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RESEARCH ARTICLE

S100A1 gene transfer: a strategy to strengthen engineered cardiac grafts

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Abstract

Background Cardiac tissue replacement therapy, although a promising novel approach for the potential treatment of heart failure, still suffers from insufficient contractile support to the failing myocardium. Here, we explore a strategy to improve contractile properties of engineered heart tissue (EHT) by S100A1 gene transfer.

Methods EHTs were generated from neonatal rat cardiomyocytes and transfected (MOI 10 PFU) with the S100A1 adenovirus (AdvS100A1, $n = 25$) while an adenovirus devoid of the S100A1 cDNA served as a control (AdvGFP, $n = 30$). Contractile properties of transfected EHTs were measured 7 days following gene transfer.

Results Western blot analysis confirmed a 8.7 ± 3.6-fold S100A1 protein overexpression in AdvS100A1-transfected EHTs ($n = 4$; $P < 0.01$) that increased maximal isometric force (mN; AdvGFP 0.175 ± 0.03 vs. AdvS100A1 0.47 ± 0.06; $P < 0.05$) at 0.4 mmol/L extracellular calcium concentration $[Ca^{2+}]_e$. In addition, S100A1 overexpression enhanced both maximal Ca^{2+}-stimulated force generation (+81%; $P < 0.05$) and Ca^{2+}-sensitivity of EHTs (EC50% $[Ca^{2+}]_e$ mM; AdvGFP 0.33 ± 0.04 vs. AdvS100A1 0.21 ± 0.0022; $P < 0.05$). The S100A1-mediated gain in basal graft contractility was preserved throughout a series of isoproterenol interventions (10^{-9} to 10^{-6} M). Physiological properties of EHTs resembling intact heart preparations were preserved.

Conclusions S100A1 gene transfer in EHT is feasible and augments contractile performance, while characteristic physiological features of EHT remain unchanged. Thus, specific genetic manipulation of tissue constructs prior to implantation should be part of an improved tissue replacement strategy in heart failure. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords cardiac tissue engineering; gene transfer; contractile function; S100A1; cardiac tissue replacement therapy

Introduction

Loss of myocardium due to acute myocardial infarction (AMI) triggers a sequence of molecular, cellular and physiological responses leading to left ventricular (LV) remodelling and eventually heart failure [1–3]. Recent advances in clinical therapy with the aim of preventing progressive deterioration of ventricular function, however, failed *to regenerate* infarcted myocardial tissue. Therefore, there is a need to develop novel therapeutic approaches. One experimental attempt, the replacement of defective cardiac tissue by

in vitro designed, functioning myocardium, offers an exciting option in cardiovascular medicine [4–6]. Based on the capacity of isolated immature cardiac myocytes to reconstitute three-dimensional (3D) cardiac tissue-like structures, Eschenhagen *et al*. developed a model of contracting syncytoid tissue consisting of cardiac myocytes [7]. This engineered heart tissue (EHT) preparation, as it is termed, exhibits morphological and functional properties of myocytes within native differentiated myocardium including sarcomeres, desmosomes, gap junctions and a T-tubular system with dyad formation [7,8].

Recently, Zimmermann *et al*. demonstrated the applicability of EHT grafting on the heart of syngenic rats [9]. Transplanted EHT remained viable and beat for at least 28 days, became strongly vascularized, innervated and revealed a higher degree of differentiation than observed before implantation [9]. Despite these promising results, the clinical implementation of cardiac tissue replacement therapy is still confronted with obstacles. These hurdles include limited electrical coupling with the host myocardium [10], immunogenicity of allogenic transplant [9] and insufficient contractile performance [8]. The combinative approach of cardiac tissue engineering and adenoviral vector based gene transfer may offer a potential strategy to overcome these obstacles. Additionally, the expectation of safety indicates that *ex vivo* gene transfer to cardiac grafts prior to implantation has a relatively wide therapeutic 'window' that can be exploited to sustain therapeutic protein overexpression.

In the current study, we therefore sought to further improve the strategy of cardiac tissue engineering by the S100A1 gene transfer approach. S100A1, a Ca^{2+}-binding protein of the EF-hand type, is preferentially expressed in myocardial tissue and has recently been shown to enhance cardiac contractile performance by regulating both sarcoplasmic reticulum (SR) Ca^{2+}-handling and myofibrillar Ca^{2+}-responsiveness independent of β-adrenergic stimulation [11,12]. Likewise, S100A1 has also been shown to enhance contractile performance in skeletal muscle due to improved SR Ca-release [26]. Importantly, *in vivo* overexpression of the positive inotropic cardiac regulator S100A1 was not accompanied by detrimental effects on cardiac morphology and function (7 month) [13]. This feature is probably due to the recently discovered cardioprotective effects of S100A1 and the S100A1-mediated maintenance of normal gene expression in the adult heart [14,27]. Moreover, data generated from S100A1 knock-out mice demonstrated that high S100A1 protein levels are essential for the cardiac reserve to adapt to acute and chronic hemodynamic stress *in vivo* [15]. Thus, EHT genetically modified by S100A1 gene transfer might more efficiently supplement the function of a weakened heart than genetically naive cardiac grafts and this type of genetic modification may overcome contractile limitations of using these grafts clinically.

