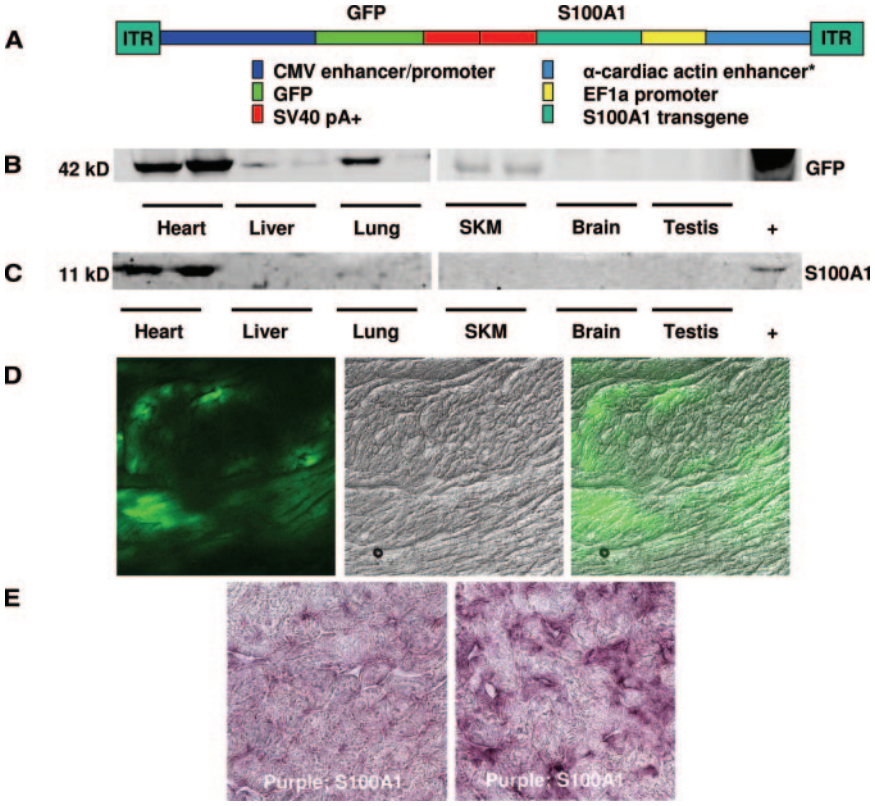

Figure 1. Cardioselective rAAV6-mediated in vivo gene transfer. A, AAV6/S100A1 construct used. Shown are the positions of the 2 independent transgenic cassettes: GFP driven by the CMV promoter and S100A1 driven by the α-cardiac actin enhancer/EF1α promoter. B, Representative Western blot analysis of GFP expression in rat tissues 8 weeks after in vivo intracoronary delivery with AAV6/S100A1. C, Representative Western blot of human S100A1 protein present only in the heart and absent in other rat tissues harboring the GFP transgene 8 weeks after AAV6/S100A1 delivery. D, Representative GFP fluorescence microscopy (left), light microscopy (middle), and overlay of both (right) of LV myocardium 8 weeks after intracoronary AAV6/S100A1 delivery. Magnification, $\times$40. E, S100A1 immunohistochemistry in LV tissue from AAV6/GFP- (left, control) and AAV6/S100A1-treated (right) rats (8 weeks after gene delivery). Magnification, $\times$40.

S100A1 is highly and preferentially expressed in the healthy heart, whereas it is found to be significantly downregulated in HF.[12,13,16] Previous studies by our laboratory have shown that targeting S100A1 expression in HF is a promising strategy to recover deranged intracellular Ca^{2+} cycling and to improve contractile function of failing cardiomyocytes.[13,17a] Cardiac adenoviral-mediated S100A1 gene therapy rescued myocardial performance in a rat HF model in vivo and ex vivo.[13] These data suggest that chronic S100A1 gene delivery to failing myocardium may be therapeutic. However, before this potential target and others for HF gene therapy are realized,[17] safe, efficient, and reproducible gene therapy vector systems must be established and tested. It is becoming increasingly clear that recombinant adeno-associated viral (rAAV) vectors have properties amenable to future human use and that S100A1 delivered to myocardium using these vectors may indeed fulfill the currently unmet promise of HF gene therapy. Accordingly, in this study, we tested the chronic effects of S100A1 gene therapy in HF and selectively targeted S100A1 gene expression to myocardium using a rAAV6 vector engineered with a novel cardiac-specific enhancer/promoter element. Finally, to further enhance potential clinical relevance, S100A1 HF gene therapy was compared with and added to pharmacological βAR-blocker treatment.

Methods

Construction of α-Cardiac Actin Enhancer/Elongation Factor 1α Promoter

Genomic DNA was isolated from mouse (C57/BL6) muscle, and polymerase chain reaction amplification was performed using PfuUltra (Stratagene, La Jolla, Calif) with the following primer pair: forward, 5′-A G G A A T T C T A A A T T T A C G T C T G C T T C C T G T C A A T G G G C-3′; and reverse, 5′-C C A G A C T A G T C A G C T G C T T T T C C T T C A G T T C A C A C C A G-3′. This resulted in a 324-bp fragment containing the α-cardiac actin myocyte-enhancer factor-2 (MEF2) domain.[18] The fragment contained an extra MEF2 element built into the forward primer and 2 enhancer myogenic differentiation antigen consensus sequences. This fragment was cloned in place of the cytomegalovirus (CMV) enhancer sequence in pCpGLacZ (InvivoGen, San Diego, Calif), resulting in pCpGa-cardLacZ. The human S100A1 gene was cloned by polymerase chain reaction from the plasmid (pBS^{SK-}) with PfuUltra (Stratagene) using the following primer pair: forward, 5′-CTGCCATGGGCTCTGAGCTGGAGACGGCG-3′; and reverse, 5′- C A G C T A G C T C A T T C A A C T G T T C T C C C C A G A A G A A A T T-3′. This resulted in a 290-bp fragment that replaced the LacZ gene in pCpGa-cardLacZ, resulting in pCpGα-cardS100. This plasmid was digested with *Eco*RI, and the 5′ overhang was filled in with Klenow (New England Biolabs, Beverly, Mass) and cloned into pTRUFR in place of the HSVtk enhancer/promoter neo gene, resulting in a packaging construct containing CMV-driving green fluorescent protein (GFP) and α-cardiac actin enhancer/elongation factor 1α (EF1α) promoter–driving S100A1 (see Figure 1A). Detailed procedures for production and purification of AAV constructs are given in the online-only Data Supplement.

