S100A1 Genetically Targeted Therapy Reverses Dysfunction of Human Failing Cardiomyocytes

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Objectives
This study investigated the hypothesis whether S100A1 gene therapy can improve pathological key features in human failing ventricular cardiomyocytes (HFCMs).

Background
Depletion of the Ca²⁺-sensor protein S100A1 drives deterioration of cardiac performance toward heart failure (HF) in experimental animal models. Targeted repair of this molecular defect by cardiac-specific S100A1 gene therapy rescued cardiac performance, raising the imminent question of its effects in human failing myocardium.

Methods
Enzymatically isolated HFCMs from hearts with severe systolic HF were subjected to S100A1 and control adeno-viral gene transfer and contractile performance, calcium handling, signaling, and energy homeostasis were analyzed by video-edge-detection, FURA2-based epifluorescent microscopy, phosphorylation site-specific antibodies, and mitochondrial assays, respectively.

Results
Genetically targeted therapy employing the human S100A1 cDNA normalized decreased S100A1 protein levels in HFCMs, reversed both contractile dysfunction and negative force-frequency relationship, and improved contractile reserve under beta-adrenergic receptor (β-AR) stimulation independent of cAMP-dependent (PKA) and calmodulin-dependent (CaMKII) kinase activity. S100A1 reversed underlying Ca²⁺ handling abnormalities basally and under β-AR stimulation shown by improved SR Ca²⁺ handling, intracellular Ca²⁺ transients, diastolic Ca²⁺ overload, and diminished susceptibility to arrhythmogenic SR Ca²⁺ leak, respectively. Moreover, S100A1 ameliorated compromised mitochondrial function and restored the phosphocreatine/adenosine-triphosphate ratio.

Conclusions
Our results demonstrate for the first time the therapeutic efficacy of genetically reconstituted S100A1 protein levels in HFCMs by reversing pathophysiological features that characterize human failing myocardium. Our findings close a gap in our understanding of S100A1’s effects in human cardiomyocytes and strengthen the rationale for future molecular-guided therapy of human HF. (J Am Coll Cardiol 2011;58:966–73) © 2011 by the American College of Cardiology Foundation

Heart failure (HF) remains a major cause of morbidity and mortality (1), and a currently unmet need for novel therapeutic strategies requires clinically applicable treatment options targeting the underlying molecular causes of the...
disease. A promising innovation entering the clinical stage is genetically targeted therapy of human HF (2). Ongoing early stage phase I/II human clinical trials by Hajjar et al. (3) determined safety and therapeutic efficacy of gene-based restoration of an enzyme improving cardiomyocyte Ca2+ handling in patients with advanced HF. These studies, therefore, pave the way for the development of molecular-guided treatments for HF aiming at the roots of the disease.

The EF-hand Ca2+-sensor protein S100A1 has emerged as an attractive target for genetically targeted HF therapy because the association between decreased S100A1 expression in cardiomyocytes and the progression to systolic HF has been well documented. Translational studies in small- and large-animal HF models identified S100A1 as a unique regulator of an integrative and Ca2+-controlled network in cardiomyocytes improving sarcoplasmic reticulum (SR), sarcomeric, and mitochondrial function (reviewed in Rohde et al. [4]). Comprehensive actions of S100A1 are modulation of ryanodine receptor (RyR2), SR Ca2+-ATPase (SERCA2), cardiac titin, and mitochondrial function (reviewed in Rohde et al. [4]). As a result, hearts with increased S100A1 expression exhibit superior systolic and diastolic performance (6,10,11), as well as mitochondrial high-energy phosphate generation (8,9), whereas mice with cardiac S100A1 deficiency show accelerated HF and fatal arrhythmias in response to chronic hemodynamic stress and ischemic damage (12–15). Mechanistically, S100A1 improves cardiomyocyte performance by augmenting Ca2+ transient amplitudes and decreasing diastolic Ca2+ overload based on increased Ca2+-induced SR Ca2+ release together with decreased diastolic SR Ca2+ leak and accelerated Ca2+ resequestration (5–11,14,16).