Methods

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Production of replication-deficient S100A1 adenovirus

First-generation early gene 1/3 deleted S100A1 adenovirus (AdvS100A1) was obtained by the use of the pAdTrack-CMV/pAdEasy-1 system as previously described [11,16]. Expression of S100A1 cDNA and green fluorescent protein (GFP) reporter gene was driven by a cytomegalovirus (CMV) promoter. The same adenovirus devoid of S100A1 cDNA served as control (AdvGFP). Both replication-deficient adenoviruses were amplified in human embryonic kidney 293 cells (HEK 239), purified and enriched by cesium chloride centrifugation as described [16].

Generation and adenoviral transfection of rat engineered heart tissue (EHT)

Engineered heart tissues (EHTs) from neonatal rat cardiomyocytes were created as previously published [7]. Hearts from 2–3-day-old neonatal rats were rapidly excised and atrial tissue was dissected. Cardiomyocytes were isolated from ventricular tissue by trypsin digestion and cells were preplated in supplemented Dulbecco's modified Eagle's medium (sDMEM) containing 10% horse serum (GIBCO), 2% chick embryo extract (GIBCO), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C and 5% CO_2/95% O_2 for 2 h. Nonattached cells were counted and diluted in sDMEM to a final concentration of 20×10^6 cells/mL. To obtain a series of four EHTs, 8×10^6 cells (0.4 mL) were suspended in 2.4 mL ice-cold matrix solution consisting of 0.88 mL 2× sDMEM, 0.8 mL rat collagen type I (3.7 mg/mL) (Upstate Biotechnologies), 0.28 mL extracellular matrix from Engelbreth-Holm-Swarm tumor (TEBU) and 0.44 mL 0.1 M NaOH. For each EHT, 0.7 mL was pipetted into a standardized rectangular casting mold (17 × 10 × 5 mm; length × width × height) holding one set of Velcro-coated silicone tubes kept at a fixed distance with a metal wire spacer (13 × 8 × 8 mm; length × width × height). EHTs were allowed to harden at 37 °C and 5% CO_2/95% O_2 for 30 min before sDMEM was added. Culture medium was changed every day. At day 7, EHTs were transfected adenovirally (10 PFU/cell) in DMEM with AdvS100A1 or AdvGFP for 30 min. On day 14, EHTs were transferred into a thermostated organ bath at 37 °C containing 5% CO_2/95% O_2 equilibrated Tyrode solution (in mmol/L: NaCl 119.8, KCl 5.4, $CaCl_2$ 0.1, $MgCl_2$ 1.05, NaH_2PO_4 0.42, $NaHCO_3$ 22.6, Na_2 EDTA 0.05, ascorbic acid 0.28, glucose 5.0) and subjected to isometric force measurement as described [7,8]. EHTs

were adjusted to the original spacer length and electrically stimulated with bipolar rectangular pulses (5 ms, 30 V) at 1.5 Hz for 15 min to reach a stable value that we defined as baseline. Data acquisition and analyses were performed with a custom-made PC-assisted system (BMON-2; Ingenieurbüro Jäckel, Hanau, Germany).

Experimental protocol 1: measurement of maximal Ca^{2+}-dependent isometric force

EHT length was stepwise (0.1 mm) adjusted to L_{max}, the length at which EHT developed maximal force. After steady-state values had been reached (8 min), the following contractile parameters were measured in response to increasing extracellular calcium concentrations $[Ca^{2+}]_e$ (in mmol/L: 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4) at 1.5 Hz stimulation frequency: maximal twitch amplitude of isometric force (TA), resting tension (RT), time to 90% of peak force development (TTP), time to reach 50% decay of maximal force development (TD), the first derivative of force development (+dP/dt) and decline (−dP/dt). EC_{50} was obtained by the best fit of a three-coefficient Hill equation to Ca^{2+}-sensitivity measurements (sigma plot).

Experimental protocol 2: frequence dependency of isometric force development

EHT length was adjusted to L_{max} at 0.4 mmol/L $[Ca^{2+}]_e$ and 60 beats/min stimulation frequency to define baseline values. Force-frequency relationship of contractile parameters were evaluated at 60, 90, 120, 200 and 300 beats/min under steady-state conditions. Finally, stimulation frequency was reduced to 90 beats/min to exclude any functional impairment by the stimulation protocol.

Experimental protocol 3: influence of isoproterenol on isometric force generation

To assess the influence of β-adrenergic stimulation, EHTs were challenged with incremental doses of isoproterenol (in mol/L: 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}). Contractile indices as described above were investigated at L_{max}, 0.4 mmol/L $[Ca^{2+}]_e$ and 1.5 Hz stimulation frequency under steady-state conditions.