Myocardial Infarction and Evaluation of In Vivo Cardiac Function

Procedures were performed as described previously.[13,17a] The detailed experimental procedures for left ventricular (LV) cryoinfarction, echocardiography, and hemodynamic analysis of cardiac function are given in the online-only Data Supplement.

Myocardial In Vivo Gene Delivery

All animal procedures and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Thomas Jefferson University. Myocardial gene trans-

fer to 10-week post–myocardial infarction (MI) rats was achieved as previously described[13,17a] with some modifications. Briefly, under general anesthesia (2% isoflurane, vol/vol), a midline cervical incision was made, and the animal was cooled to 29°C with ice packs. A P-50 catheter (Becton Dickinson, Sparks, Md) was advanced into the aortic root via the right carotid artery, and the ascending aorta was looped with 2-0 silk. Then, 1.2 mg adenosine was injected into the right ventricle with a 30½-gauge needle. The ascending aorta was clamped, and 2.5×10^{11} particles of the AAV construct and 8 μg of substance P (Sigma Chemical Co, St Louis, Mo) were rapidly injected into the aortic root, allowing coronary perfusion. After 2 minutes, the aortic clamp was released, a bolus of dobutamine (30 μg IA) was administered, and the animal was rewarmed with a heating pad.

Histology, Western Blotting, and Real-Time Polymerase Chain Reaction

Immunohistochemistry, assessment of infarct size, Western blot analysis, and quantitative real-time polymerase chain reaction were performed as previously reported.[13,17a]

Ca^{2+} Transient Analysis and Contractile Parameters of Isolated Adult Rat Cardiomyocytes

Isolation of adult rat cardiomyocytes, assessment of contractile parameters, and Ca^{2+} transients of isolated cardiomyocytes were done as described.[13,17a]

Statistical Analysis

Data are summarized as mean±SEM. Comparisons were made using *t* tests or ANOVA as appropriate. When multiple observations exist per animal, we used a mixed-effects model with random intercept for each animal to account for the repeated measurements. The statistical significance of each effect was then determined from Wald statistics derived from the mixed-effects models. A Bonferroni correction was applied to the probability values whenever multiple comparisons arose. Statistical analysis was performed with the Graph PadPrism software and the R statistical package (www.r-project.org). For all tests, $P<0.05$ is considered statistically significant after Bonferroni corrections if needed.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Cardioselective S100A1 Gene Delivery With a Novel α-Cardiac Actin Enhancer

To engineer a putative myocardium-selective promoter, a 316-bp fragment of the α-cardiac actin gene enhancer containing 2 MEF2 sequences and 2 enhancer MyoD consensus sequences was amplified from mouse genomic DNA and ligated to the EF1α promoter within an AAV shuttle plasmid (Figure 1A). The human S100A1 cDNA was then cloned into this plasmid. The final construct (AAV6-S100A1) also contains a separate transgene cassette with the CMV promoter driving expression of the green fluorescent protein (GFP) marker gene (Figure 1A). To first examine whether this vector supports stable cardiac expression in vivo, AAV6-S100A1 was delivered to normal rats (n=4) via intracoronary delivery.[13,17a] Consistent with a CMV-driven transgene, 8 weeks after gene delivery, GFP expression was found outside the heart with appreciable levels in liver, lung, and skeletal muscle (Figure 1B). In contrast to these findings, the human isoform of S100A1 was detectable only in cardiac homogenates, indicating that S100A1 expression driven by the combination of the α-cardiac actin enhancer and the EF1α promoter is cardioselective (Figure 1C).

Infection of myocardium by our novel AAV6 vector as assessed by GFP fluorescence of cardiac sections was global in nature but not homogeneous throughout the heart (Figure 1D), which is consistent with previous gene delivery studies using this intracoronary delivery method.[13,17a] This distribution pattern also was evident with S100A1 expression as confirmed by immunohistochemistry using an antibody specific for the human isoform of S100A1 (Figure 1E).

Characterization of Cardiac Dysfunction After MI and Before Gene Delivery

To induce chronic HF in rats, we used a cryoinfarct model, which leads to HF in 10 to 12 weeks.[13] Ten weeks after MI, cardiac function was assessed to determine pre–gene therapy status in these rats compared with sham animals. All MI rats were found to have significant LV dysfunction and were randomized into 5 treatment groups (Figure 2). Global HF was evident because of significantly diminished ejection fraction compared with sham animals (Figure 2A). Moreover, significant post-MI remodeling was apparent as determined by LV dilatation (Figure 2D). Importantly, all post-MI rats had similar HF (Figure 2 and data not shown); thus, all randomized groups had equal pre–gene therapy status. In addition to treating HF rats with AAV6-S100A1, separate groups also were treated with the β_1AR antagonist, metoprolol (250 $mg \cdot kg^{-1} \cdot d^{-1}$ in the drinking water) beginning 10 weeks after MI. All groups were then followed up over 2 months.

S100A1 Gene Therapy Improves In Vivo Cardiac Function Long Term and Reverses LV Remodeling in HF

The in vivo functional consequences of chronic cardioselective S100A1 gene therapy in HF were determined by echocardiography and with closed-chest cardiac catheterization. All HF groups still had significantly impaired cardiac function compared with sham rats (Figure 2B, 2E, and 2F) 18 weeks after MI; however, rats treated at 10 weeks after MI with S100A1 had significantly improved cardiac function as assessed by percent ejection fraction (Figure 2B and 2C). Representative M-mode echocardiographic recordings are shown in Figure 2G. Of interest, the significant ≈30% improvement in global cardiac function after 8 weeks of cardiac-selective S100A1 expression was seen with or without metoprolol (Figure 2C). In contrast to this improvement with S100A1, HF rats treated with only GFP or saline had further deterioration of cardiac function over this period (Figure 2C), whereas metoprolol induced no improvement but also no further functional decline (Figure 2C).