Preclinical studies have clearly documented the association of decreased cardiomyocyte S100A1 expression with control cells. Normalization of S100A1 even reversed the negative force-frequency response (FFR) seen in control HFCMs, whereas the Ad-GFP control virus did not change diminished S100A1 protein levels (Fig. 1B). Our in vitro gene therapy protocol apparently restored S100A1 protein levels to approximately normal levels in HFCMs, whereas mice with cardiac S100A1 deficiency show accelerated HF and fatal arrhythmias in response to chronic hemodynamic stress and ischemic damage (12–15). Mechanistically, S100A1 improves cardiomyocyte performance by augmenting Ca2+ transient amplitudes and decreasing diastolic Ca2+ overload based on increased Ca2+-induced SR Ca2+ release together with decreased diastolic SR Ca2+ leak and accelerated Ca2+ resequestration (5–11,14,16).

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**Results**

Genetically targeted therapy reconstitutes S100A1 expression in human failing cardiomyocytes. S100A1 protein and mRNA levels were decreased in ischemic cardiomyopathy by 3.5-fold (Fig. 1A) and 5.0-fold (data not shown), respectively, compared with healthy normal myocardium. These findings suggest that abnormal transcriptional regulation causes aberrant S100A1 protein expression in advanced stages of HF, and corroborate earlier results by Remppis et al. (19). Human S100A1 cDNA was delivered to isolated HFCMs by adenoviral infection (Ad-S100A1), resulting in significantly enhanced S100A1 protein levels 24 h after transfection (Fig. 1B) (+3.2-fold, p < 0.05, n = 5). In our in vitro gene therapy protocol apparently restored S100A1 protein levels to approximately normal levels in HFCMs, whereas the Ad-GFP control virus did not change diminished S100A1 protein levels (Fig. 1B). Expression of other Ca2+-handling proteins in HFCMs, such as down-regulated SERCA2a and increased NCX were unchanged (Online Fig. 1).

**S100A1 gene replacement improves contractility and reverses negative force-frequency relation.** HFCMs with restored S100A1 protein levels showed significant improvement of fractional shortening (FS) (Figs. 2A and 2B) at all tested frequencies (0.2 to 2 Hz), indicating concurrent enhancement of systolic and diastolic function compared with control cells. Normalization of S100A1 even reversed the negative force-frequency response (FFR) seen in control HFCMs, which is a hallmark of human failing hearts (20). In vitro S100A1 therapeutic effects were most prominent at

**Materials and Methods**

Human ventricular myocardium was obtained from 27 patients with severe ischemic systolic HF undergoing heart transplantation. Protocols for cardiomyocyte isolation, generation of the adenovirus used to express S100A1 in
cultured HFCMs, analysis of cardiomyocyte contractility and Ca2+ handling, RNA and protein expression, SR Ca2+ fluxes, mitochondrial function, and high-energy metabolites are detailed extensively in the Online Appendix. Results are presented as mean ± SEM. Comparisons between treatment groups were made using unpaired 2-tailed Student t test and analysis of variance followed by the Student-Newman-Keuls method for post hoc analysis. Fisher exact test was used to compare percentage of diastolic Ca2+ waves between groups by comparing paced cardiomyocytes without, to cardiomyocytes with diastolic Ca2+ waves. For all tests, a probability value <0.05 was considered significant.

**Abbreviations and Acronyms**

- βAR = beta-adrenergic receptor
- Ad = adenoviral
- CaMKII = calmodulin-dependent protein kinase II
- FFR = force-frequency response
- FS = fractional shortening
- HF = heart failure
- HFCM = human failing cardiomyocyte
- PC/ATP = phosphocreatine/adenosine-triphosphate
- PKA = cAMP-dependent protein kinase A
- RyR2 = Ryanodine Receptor 2
- SERCA2 = Sarcoplasmic Reticulum Ca2+-ATPase 2
- SR = sarcoplasmic reticulum
1 and 2 Hz, reflecting clinically relevant human heart rates in vivo (Figs. 2A and 2B).