Western blot analysis

Western blots were performed as described previously [17] to assess protein levels of S100A1, green fluorescent protein (GFP), calsequestrin (CSQ), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a), phospholamban (PLB) and cardiac α-actin. EHTs were homogenized at 4 °C in 3 w/v phosphate-buffered saline (PBS) with 5 mM EGTA and protease inhibitor mixture (1836170, complete Mini EDTA free; Roche Diagnostics GmbH, Germany) and centrifuged at 10 000 *g* for 15 min. Supernatant protein was subjected to electrophoresis, transferred to a PVDF membrane, and probed with either anti-S100A1-Ab (SA 5632, custom-made), anti-GFP (Clontech), anti-PLB-Ab (MA3-922; Affinity Bioreagents), anti-SERCA2a-Ab (sc-8095; Santa Cruz Biotechnology), anti-CSQ-Ab (208915; Calbiochem) or anti-cardiac α-actin-Ab (Ac1-20.4.2; Progen). Blots were developed with the Avidix chemoluminescence detection system (Tropix; Applied Biosystems, Foster City, CA, USA), quantified by densitometry and normalized to actin.

Histological studies and determination of cell number

EHTs were embedded in tissue freezing medium (Jung, Nussloch, Germany) at original spacer length and deep-frozen at −80 °C. Sections (10 µm) were cut parallel to the plane of the tissue, mounted on glass slides and stained for hematoxylin-eosin and trichrome, respectively. Pictures (magnification 10× and 40×) were taken with an inverse Olympus IX 70 microscope.

For evaluation of cell number, EHTs were digested with 500 µL 0.1% collagenase in PBS, pH 7.4, for 1 h at 37 °C under constant shaking as previously described [7]. Cells were counted microscopically in a 10 µL aliquot.

RNA isolation and real-time RT-PCR

Total RNA was isolated from EHTs by the Trizol® method according to the recommendations of the manufacturer (Life Technologies, Eggenstein, Germany). cDNA was synthesized by reverse transcription of the RNA with Superscript II® (Life Technologies) as recommended. Real-time RT-PCR was performed in triplicate with serial 10-fold dilutions of the cDNA on an ABI 7000 sequence detection system with the SYBR® Green PCR master mix from Applied Biosystems (Darmstadt, Germany). Expression of the atrial natriuretic peptide precursor (NPPA) gene (GenBank Acc. No. NM_012612) was examined with primers CCCGACCCACGCCAGCATGG (forward) and CAACTGCTTTCTGAAAGGGGT (reverse) flanking 700 bp. For normalization, 18S rRNA was used with primers TCAAGAACGAAAGTCGGAGG (forward) and GGACATCTAAGGGCATCAC (reverse) flanking 489 bp. PCR conditions were 95 °C, 10 min, and 40 cycles of 94 °C, 15 s; 57 °C, 15 s; 72 °C, 1 min. Fold changes between samples in NPPA expression, normalized to 18S rRNA, were calculated using the differences in ΔC_t values between the two samples ($\Delta\Delta C_t$) and the equation: fold change $= 2^{-\Delta\Delta Ct}$ (Applied Biosystems User Bulletin 2). The threshold cycle C_t indicates the cycle number during exponential amplification at which the amount of amplified target reaches a fixed threshold. ΔC_t is a

relative value and defined as the C_t for NPPA minus C_t for 18S rRNA.

Statistical analysis

All values presented are arithmetic means ± SEM. Statistical analysis was performed by using ANOVA. A value of $P < 0.05$ was accepted as statistically significant.

Results

Assessment of adenoviral transfection and influence of S100A1 gene transfer on protein expression in EHT

Figure 1A depicts a representative 14-day-old engineered heart tissue (EHT) displaying a biconcaval shape. Sections parallel to the plane of the tissue, stained with hematoxylin-eosin and trichrome (Figures 1B and 1C), show the characteristic concentration of longitudinally orientated cells at the free lateral edges of the tissue as previously reported [7]. Application of 10 pfu/cell for both S100A1 adenovirus (AdvS100A1) and control virus (AdvGFP) revealed a robust adenoviral transfection of EHTs as assessed by GFP fluorescence (Figure 1D). Figure 2A illustrates representative Western blot analysis carried out for S100A1 and GFP from EHT homogenates. Transfection with AdvS100A1, normalized to actin, resulted in a 8.7 ± 3.6-fold increase in total S100A1 protein levels compared with AdvGFP or wild-type (WT) EHTs ($P < 0.01$). Importantly, we found no difference in S100A1 protein levels in AdvGFP-treated compared with untreated WT EHTs (1.41 ± 0.27 vs. 1.3 ± 0.196, $P =$ n.s.). Moreover, S100A1 overexpression in reconstituted heart tissue did not alter protein levels of representative contractile and sarcoplasmic proteins as assessed by actin, CSQ, SERCA2a and PLB expression (Figure 2A). Although equal adenoviral dosage was used for either AdvS100A1 or AdvGFP, GFP expression was found to be significantly higher in AdvGFP-infected compared with AdvS100A1-treated EHTs (AdvGFP: 10.03 ± 7.1 vs. AdvS100A1: 2.8 ± 2.02, $P < 0.05$) while GFP expression was not detectable in WT EHTs. Determination of cell number in AdvS100A1 (1.3×10^6 cells) and AdvGFP (1.31×10^6 cells) transfected as well as in WT (1.36×10^6 cells) EHT revealed no statistically significant difference (Figure 2B). Moreover, the atrial natriuretic peptide precursor (NPPA) mRNA level as a molecular hypertrophy marker was not significantly altered in AdvS100A1-transfected EHTs compared with the AdvGFP control group. We calculated a ΔC_t of 9.61 for AdvGFP- and 9.58 for AdvS100A1-transfected EHTs at the 10^{-1} dilutions of the cDNA samples resulting in a fold change of 1.02