Both cardiac S100A1 gene therapy and β-blocker treatment significantly attenuated further LV chamber dilatation as measured by 18-week post-MI LV diastolic dimension compared with pretreatment (10 week after-MI) values, whereas progressive dilatation occurred in saline- and GFP-treated HF rats (Figure 2D and 2E). S100A1 expression with concurrent metoprolol treatment did not further attenuate LV remodeling over either treatment alone (Figure 2E). Finally,

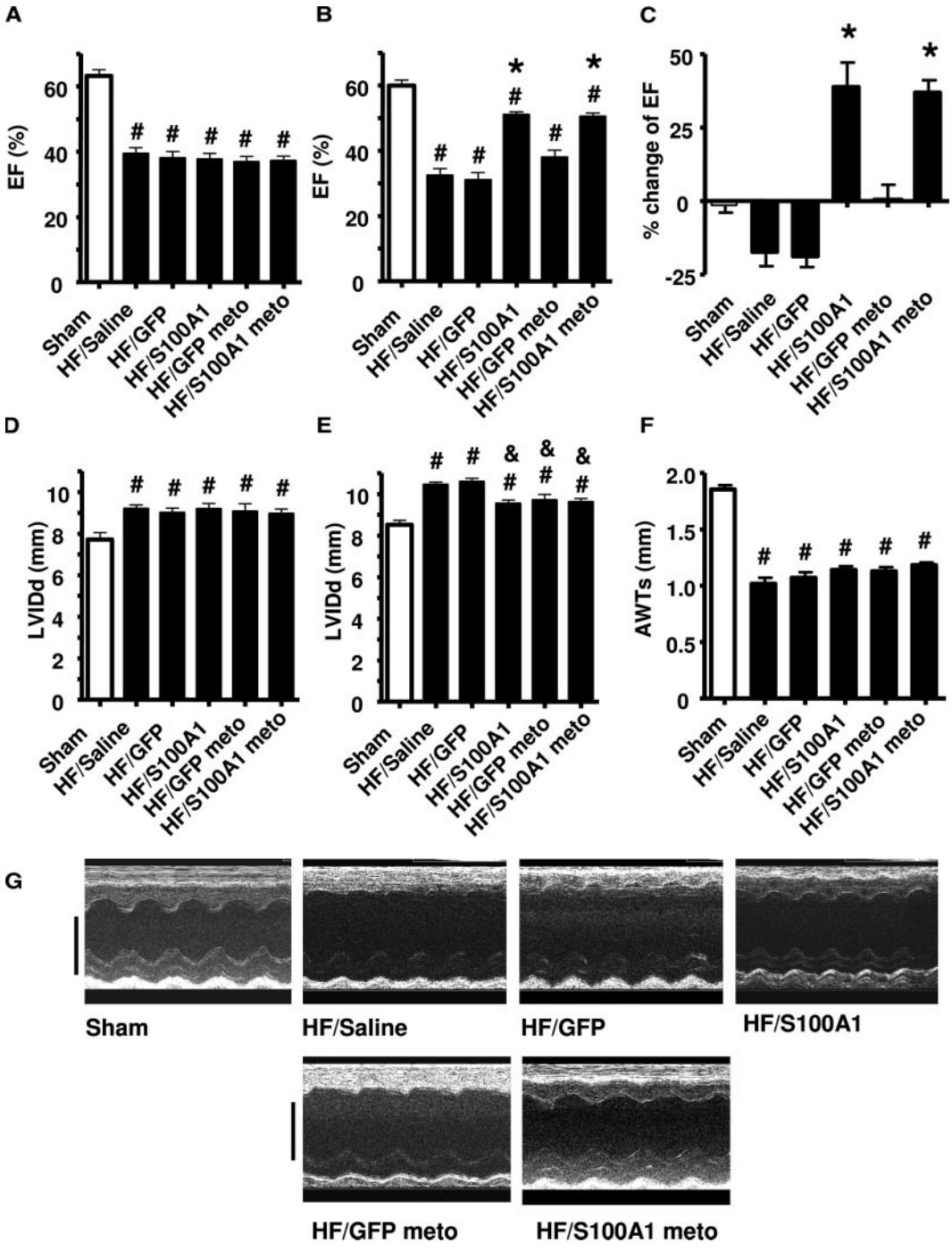

Figure 2. S100A1 gene therapy recovers cardiac function in HF. A, Ejection fraction (EF%) 10 weeks after MI before gene treatment and (B) 8 weeks after in vivo intracoronary AAV6/S100A1 gene delivery with or without metoprolol treatment. C, Percent change in alteration of EF after 8 weeks. D, LV chamber dimensions were similar before gene delivery in all HF groups. E, Effect of AAV6/S100A1 and/or β-blocker treatment on LV chamber dimensions after 8 weeks of treatment. F, Anterior wall thickness (AWT_S) 18 weeks after MI. Sham, n=11; HF/saline, n=14; HF/GFP, n=11; HF/S100A1, n=12; HF/GFP-metoprolol (β), n=9; and HF/S100A1-metoprolol (β), n=9. Data are presented as mean±SEM. Bar=8 mm. G, Representative raw traces of M-mode echocardiography 8 weeks after gene delivery in the 6 experimental groups. *$P<0.05$ vs HF/saline, HF/GFP, or HF/GFP-metoprolol groups; #$P<0.05$ vs sham; &$P<0.05$ vs HF/saline or HF/GFP groups, ANOVA analysis and Bonferroni test between all groups.

the anterior wall (at the site of the infarct) was similarly thinned in all HF groups at 18 weeks after MI and was unchanged after treatment (Figure 2F), which is not surprising because gene delivery was performed 10 weeks after MI when expansion and scaring of the infarct are complete.[19]

After echocardiographic assessment of cardiac function, terminal cardiac catheterization was performed to measure 18-week post-MI hemodynamics. As expected, LV contractility and relaxation as measured by the maximal rate of LV pressure rise (dP/dt) and fall (−dP/dt), respectively, was