**S100A1 gene-based therapy improves cellular Ca
2+
 handling and SR Ca
2+
 load in HFCMs.** Ca
2+
 measurements in field-stimulated HFCMs revealed significantly increased systolic Ca
2+
 transient amplitudes (control 152 ± 31 nmol/l vs. S100A1 389 ± 45 nmol/l, p < 0.05) and enhanced SR Ca
2+
 load (control 314 ± 22 nmol/l vs. S100A1 468 ± 36 nmol/l, p < 0.05) in S100A1-treated cells (Figs. 3A and 3B). Accordingly, diastolic cytosolic Ca
2+
 concentrations (control 302 ± 44 nmol/l vs. S100A1 187 ± 16 nmol/l, p < 0.05) and the normalized Ca
2+
 50% transient decay (control 413 ± 23 ms vs. S100A1 301 ± 24 ms, p < 0.05) were significantly lower in S100A1-expressing HFCMs, which was accompanied by significantly greater enzymatic SERCA2 activity (+1.51-fold vs. control HFCMs, p < 0.05) (Online Fig. 2). In SR vesicles treated with 100 nmol/l hrS100A1 protein (p < 0.05 vs. vehicle; n = 5), which was associated with a 2.1-fold significantly enhanced S100A1/SERCA2 interaction (Online Fig. 3), Ca
2+
 uptake and Ca
2+
 ATPase activity improved by 1.9- and 2.4-fold, respectively. Overall, these data point toward a direct effect of S100A1 on SERCA2 to stimulate accelerated SR Ca
2+
 resequestration and improve diastolic function in HFCMs.

**S100A1 improves contractile reserve mechanisms independent of cAMP-dependent and calmodulin-dependent kinase activity.** In light of previous studies showing preserved effects of S100A1 in failing myocardium with concurrent beta-adrenergic receptor (β-AR) stimulation, we determined Ca
2+
 handling in the presence of the β-AR agonist isoproterenol. As shown in Figures 4A and 4B, β-AR-stimulated S100A1-expressing HFCMs exhibited a significantly greater relative increase in systolic Ca
2+
 transient amplitudes (S100A1 +52 ± 4% vs. control 23 ± 6%, p < 0.05) and preserved gain in SR Ca
2+
 load over controls (nmol/l; control 367 ± 35 vs. S100A1 551 ± 39, p < 0.05). Despite increased β-AR responsiveness, subsequent analysis of downstream cAMP-dependent protein kinase A (PKA) and calmodulin-dependent protein kinase II (CaMKII) activity revealed a similar increase in phosphorylation levels of phospholamban (PLB) in S100A1-expressing and control HFCMs from equal baseline phosphorylation (relative n-fold increase; PLB-ser 16/PLB-thr 17 2.2 ± 0.6 control, p = NS; PLB-ser 16/PLB-thr 17 1.7 ± 0.6 S100A1 vs. +1.8 ± 0.5 control, p = NS) (Figs. 5A and 5B). These results indicate that S100A1 therapeutic effects under basal and β-AR stimulation, at least on diastolic SR Ca
2+
 uptake, do not emanate from altered PKA and CaMKII activity.

**S100A1 genetically targeted therapy prevents arrhythmogenic SR Ca
2+
 leak in HFCMs.** Given the risk of arrhythmogenic Ca
2+
 leak by reloading the SR with Ca
2+
, we assessed whether S100A1 effects render HFCMs susceptible to diastolic Ca
2+
 wave formation. Eighty percent (20 of 25) of control but only 13% (3 of 25) of S100A1-expressing field-stimulated (2 Hz) HFCMs exhibited diastolic Ca
2+
 waves in response to β-AR stimulation (Fig. 6A) (p < 0.05 control vs. S100A1), indicating protection from a potential arrhythmogen-
genic substrate for delayed after-depolarization (21). The protective effect of S100A1 in HFCMs was associated with augmented binding to the human RyR2 (S100A1/RyR2 ratio; control 0.42 vs. S100A1/RyR2 ratio 0.86, p < 0.05) (Fig. 6B) and occurred despite a significantly higher SR Ca\(^{2+}\) load than control cells (Fig. 4B). Enhanced S100A1/RyR2 interaction (S100A1/RyR2 ratio; control 0.42 vs. S100A1/RyR2 ratio 0.86, p < 0.05) in treated SR vesicles suggests a direct inhibitory effect of S100A1 on RyR2 dysfunction, given a 2.8-fold decrease in Ca\(^{2+}\) leak from S100A1-treated SR vesicles (Online Fig. 3).