S100A1 overexpression increases maximal Ca^{2+}-dependent isometric force development and Ca^{2+}-sensitivity of EHT

Figure 3A depicts original tracings of representative EHTs transfected with either AdvGFP (lower tracing)

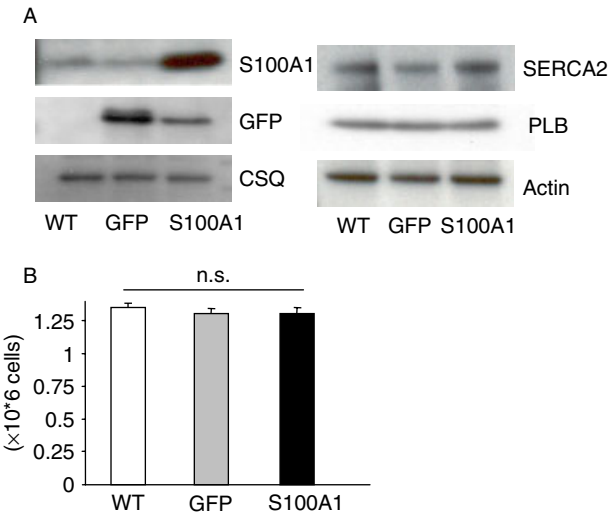

Figure 2. (A) Effect of S100A1 adenoviral-mediated gene transfer on S100A1, GFP, calsequestrin (CSQ), sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), phospholamban (PLB) and actin protein expression 7 days following gene delivery. Representative SDS-PAGE analysis of extracts from wild-type (WT), AdvGFP- and AdvS100A1-transfected EHTs. (B) Depiction of cell yield of EHTs 14 days after tissue reconstitution as analyzed after collagenase digestion in WT, AdvGFP- and AdvS100A1-transfected EHT

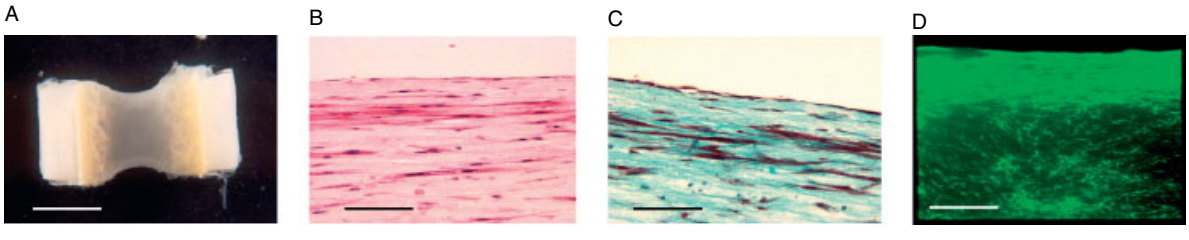

Figure 1. Morphology of engineered heart tissue (EHT). (A) Photograph of a representative EHT 14 days after reconstitution. Note the concentration of cells (white appearance) at the biconcaval edges (bar = 5 mm). Hematoxylin-eosin (H&E) staining (B) and trichrome staining (C) of 10 μm paraffin sections illustrate the longitudinally orientated cells at the free lateral edge of the EHT. (D) Coexpression of the green fluorescent protein (GFP) demonstrates S100A1 overexpression in >99% of the cells. Infection with AdvGFP resulted in similar transfection efficiency (data not shown). Magnifications: 2× (A), 40× (B, C), 10× (D)