S100A1 Gene Therapy Rescues Chronic Heart Failure

	Sham	HF/Saline	HF/GFP	HF/S100A1	HF/GFP-Metoprolol	HF/S100A1-Metoprolol
LV catheterization, basal						
HR, bpm	292±6	295±9	288±7	288±9	264±8*	269±5*
LV dP/dt, mm Hg/s	6559±281	5205±295†	5066±262†	6720±353‡	4948±225†	6390±236‡
LV −dP/dt, mm Hg/s	6565±277	4740±284†	4416±136†	5616±461‡	4418±223†	5450±130‡
LVEDP, mm Hg	2.5±0.4	8.2±0.7†	6.8±0.3†	3.2±0.4§	4.6±0.5§	2.4±0.4§
LVESP, mm Hg	128±2.0	113±2.9†	112±2.5†	124±4.4‡	109±3.1†	118±1.3
Isoproterenol (333 ng/kg BW)						
HR, bpm	341±5	334±4	342±7	349±5	308±6*	310±4*
LV dP/dt, mm Hg/s	14 673±215	9388±215†	9105±381†	11 887±660‡†	9403±491†	10 808±686†
LV −dP/dt, mm Hg/s	8039±317	5543±274†	5490±378†	7566±368‡	5571±333†	6278±302†
LVEDP, mm Hg	1.4±0.2	6.8±1.1†	6.1±0.8†	1.3±0.3§	2.8±0.4§	2.2±0.3§
LVESP, mm Hg	126±1.9	103±2.3†	105±4.3†	121±4.6‡	105±3.7†	113±3.1
HW/BW ratio, g/kg	2.5±0.05	3.1±0.05†	3.06±0.04†	2.76±0.08§	2.77±0.05§	2.81±0.04§
Cell length, μm	104.1±2.4	125.9±3.5†	127.8±3.1†	113.9±2.4‡	118.9±2.3†	110.8±2.3‡

Effect of S100A1 gene therapy in heart failure. Eight weeks after gene therapy in HF, in vivo LV dP/dt, −dP/dt, EDP, end-systolic pressure (ESP), and heart rate (HR) were assessed in sham (n=11), HF/saline (n=12), HF/GFP (n=10), HF/S100A1 (n=11), HF/GFP-metoprolol (n=10), and HF/S100A1-metoprolol (n=10) rats under basal conditions and after maximal isoproterenol stimulation. Ratio of heart weight to body weight (HW/BW) was also assessed in all groups. Also included is the diastolic cell length measured 2 hours after cardiomyocyte isolation from 3 animals per group. Sham (n=34), HF/saline (n=41), HF/GFP (n=43), HF/S100A1 (n=56), HF/GFP-metoprolol (n=44), HF/S100A1-metoprolol (n=65). ANOVA analysis and Bonferroni test were used between all groups. Data are presented as mean±SEM.

*$P<0.05$ vs each non–βAR-blocker–treated group; †$P<0.05$ vs sham; ‡$P<0.05$ vs HF/saline, HF/GFP, or HF/GFP-metoprolol groups; §$P<0.05$ vs HF/saline or HF/GFP.

significantly reduced in failing hearts treated with either saline or GFP compared with the sham group (the Table). Moreover, LV systolic pressure (LVSP) was significantly reduced in the HF/saline and HF/GFP groups, whereas end-diastolic pressure (EDP) was significantly increased in these HF rats (the Table). Representative in vivo LV dP/dt tracings from each group are shown in Figure 3A. After 8 weeks of increased cardiac S100A1 expression, significant improvement can be observed in LV contractile function (Figure 3A and the Table). Interestingly, β-blocker treatment (HF/GFP-metoprolol) significantly reduced LVEDP, although LV dP/dt and LV −dP/dt values were similar to those in the HF/saline and HF/GFP groups (the Table).

S100A1 gene therapy in HF without or with metoprolol increased all measures of cardiac contractile function, including LV systolic pressure and LVEDP, over the 8-week period compared with other HF groups, and S100A1 alone had the largest improvement (the Table). Moreover, S100A1 gene therapy led to a restoration of global myocardial function of failing hearts in vivo because dP/dt, −dP/dt, and end-systolic

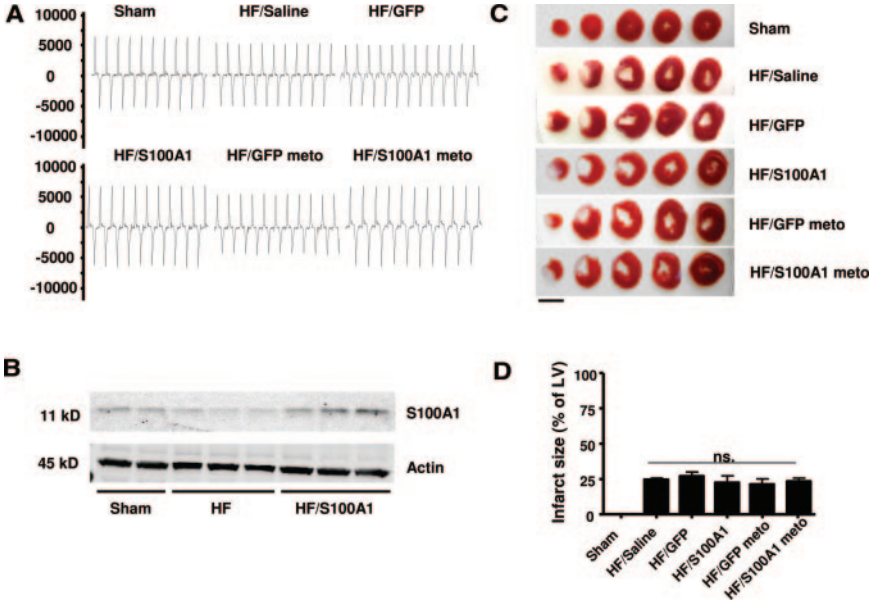

Figure 3. AAV6/S100A1 gene therapy rescues cardiac function in HF. A, Representative raw traces of dP/dt values 8 weeks after intracoronary delivery of AAV6/S100A1 in the 6 experimental groups (18 weeks after MI). B, Western blot from representatives of sham, HF, and HF/S100A1-treated groups showing S100A1 protein expression levels significantly reduced in HF, but delivery of the AAV6/S100A1 construct resulted in S100A1 overexpression. C, Representative triphenyltetrazolium chloride–stained cardiac cross sections 18 weeks after MI. Scale bar=10 mm. D, Average LV infarct size in the 5 HF groups (n=4 for each group).