Compromised energy homeostasis and mitochondrial function in HFCMs is ameliorated through S100A1 gene replacement. Since elevated diastolic Ca\(^{2+}\) levels and SR Ca\(^{2+}\) leak can disturb cardiomyocyte energy homeostasis (22), we assessed the impact of S100A1 on high-energy phosphate content. The phosphocreatine/adenosine-triphosphate (PC/ATP) ratio was significantly elevated after S100A1 gene delivery (2.4 ± 0.05) to previously published near-normal levels (5,23) as opposed to control with a ratio of 1.1 ± 0.2 (p < 0.05 vs. S100A1) (Fig. 7A). Functional analysis of isolated mitochondria from S100A1-expressing HFCMs revealed superior electron flow even under Ca\(^{2+}\) stress (Fig. 7B) and enhanced mitochondrial S100A1 protein content (Online Figure 4) compared with controls. S100A1 restoration in HFCMs was further accompanied by preserved ΔΨm basally and in response to Ca\(^{2+}\) treatment (Online Fig. 5). Isolated mitochondria from S100A1-expressing HFCMs further unveiled diminished susceptibility to mitochondrial permeability transition in response to experimental Ca\(^{2+}\) overload and phosphate stress (Online Fig. 6). Overall, these findings suggest a potentially direct effect of S100A1 and further corroborate S100A1’s beneficial actions on mitochondrial function and energy homeostasis in HFCMs.

Discussion

This study shows the profound effects of S100A1 genetically targeted therapy in HFCMs and translates previous mechanistic and therapeutic findings in animal HF models towards S100A1 gene-based treatment of human failing myocardium in vitro (for synopsis, see Online Fig. 7).

Therapeutic efficacy of restored S100A1 protein expression in human HF. A key feature of HF seems to be depletion of S100A1 in cardiomyocytes, which results in defective intracellular Ca\(^{2+}\) cycling and poor contractility (4). Our...
study shows that therapeutic actions of S100A1 in HFCMs might require only modest changes in S100A1 expression obtainable by myocardial-targeted, viral-based gene addition. Sustained therapeutic consequences of similarly modest changes in cardiomyocyte S100A1 expression in various experimental HF models (3,4) prompt speculation that even a moderate S100A1 gene dosage may achieve therapeutically effective changes in human failing myocardium in vivo. Further proof of in vitro therapeutic efficacy stems from the observation that S100A1 can rescue defective SR function and Ca^{2+} handling in HFCMs despite continued abnormalities in the expression of key sarcoplasmic (SERCA2) and sarcolemmal (NCX) Ca^{2+}-regulator proteins. Similar findings in experimental HF (5,17,18) corroborate the notion that S100A1 neither requires nor depends on normalized SERCA2 and NCX expression, at least for acute therapeutic actions in vitro.

**Key role of restored SR function and Ca^{2+} homeostasis.** The SR is critical in the regulation of human cardiomyocyte Ca^{2+} homeostasis (24). S100A1 seems capable of restoring SR function in HFCMs, which is most likely key to improved systolic SR Ca^{2+} release being translated into augmented intracellular Ca^{2+} transients with accelerated removal of cytosolic Ca^{2+} and reversal of diastolic Ca^{2+} overload. These results are in line with previously characterized molecular mechanisms in animal HF models relating S100A1 salutary actions to improved RyR2 and SERCA2 function with subsequent rescue of systolic and diastolic contractile function, prevention of arrhythmogenic Ca^{2+} leak, and ameliorated energy homeostasis (5,6,10,14,17,25).