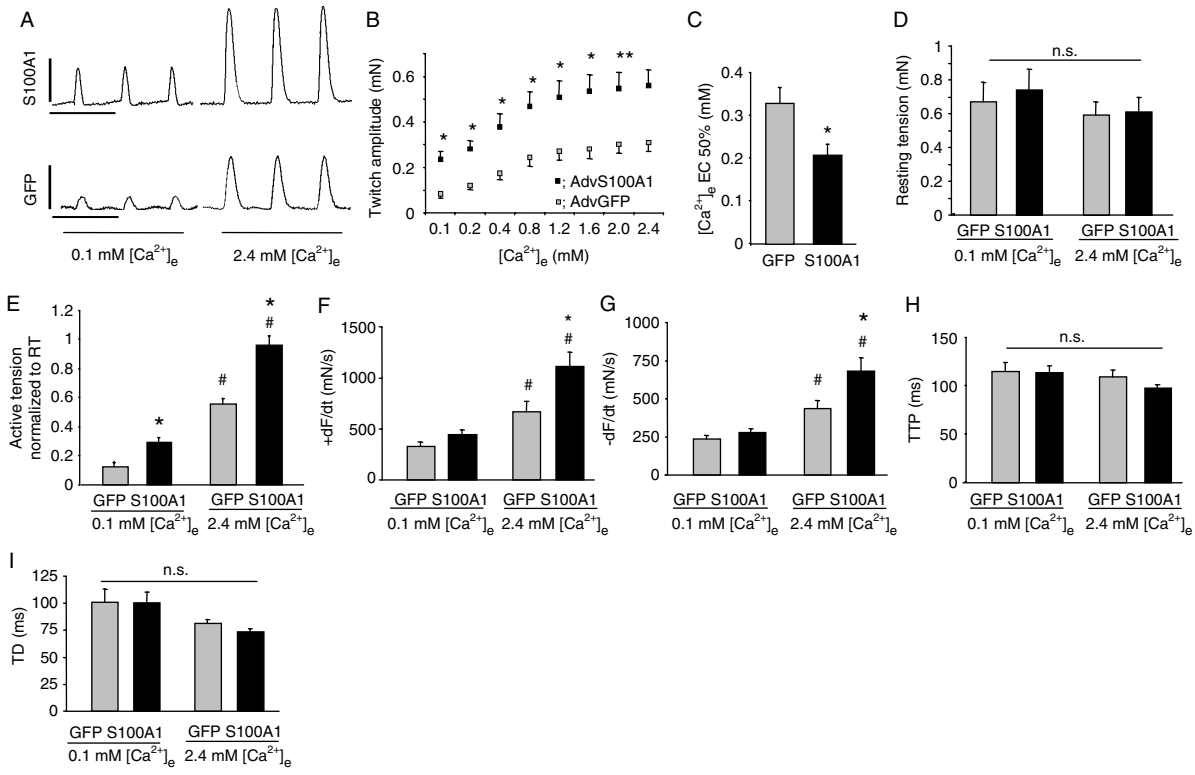

Figure 3. Effect of S100A1 gene delivery on contractile properties of EHT with incremental extracellular calcium concentrations ($[Ca^{2+}]_e$). (A) Original tracings of a representative experiment. AdvS100A1 (upper tracings) and AdvGFP (lower tracings) infected EHTs recorded at 0.1 mmol/L $[Ca^{2+}]_e$ (left) and 2.4 mmol/L $[Ca^{2+}]_e$ (right) at L_{max} after equilibration at 1.5 Hz. (upright bar = 0.25 mN; horizontal bar = 1 s). (B) Illustrates enhanced twitch amplitude of AdvS100A1-transfected EHTs (■ $n = 25$) over the whole range of tested $[Ca^{2+}]_e$ (0.1–2.4 mmol/L) compared with AdvGFP control (□ $n = 30$). Stepwise increase of $[Ca^{2+}]_e$ augments twitch amplitude of both, AdvGFP- and AdvS100A1-infected EHTs. (C) Decrease in $[Ca^{2+}]_e$ EC50% in S100A1-overexpressing EHTs. (D–I) Effect of S100A1 gene transfer and $[Ca^{2+}]_e$ on resting tension (RT), the ratio of active tension (AT) to resting tension (RT), +dF/dt, −dF/dt, time to peak (TTP) and time to decay (TD) of EHT. Data are mean ± SEM. #, $P < 0.05$ 0.1 mol/L $[Ca^{2+}]_e$ vs. 2.4 mol/L $[Ca^{2+}]_e$. *, $P < 0.05$ AdvS100A1 vs. AdvGFP

or AdvS100A1 (upper tracing) after equilibration and stretching to L_{max} at 0.1 mmol/L $[Ca^{2+}]_e$ (left) and 2.4 mmol/L $[Ca^{2+}]_e$ (right) (1.5 Hz). For all contractile and physiological parameters assessed by the experimental protocols, we found no significant difference between wild-type (WT) EHTs and AdvGFP-transfected EHTs. Data of WT controls is not shown.

Exposing both AdvGFP- and AdvS100A1-treated EHTs to cumulatively increasing extracellular calcium concentrations ($[Ca^{2+}]_e$ from 0.1–2.4 mmol/L) resulted in a significantly enhanced generation of isometric tension (Figure 3B). The 8.7-fold S100A1 protein overexpression significantly increased maximal isometric tension of EHTs by 182% (AdvS100A1: 0.2375 ± 0.031 mN vs. AdvGFP: 0.084 ± 0.015 mN) at 0.1 mmol/L $[Ca^{2+}]_e$ and by 81% (AdvS100A1: 0.56 ± 0.068 mN vs. AdvGFP: 0.31 ± 0.039 mN) at 2.4 mmol/L $[Ca^{2+}]_e$ compared with AdvGFP-transfected controls (Figure 3B). Moreover, S100A1 gene delivery significantly reduced $[Ca^{2+}]_e$ at half-maximal tension development (EC50%) (0.2075 ± 0.022 $[Ca^{2+}]_e$ (AdvS100A1) vs. 0.329 ± 0.037 $[Ca^{2+}]_e$ (AdvGFP); $P < 0.05$) (Figure 3C). At increasing $[Ca^{2+}]_e$, no significant alterations in resting tension were observed in AdvGFP- and AdvS100A1-transfected EHTs (Figure 3D). The increased developed active tension (AT) in S100A1-overexpressing EHTs resulted in a significantly augmented active tension (AT) to resting tension (RT) ratio at all values of $[Ca^{2+}]_e$ tested, from 0.1 mmol/L $[Ca^{2+}]_e$ (0.32 ± 0.036 (AdvS100A1) vs. 0.125 ± 0.012 (AdvGFP); $P < 0.01$) to 2.4 mmol/L $[Ca^{2+}]_e$ (0.96 ± 0.065 (AdvS100A1) vs. 0.55 ± 0.03 (AdvGFP); $P < 0.01$) (Figure 3E). Higher extracellular calcium concentration resulted in a significant increase in +dF/dt and −dF/dt in both, AdvS100A1- and AdvGFP-transfected EHTs (Figures 3F and 3G). Notably, the S100A1-mediated gain in developed isometric force was accompanied by a significantly enhanced +dF/dt and −dF/dt in the range 0.4–2.4 mmol/L $[Ca^{2+}]_e$ compared with AdvGFP controls (Figures 3F and 3G). In contrast, S100A1 gene transfer in EHTs neither resulted in an alteration of time to peak (TTP) (Figure 3H) nor of time to decay (TD) (Figure 3I) compared with AdvGFP controls.