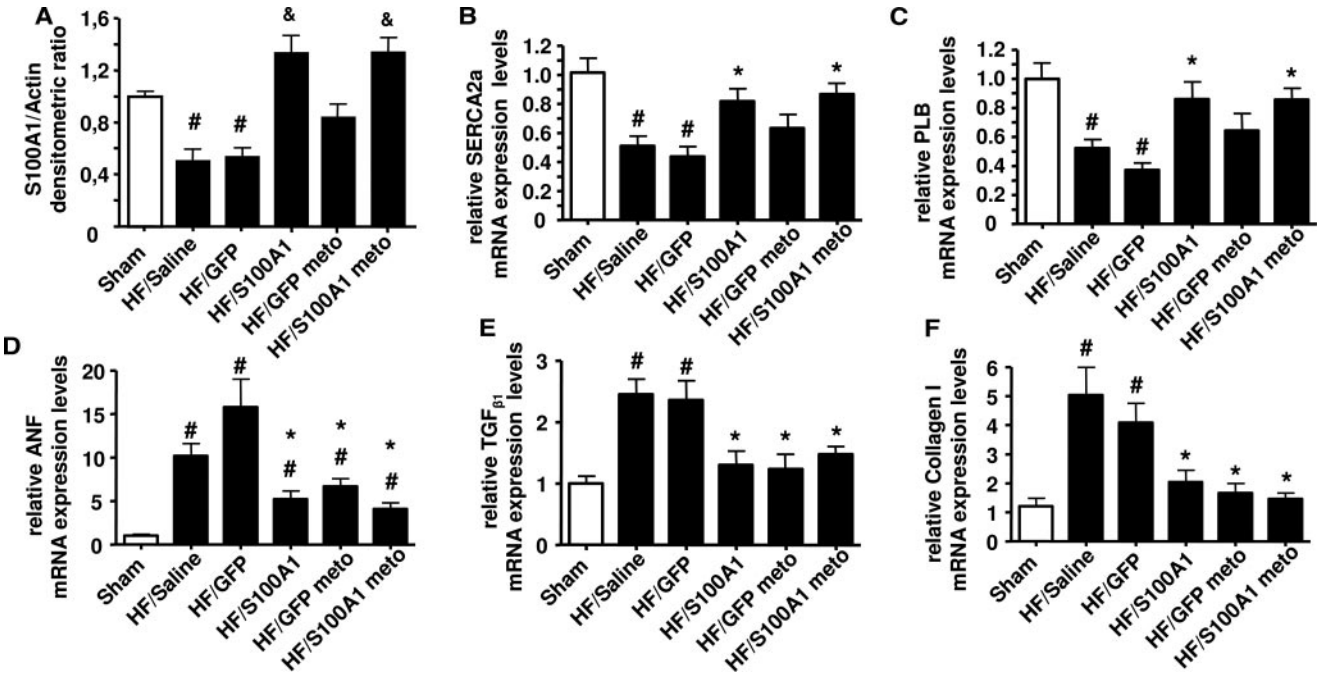

Figure 4. Effect of S100A1 gene therapy and/or β-blocker treatment on cardiac expression of key genes. A, Densitometric quantitative analysis of the ratio of S100A1 to actin of Western blots from LV homogenates in all 6 groups. Relative LV (B) SERCA2a mRNA, (C) phospholamban (PLB) mRNA, (D) atrial natriuretic factor (ANF) mRNA, (E) transforming growth factor-β1, and (F) collagen I mRNA expression in the 6 groups measured via real-time polymerase chain reaction. Sham (n=6), HF/Saline (n=8), HF/GFP (n=8), HF/S100A1 (n=7), HF/GFP-metoprolol (n=6), and HF/S100A1-metoprolol (n=6) were standardized to amplified 18S rRNA and HF levels vs sham values as the control. Data are presented as mean±SEM. *$P<0.05$ vs HF/saline or HF/GFP groups; #$P<0.05$ vs sham; &$P<0.05$ vs sham, HF/saline, HF/GFP, or HF/GFP-metoprolol groups, ANOVA analysis and Bonferroni test between all groups.

pressure could not be statistically distinguished from healthy, sham-operated animals, although the EDP remained elevated (the Table). When failing hearts were challenged with a maximal dose of isoproterenol, chronic S100A1 overexpression continued to improve cardiac performance in vivo (the Table). Heart rate was not affected by MI or in gene therapy groups; therefore, heart rate was not responsible for the functional improvements seen with S100A1 but, as expected, was significantly reduced by metoprolol (the Table).

Cardioselective S100A1 expression was confirmed by Western blotting, and levels from whole-heart homogenates can be seen in Figure 3B. In HF (18 weeks after MI), significant loss of cardiac S100A1 protein levels compared with sham levels occurs, and AAV6-S100A1 gene delivery driving S100A1 expression only in the heart with the α-cardiac actin enhancer/EF1α promoter not only restores normal S100A1 levels but also increases S100A1 protein expression (Figures 3B and 4A). S100A1 protein overexpression was not evident in other tissues in HF rats, whereas GFP had the same expression pattern in rat tissues outside the heart as in Figure 1 (data not shown).

To determine whether all hearts had similar injury, we assessed LV infarct size within a subset of hearts from each group (n=4) via triphenyltetrazolium chloride (TTC) staining. Representative TTC-stained cardiac sections from each group are shown in Figure 3C. Analysis revealed an average infarct size of 22.9±0.9% of the LV (33.2±1.4% of the LV free wall), which was similar in all groups (Figure 3D).

Analysis of Representative Ca^{2+}-Cycling Proteins in HF and Treated Rats

Hearts removed at the end of the 18-week study made it possible to examine gene expression of key Ca^{2+}-cycling molecules associated with cardiac function/dysfunction within our 6 experimental groups. Chronic S100A1 gene therapy significantly increased cardiac S100A1 mRNA and protein as expected, and expression was indeed significantly decreased in the HF groups (Figures 3B and 4A; mRNA data not shown). In addition, SERCA2a and PLB mRNA levels were significantly decreased in the HF/saline and HF/GFP groups 18 weeks after MI compared with sham, and both β-blocker and AAV6/S100A1 treatment attenuated the downregulation of these key SR Ca^{2+}-cycling molecules in HF (Figure 4B and 4C). In fact, AAV6/S100A1 gene therapy resulted in elevated SERCA2a and phospholamban levels that were statistically indistinguishable from sham levels (Figure 4B and 4C), whereas metoprolol administration attenuated the downregulation of SERCA2a, phospholamban, and S100A1 in HF to a lesser degree (Figure 4A, 4B, and 4C).