**Therapeutic impact on contractile dysfunction.** The ability of the SR to transport larger amounts of Ca^{2+} appears critical for frequency-dependent enhancement in contractile strength (20). Hence, reversal of the negative FFR in HFCMs may be attributed to S100A1-mediated frequency-dependent improvement of SR Ca^{2+} load and, subsequently, augmented intracellular systolic Ca^{2+} transient amplitudes. Of note, the S100A1-mediated effect on peak systolic Ca^{2+} levels did not prolong relaxation but rather improved diastolic function despite persistent abnormalities in SERCA2 and NCX expression, likely due to its concurrent impact on diastolic SR Ca^{2+} uptake in HFCMs. The latter indicates that a previously described interaction of S100A1 with SERCA2 both in human (7) and rodent myocardium (5,10) might actually reflect a relevant stimulus of diastolic SR Ca^{2+} resequstration.

The therapeutic effect of S100A1 on contractile performance of HFCMs appeared most prominent at frequencies...
approximating actual beating rates of normal and diseased human hearts (60 to 120 beats/min). Since rodent myocardium per se exhibits a negative contractile response at human heart rates (26) and does not faithfully mirror the human phenotype with respect to Ca²⁺ handling, discovery of this potentially relevant in vitro therapeutic effect is dependent on the use of HFCMs.

**Prevention of arrhythmogenic SR Ca²⁺ leak by S100A1 gene-based therapy.** A potentially fatal side effect of drugs or molecular interventions that reload the SR with Ca²⁺, such as β-AR agonists, is arrhythmogenic diastolic Ca²⁺ leak (21). Our data show that S100A1 effectively prevents diastolic Ca²⁺ wave formation in experimentally forced SR Ca²⁺ leakage despite enhanced SR Ca²⁺ load. Increased S100A1 interaction with the RyR2 under these conditions prompts speculation that it directly modulates RyR2 diastolic function, because similar findings in animal HF models corroborate these data (5,6,10,14,17,25). Thus, although the molecular mechanism remains to be determined, S100A1 seems to be a unique molecular therapeutic with respect to SR Ca²⁺ handling, potentially superior to β-AR inotropic agonists by combining enhanced contractile function with prevention of arrhythmogenic Ca²⁺ leak.

**β-AR–independent improvement of contractile reserve.** The S100A1-mediated gain in contractile performance and SR Ca²⁺ handling in HFCMs was preserved under β-AR stimulation and, subsequently, improved β-AR reserve. Further investigation of putative downstream signaling seems to suggest that S100A1 therapeutic actions under basal and stimulated conditions in HFCMs neither depend on nor include detrimental and pro-arrhythmogenic PKA and CaMKII activity. This is in line with previous studies showing that S100A1-mediated improvement of cardiac function in experimental HF models is additive to stimulation with β-AR agonists but independent of downstream PKA and CaMKII activity (5,6,10,14,17,18). These findings support the notion that targeted S100A1 restoration in failing myocardium acts synergistically with catecholamine therapy and could even lower the dosage and duration of toxic, pro-arrhythmogenic cAMP-targeted therapies in sys-
Clinical implications. Our study demonstrates that modest changes of S100A1 protein by genetically targeted gene addition are actually therapeutic in failing human myocardium in vitro. The unique molecular profile of S100A1 originates from reversing clinically relevant key features in HFCMs. S100A1 might, therefore, enable long-term therapy of human failing myocardium without pro-arrhythmic side effects and increased energy consumption typically seen with the use of conventional inotropic agents such as sympathomimetic agents and phosphodiesterase inhibitors. Given the proven therapeutic efficacy of S100A1 in pre-
clinical studies, these findings might well present the rationale for a new treatment targeting a key deficiency in advanced human HF. Since synergistic effects of S100A1 with β-AR blockers were observed in experimental HF (4), it is tempting to speculate that future targeted S100A1 gene therapy may complement conventional drug regimens for systolic HF treatment.

Conclusions

This study is the first to document the therapeutic effectiveness of restored S100A1 protein expression in human failing myocardium in vitro. Given the recent evolution of increasingly efficient gene transfer technologies, which have placed congestive HF within the reach of gene-based clinical therapy, these promising results provide the necessary insight into the molecular and cellular mechanisms of S100A1 action in isolated cardiomyocytes, building a strong rationale for its further translation.

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APPENDIX

For an expanded Methods section and supplemental figures and their legends, please see the online version of this article.