S100A1 gene transfer did not alter force-frequency relationship of EHT

EHTs casted with rat neonatal cardiomyocytes showed a significant negative staircase in the range 60–300

beats/min, resembling that of intact rat cardiac tissue (Figure 4). S100A1 gene transfer did not alter the negative force-frequency relationship of rat EHT (Figure 4). The observed S100A1-mediated gain in EHT contractility was preserved at increased heart frequencies (frequency of 200 beats/min): 0.301 ± 0.041 mN (AdvS100A1) vs. 0.152 ± 0.03 mN (AdvGFP); $P < 0.05$. The response to frequency changes was completely reversible for S100A1-overexpressing and control EHTs (AdvGFP) (Figure 4). Moreover, at higher pacing rates, RT, TTP and TD remained the same between the AdvS100A1-transfected EHTs and the control group.

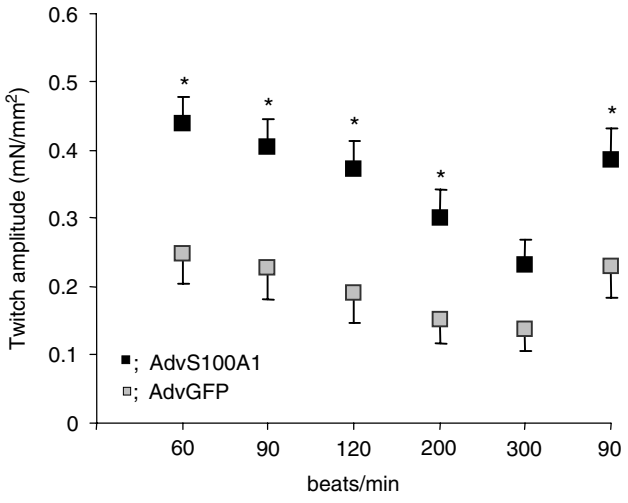

Figure 4. Effect of S100A1 gene transfer on twitch amplitude of EHT at increasing pacing frequencies. (A) Depicts enhanced twitch amplitudes in S100A1-overexpressing EHTs (■ $n = 13$) over the whole range of tested pacing frequencies (60–300 beats/min) as compared with AdvGFP control (□ $n = 17$). A negative force-frequency relationship was observed in both, AdvGFP- and AdvS100A1-infected EHTs. *, $P < 0.05$ AdvS100A1 vs. AdvGFP

Preservation of increased isometric force development of AdvS100A1-treated EHT under β-adrenergic stimulation

Figure 5A depicts original tracings illustrating the contractile response of AdvS100A1 (upper tracing) and AdvGFP (lower tracing) infected reconstituted heart tissue to isoproterenol (1 μmol/L). At 0.4 mM $[Ca^{2+}]_e$, a maximally effective concentration of the β-adrenergic agonist isoproterenol (1 μmol/L) significantly increased isometric force development of both AdvS100A1 (from 0.43 ± 0.05 to 0.56 ± 0.06 mN) and AdvGFP (from 0.26 ± 0.047 to 0.35 ± 0.043 mN) treated EHTs (Figure 5B). S100A1 gene transfer to EHTs resulted in a 60% enhancement of isometric force development upon β-adrenergic stimulation compared with AdvGFP controls. Furthermore, the S100A1-mediated gain of AT/RT ratio was preserved upon β-adrenergic stimulation (2.0 ± 0.1 (AdvS100A1) vs. 1.3 ± 0.04 (AdvGFP); $P < 0.05$) (Figure 5C). The first derivatives of maximal pressure rise (+dF/dt) and fall (−dF/dt) were significantly elevated in AdvS100A1-overexpressing EHTs compared with AdvGFP controls (Figures 5D and 5E).