Cardioselective AAV6/S100A1 Treatment in HF Also Reduces Cardiac Hypertrophy

We also investigated the parameters of post-MI cardiac hypertrophy in the 6 experimental groups. First, the ratio of heart weight to body weight was found to be significantly increased in all HF groups compared with sham, but this was significantly attenuated with metoprolol treatment and

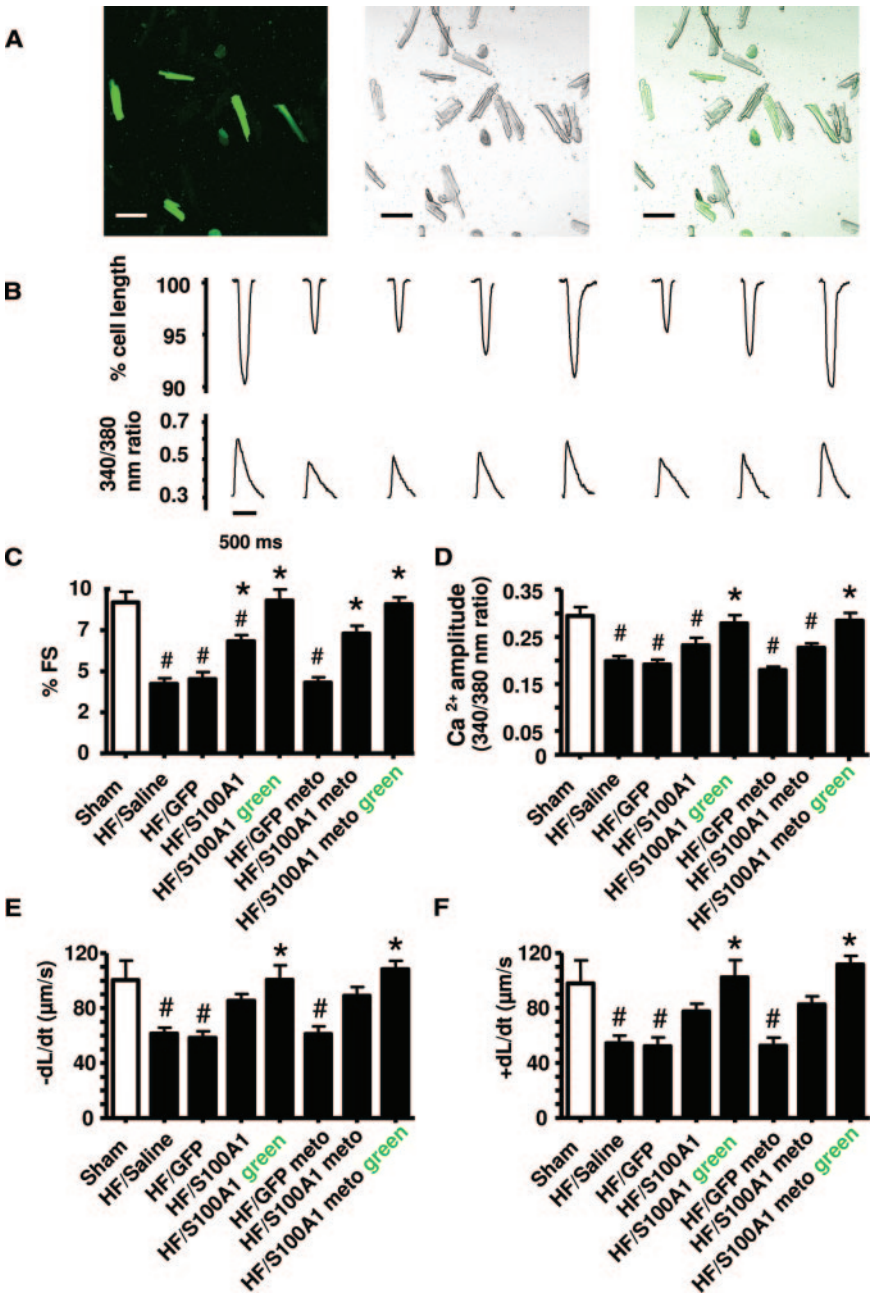

Figure 5. Cardioselective AAV6/S100A1 gene therapy in HF increases contractility and intracellular Ca^{2+} transients of isolated cardiomyocytes. A, GFP expression was used to identify in vivo infected cells. GFP fluorescence (left), light microscopy (middle), and overlay of both (right). Scale bar=100 μm. B, Representative raw traces of fractional shortening (percent change in cell length, shown as downward deflection, top) and original traces of representative $[Ca^{2+}]_i$ transients (shown as upward deflection, bottom) of (from left to right) sham (n=39), HF/saline (n=50), HF/GFP (n=41), HF/S100A1-nongreen (n=29), HF/S100A1-green (n=22), HF/GFP-metoprolol (n=49), HF/S100A1-metoprolol nongreen (n=27), and HF/S100A1-metoprolol green (n=24). C, Percentage of cell shortening (%FS). D, Ca^{2+} amplitude (340/380-nm ratio), (E) rate of cell shortening (−dL/dt), and (F) rate of cell relengthening (dL/dt). Cells were isolated from 3 different rats per group. Measurements in AAV6/S100A1 and AAV6/S100A1+metoprolol were taken from both green (infected) and nongreen (noninfected) myocytes. Data are presented as mean±SEM. To address multiple observations per animal (nongreen versus green cells from the same animal and within the same group), we used a mixed effects model with random intercept for each animal to account for the repeated measurements. The statistical significance of each effect was then determined based on Wald statistics derived from the mixed-effects models. *$P<0.05$ vs HF/saline, HF/GFP, or HF/GFP-β-blocker; #$P<0.05$ vs sham.