In contrast, S100A1 overexpression did not affect TTP, TD and RT as isoproterenol significantly shortened

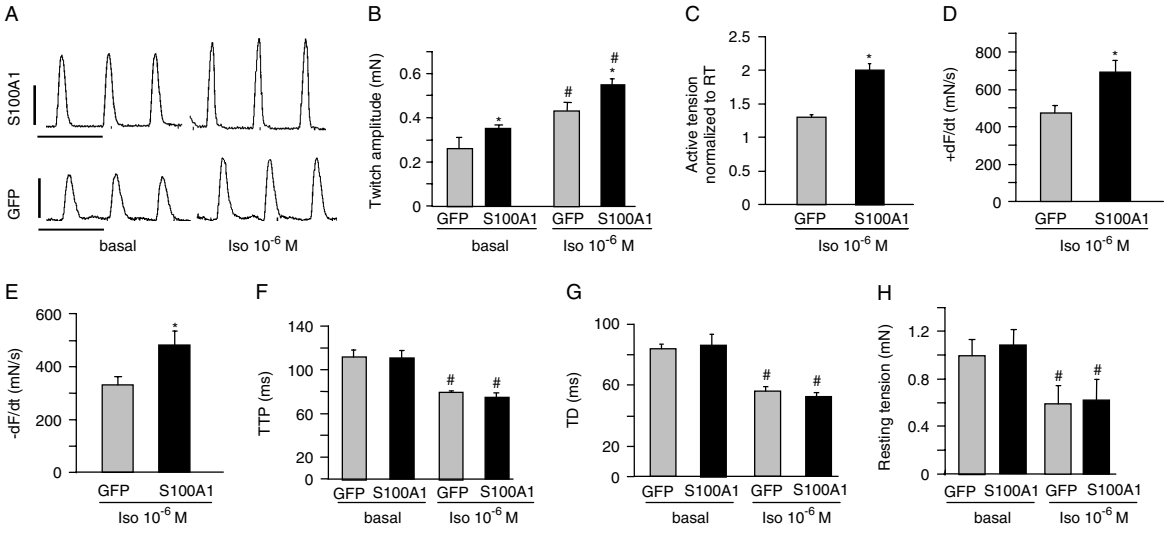

Figure 5. Inotropic response of S100A1 gene delivered and control EHT to a maximal effective isoproterenol dose. (A) Original tracings of a representative experiment. AdvS100A1 (upper tracings) and AdvGFP (lower tracings) infected EHTs recorded under basal conditions (left) and at isoproterenol stimulation (1 μmol/L) (right) at L_{max} after equilibration (1.5 Hz). (upright bar = 0.25 mN; horizontal bar = 1 s). (B) 1 μmol/L isoproterenol increases twitch amplitude of both, AdvGFP- and AdvS100A1-infected EHTs. S100A1 gene transfer mediated enhanced twitch amplitude (■ $n = 15$) is preserved under a maximally effective isoproterenol concentration at 0.4 mmol/L $[Ca^{2+}]_e$ compared with AdvGFP control (□ $n = 16$). (C–H) Effect of S100A1 gene transfer and isoproterenol stimulation (1 μmol/L) on the ratio of active tension (AT) to resting tension (RT), −dF/dt, +dF/dt, time to peak (TTP), time to decay (TD) and resting tension (RT) of EHT. Data are mean ± SEM. #, $P < 0.05$ basal (0.4 mol/L $[Ca^{2+}]_e$) vs. 1 μmol/L isoproterenol (0.4 mol/L $[Ca^{2+}]_e$). *, $P < 0.05$ AdvS100A1 vs. AdvGFP

TTP (Figure 5F), TD (Figure 5G) and RT (Figure 5H) in AdvS100A1- and AdvGFP-transfected EHT.

Discussion

Engineered heart tissue (EHT) represents a promising tool for potential tissue replacement therapy as the implanted rat cardiac tissue constructs are contracting, strongly vascularized and surpass the degree of differentiation reached before implantation [9]. Moreover, Zimmermann *et al*. have demonstrated that EHTs were almost completely incorporated into the native rat myocardium 14 days after implantation, thus proving the applicability of EHT for cardiac engraftment [9]. The insufficient contractile force generated by EHTs however represents a major obstacle to the clinical implementation of cardiac engraftment. Our current results now give evidence that S100A1 gene manipulation effectively increases Ca^{2+}-sensitivity and contractile properties of EHTs. This novel approach to technically alter EHT characteristics may aid in the potential therapeutic application of such grafts to repair the failing heart.

Interestingly, previous studies have shown that adenoviral-mediated overexpression of the S100A1 protein in isolated myocardial cells resulted in a marked increase in the rate of unloaded contraction and relaxation that was associated both with increased intracellular Ca^{2+}-transients and decreased Ca^{2+}-sensitivity of myofilaments [11,12]. Since in S100A1 transgenic mice a marked increase in the cardiac contractile state was observed *in vivo* [13], we sought to exploit the S100A1 gene transfer to genetically manipulate and improve contractile properties of EHT.

We recently found that S100A1 gene transfer increases force generation of EHTs [11]. To date the impact of this positive inotropic intervention on clinically important features of isometric contractility is not known. As it is a prerequisite of any therapeutic genetic manipulation not to interfere with basic regulatory mechanisms of muscular contractility, we investigated the effects of S100A1 gene transfer on typical features of myocardial contractility.

The major mechanism by which myocardial function is altered is the change in the inotropic state of the muscle [18] based on an enhanced delivery of Ca^{2+} to the contractile system so as to increase force and shortening of the cardiac myocyte. We thus tested the inotropic state of EHTs after S100A1 gene transfer under basic conditions as well as under different positive inotropic conditions like incremental extracellular Ca^{2+}-concentration, β-adrenergic stimulation, and a stepwise increase in resting tension, all of which are known to increase systolic Ca^{2+}-supply to the contractile apparatus. In the current study we found that the manipulation of S100A1 expression in EHTs significantly increases isometric force generation, $+dF/dt$, $-dF/dt$ and the ratio of active tension (AT) to resting tension (RT) over a wide range of $[Ca^{2+}]_e$ values, while resting tension (RT) remained unchanged. Importantly, this positive inotropic intervention neither changed the response to Ca^{2+} or β-adrenergic stimulation inasmuch as it showed an additive effect concerning the gain in force generation.

In vivo the contractile state also depends on the force-length relationship (Frank-Starling law). This mechanism regulates myocardial contractility on a beat-to-beat basis depending on the venous return to the heart, i.e. the diastolic preload [19]. It was therefore important to investigate the impact of S100A1 on EHT force generation under increasing resting tensions. Our data show that also the tension-related increase in force generation is unaltered by S100A1 and again displays an additive inotropic gain with S100A1. The heart rate is another basic mechanism that acutely regulates the contractile state of the heart, which is referred to as the force-frequency relationship (Bowditch staircase) and which is also thought to be related to an increase in systolic Ca^{2+}-delivery [20]. In S100A1-supplemented EHTs, we found the force-frequency relationship to be preserved although the generated force was significantly higher than in controls over a wide range of pacing frequencies. Taken together, S100A1-supplemented EHTs continued to exhibit functional characteristics of rat hearts such as a positive force length, a negative force-frequency relationship, a high sensitivity to calcium, and a positive inotropic and lusitropic response to isoproterenol as previously described for EHT [7,8].

These data confirm for the first time that S100A1 gene transfer is associated with a gain in isometric force generation without interfering with clinically relevant regulatory mechanisms of myocardial contractility. Furthermore, the current results extend our findings concerning the impact of S100A1 on unloaded contractility in cultured cardiac myocytes to a more physiological model of isometric contraction in EHTs. An exciting aspect is that the augmented contractile performance was also translated to an increased inotropic cardiac reserve, i.e. an additive increase of force generation with other positive inotropic interventions, which is consistent with observations in S100A1 transgenic and knock-out mice [13,15]. Accordingly, data generated from S100A1 knock-out mice demonstrated that high S100A1 protein levels are essential for the cardiac reserve to adapt to acute and chronic hemodynamic stress *in vivo* [15]. This feature might prove to be advantageous especially during the initial stages of cell grafting, since Shimizu *et al*. have demonstrated increased cell survival and electrical coupling in higher developed, reconstituted grafts [4].

Both proliferation and hypertrophy are unlikely as underlying causes of increased contractile properties of S100A1-overexpressing EHTs as the determination of cell numbers and RNA analyses of atrial natriuretic peptide precursor (NPPA) revealed no difference between AdvGFP- and AdvS100A1-treated EHTs 7 days following gene transfer. Moreover, Western blot analyses of AdvS100A1-treated EHTs were consistent with our recent finding of unaltered cardiac Ca^{2+}-cycling proteins in S100A1-overexpressing cardiomyocytes [11]. Therefore, we believe that the proportion of cardiomyocytes to

non-myocytes remained unaltered after the S100A1 gene transfer. Even though S100A1 overexpression was much higher in this study (8.7-fold), our results are consistent with previous observations in transgenic mice in which a 4-fold S100A1 overexpression did not induce detrimental effects on cardiac morphology and physiology [13]. Accordingly, the use of the S100A1 gene might be superior to other strategies to strengthen engineered cardiac grafts, since in β_1-adrenergic receptor transgenic mice the increased myocardial contractility was followed by myocyte hypertrophy and progressive heart failure [21].

EHT can be designed in different geometrical shapes which may influence contractile parameters [22]. Here, we focused our investigation on the contractile performance of S100A1-supplemented planar EHT matrices to principally demonstrate that S100A1 gene transfer improves contractile performance of EHT and thus offers a strategy to overcome the low contractile force of *in vitro* engineered cardiac grafts. To clinically implement genetically manipulated EHTs, potential immunogenicity associated with the adenoviral gene delivery system has to be addressed and evaluated carefully [23]. Recently developed adeno-associated viral (AAV) systems are not expected to cause an immune response [24,25] and this feature might increase the duration of therapeutic gene expression *in vivo*. *In vitro*, immunological responses against adenoviral antigens are unlikely and, indeed, contractile function of AdvGFP-infected and uninfected EHT did not differ significantly.

The study reports two novel findings. The first is that the combinative approach of tissue engineering and S100A1 gene transfer is a potent strategy to develop strengthened engineered cardiac grafts. Moreover, S100A1 overexpression does not alter characteristic contractile features of engineered heart tissue nor leads to any detrimental effects. Second, these results demonstrate for the first time that the S100A1 gene transfer is an effective therapeutic means to manipulate isometric contractility. In conclusion, the S100A1 gene transfer might offer a valuable strategy to advance the recent efforts in clinical implementation of cardiac tissue replacement therapy.

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