S100A1 (the Table). Ventricular atrial natriuretic factor mRNA expression typically associated with cardiac hypertrophy also was significantly increased 18 weeks after MI in all analyzed groups compared with sham (Figure 4D); however, both β-blocker treatment and S100A1 gene therapy significantly reduced cardiac atrial natriuretic factor expression in HF (Figure 4D). On the cellular level, cardiac hypertrophy was reflected by a significantly increased length of isolated cardiomyocytes 18 weeks after MI, which interestingly was significantly reduced only in the AAV6/S100A1 groups (the Table). We also examined cardiac mRNA levels of transforming growth factor-β1 and collagen I mRNA as molecular markers of remodeling. Both were significantly elevated in the HF/saline and HF/GFP groups and were significantly reduced with metoprolol and chronic S100A1 gene therapy in HF (Figure 4E and 4F).

Long-Term S100A1 Gene Therapy Increases Contractile Properties and Ca^{2+} Transients of Cardiomyocytes After MI

To explore the mechanism of long-term S100A1 gene therapy HF rescue, we investigated the contractile performance and intracellular Ca^{2+} handling in freshly isolated LV cardiomyocytes from either sham or failing hearts 8 weeks after the various treatments. Figure 5A contains representative field-light and fluorescent images of isolated myocytes. We took advantage of GFP expression to study cells being infected (green) or not infected by in vivo AAV6 gene transfer. Figure 5B shows representative steady-state twitches (top) and Ca^{2+} transients (bottom) of cardiomyocytes isolated from all 6 experimental groups 2 months after therapeutic intervention.

Myocytes isolated from saline- and GFP-treated HF rats had significant decreases in fractional cell shortening (Figure

5C), the amplitude of the $[Ca^{2+}]_i$ transient (Figure 5D), the rate of myocyte shortening (−dL/dt; Figure 5E), and the rate of myocyte relengthening (dL/dt; Figure 5F) compared with myocytes isolated from sham hearts. Metoprolol treatment alone did not affect contractile properties of isolated failing myocytes under our conditions because $[Ca^{2+}]_i$ transients, percent fractional shortening, dL/dt, and −dL/dt were similar to the HF/saline and HF/GFP groups (Figure 5C through 5F). Data using only infected cells (green) from AAV6/S100A1-treated HF rats showed that S100A1 overexpression completely rescues myocyte dysfunction because contractile parameters and $[Ca^{2+}]_i$ transients were similar in these HF cells compared with nonfailing myocytes (Figure 5C through 5F). Furthermore, the therapeutic effect on isolated myocytes from AAV6/S100A1 gene therapy in HF was preserved under additional β-blocker administration (Figure 5C through 5F). Interestingly, noninfected cardiomyocytes (nongreen) obtained from AAV6/S100A1-treated rats showed a trend toward improved contractile properties and $[Ca^{2+}]_i$ transients compared with the HF/saline, HF/GFP, and HF/GFP–β-blocker groups (Figure 5C through 5F).

Discussion

The data presented above demonstrate the use of a novel rAAV6 vector containing a cardioselective promoter to support chronic therapeutic gene expression in the failing heart. Using this novel vector in a previously described chronic post-MI rat HF model,[13] we show that cardioselective long-term S100A1 gene therapy can reverse global in vivo cardiac dysfunction and attenuate LV remodeling. Notably, improved function in HF after cardioselective S100A1 treatment remained at least 8 weeks after in vivo gene delivery, providing evidence for a sustained therapeutic effect. This finding is in line with previous results providing proof of concept for effective adenovirus-mediated S100A1 gene therapy of HF, supporting S100A1 as a novel therapeutic target for HF.[13] Moreover, S100A1-mediated recovery of functional properties of failing myocardium was preserved with additional pharmacological βAR-blocker treatment. Thus, S100A1 gene addition could represent a viable future clinical approach for HF treatment and add to existing drug treatment.

Our choice for directing cardioselective gene expression was a sequence from the proximal enhancer region of the α-cardiac actin gene. Previously, this region that contains a MEF2 sequence was shown to direct heart but not skeletal muscle expression.[18] A second MEF2 site was added to potentially drive stronger gene expression in the heart. As our results show, the enhancer element cloned in front of the EF1α promoter produces robust expression of S100A1 that was specifically localized to the normal and failing rat heart after in vivo intracoronary delivery. Tissue selectivity was confirmed by including a second transgene cassette in this rAAV6 vector containing CMV-GFP; indeed, GFP expression was found in several extracardiac sites after intracoronary delivery. We excluded that posttranscriptional regulation limits S100A1 expression to cardiac tissue because S100A1 mRNA overexpression was not present in organs such as lung, liver, testis, and skeletal muscle, whereas S100A1 mRNA expression was increased 3-fold in isolated cardiomyocytes from rAAV6-S100A1–treated HF rats (data not shown). To the best of our knowledge, this is the first demonstration of long-term, myocardium-specific gene expression after in vivo gene delivery and represents the use of a vector that, after being tested in larger animal models, could have potential clinical utility.

In our HF model, rats were found to have significant LV dysfunction and remodeling at 10 weeks after MI, which was in line with previous results[13] and similar in all randomized treatment groups. Consistent with clinical HF development,[20–22] cardiac contractile function deteriorated further over the 2-month treatment period in control groups. However, S100A1 enhancement after cardioselective AAV6-mediated gene delivery led to functional recovery of the failing rat heart seen globally with increased percent ejection fraction and dP/dt and lower EDP; HF rescue also was seen in individual ventricular cardiomyocytes. Moreover, enhanced contractile function of S100A1-treated failing hearts was preserved under maximal βAR stimulation, further demonstrating the therapeutic potential of S100A1. Importantly, S100A1 is reduced in the failing rat heart,[13] consistent with human HF,[16] and restoration to the supranormal levels responsible for the rescue seen in this study reveals a critical role for this protein in Ca^{2+}-dependent cardiac regulation and function. These findings are in line with our recent data generated from S100A1 knockout mice demonstrating that the loss of S100A1 protein contributes significantly to the progressive deterioration of cardiac function in post-MI HF, whereas preservation of cardiac S100A1 protein is protective.[12]

Functional recovery of myocardial function in AAV6/S100A1-treated HF rats was accompanied by mitigated LV chamber dilatation and cardiac hypertrophy. These data are in line with previous findings[13] and might be explained in multiple ways. First, there may be an indirect effect resulting from enhanced contractile function of the heart; thus, a reduced biomechanical overload may result in a reverse remodeling situation. Additionally, improved $[Ca^{2+}]_i$ handling in failing cardiomyocytes resulting from rAAV6-S100A1 treatment might beneficially affect myocardial apoptosis, hypertrophy, or gene expression by modulating various Ca^{2+}-dependent signaling pathways involving calcineurin, calmodulin kinase, or protein kinase C isoforms (α, β, γ).[23] Alternatively, because the loss of S100A1 has previously been shown to be permissive for the induction of genes involved in cardiac hypertrophy,[12,13,24] increased S100A1 levels may more directly influence the silencing of these genes, leading to attenuation of hypertrophy and accompanied dilatation. Regardless of the mechanism, it is apparent that enhanced expression of S100A1 induces these beneficial effects chronically on the failing heart.

An interesting finding at the myocyte level was that when only rAAV6-S100A1–infected cardiomyocytes (green cells) were studied, a complete restoration of $[Ca^{2+}]_i$ transients occurred, and contractile parameters as S100A1-treated HF myocytes had similar values to healthy cells 8 weeks after gene therapy. Moreover, data from isolated cardiomyocytes also reveal a potential indirect therapeutic effect of cardiac S100A1 gene delivery on cells that were not infected in vivo

because these non-GFP myocytes displayed a trend toward increased functional properties. This is especially interesting because functional recovery of the failing heart globally was achieved despite inhomogeneous gene delivery being in line with previous studies.[13,17a] Our data using GFP expression in myocytes showed ≈40% infection rate; thus, there may be an indirect effect of S100A1-overexpressing myocytes to improve the function of neighboring myocytes. Furthermore, the reduced wall stress and regression of maladaptive hypertrophy may allow noninfected myocytes to recover on their own. Alternatively, in vivo AAV6/S100A1 gene delivery might be underestimated by GFP coexpression because the brightness of GFP fluorescence varied substantially between isolated myocytes (data not shown), or GFP expression might not fully match S100A1 overexpression levels because 2 different promoters were used to drive S100A1 and GFP expression.

To increase potential clinical relevancy, we added a βAR-blocker component. Metoprolol administration in HF significantly attenuated LV remodeling, reduced cardiac hypertrophy, lowered EDP, and prevented further deterioration of cardiac function in HF. However, βAR-blocker treatment did not affect functional properties of isolated failing cardiomyocytes and failed to recover functional properties of in vivo global cardiac function under our conditions. These findings are in line with observations in several rodent HF and post-MI models showing that selective β_1-blockade attenuates post-MI structural remodeling without concomitant improvement in myocardial function.[25–27]

Clinical studies such as the Metoprolol Controlled-Release Randomized Intervention Trial in Heart Failure (MERIT-HF) have proved that β-blocker therapy in patients with HF not only can attenuate pathological remodeling of the heart but also may actually improve patient outcomes.[28,29] Therefore, preservation of S100A1-mediated positive inotropic effects under β-blocker treatment, as observed in this study, suggests potentially additive action of both strategies and supports a potential future clinical application of S100A1 gene therapy in HF, which could become safer with a cardioselective approach like that described here.

Because short-term in vivo strategies already proved therapeutic effects of myocardial S100A1 gene delivery in both acute MI and in overt HF,[13,17a] a potential goal of the present study was to investigate long-term actions of S100A1 in HF. This is especially important because cytosolic Ca^{2+} overload and excess cAMP generation under chronic pharmacological inotropic treatment in HF are associated with increased mortality.[30,31] Long-term S100A1 gene therapy resulted in recovery of contractile function in HF, which was mediated, at least largely, by rescued intracellular Ca^{2+} turnover and SR Ca^{2+} cycling mechanistically similar to adenoviral S100A1 gene therapy.[13,17a] Importantly, S100A1 protein can decrease Ca^{2+} spark activity in ventricular cardiomyocytes under diastolic conditions, which might contribute to attenuate detrimental diastolic Ca^{2+} overload and SR Ca^{2+} leakage in HF through S100A1.[12,13,32] Therefore, targeting both ryanodine receptor open probability and SERCA2a activity by S100A1[12–14,32] might be advantageous compared with solely increasing SERCA2a function, which can result in impaired survival in response to ischemic events.[33]

To summarize, our study shows that long-term in vivo cardioselective S100A1 gene therapy is feasible with the α-cardiac actin enhancer/EF1α promoter in a rAAV6 vector and that it is indeed therapeutic. Moreover, effects of S100A1 gene therapy in HF are preserved under β-blocker treatment in vivo and indicate that both treatment strategies might be additive in HF.

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Disclosures

Drs Katus, Koch, Remppis, Most, Rabinowitz, and Pleger have a patent pending on the use of S100A1 in treating heart disease and on the use of the α-cardiac actin enhancer/EF1α promoter. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

In the present study, we provide proof of concept for the long-term therapeutic effectiveness of cardiac S100A1 gene therapy in the context of a clinically relevant experimental model of overt heart failure. Taking advantage of adeno-associated virus serotype 6 gene transfer in combination with a novel cardiomyocyte-specific enhancer/promoter, cardiac S100A1 gene delivery restored contractile performance and reversed cardiac remodeling of chronically failing hearts. This translational approach stems from the observation of diminished S100A1 protein levels in failing human myocardium and underscores the significant therapeutic potential of S100A1. Given the fact that S100A1-mediated therapeutic effects in our study lasted for months without detrimental effects, it is important to point out that the inotropic molecular support conveyed through S100A1 does not rely on β-adrenergic receptor signaling involving, for example, cAMP, for which long-term clinical use has been proved to be deleterious in failing human hearts. Rather, S100A1-mediated inotropy is based on balanced improvement of sarcoplasmic reticulum Ca^{2+} cycling, targeting both the cardiac ryanodine receptor isoform and the Ca^{2+}-ATPase/phospholamban complex that is directly translated into enhanced contractile performance. In the present study, this therapeutic modality exerted long-term therapeutic effects that were superior even to metoprolol, a clinically established and approved heart failure therapy in humans. Of note, metoprolol was able only to prevent but not reverse progressive deterioration of contractile function (as S100A1 gene therapy did) in our experimental setting. Thus, this study enables preclinical testing of adeno-associated virus serotype 6–S100A1 gene therapy in large-animal studies, and clinical use of S100A1 heart failure therapy is now within reach.

Stable Myocardial-Specific AAV6-S100A1 Gene Therapy Results in Chronic Functional Heart Failure Rescue

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