SMN-deficiency disrupts SERCA2 expression and intracellular Ca\(^{2+}\) signaling in cardiomyocytes from SMA mice and patient-derived iPSCs

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Abstract

Spinal muscular atrophy (SMA) is a neurodegenerative disease characterized by loss of alpha motor neurons and skeletal muscle atrophy. The disease is caused by mutations of the \(\text{SMN1}\) gene that result in reduced functional expression of survival motor neuron (SMN) protein. SMN is ubiquitously expressed, and there have been reports of cardiovascular dysfunction in the most severe SMA patients and animal models of the disease. In this study, we directly assessed the function of cardiomyocytes isolated from a severe SMA model mouse and cardiomyocytes generated from patient-derived iPSCs. Consistent with impaired cardiovascular function at the very early disease stages in mice, heart failure markers such as brain natriuretic peptide were significantly elevated. Functionally, cardiomyocyte relaxation kinetics were markedly slowed and the \(T_{50}\) for Ca\(^{2+}\) sequestration increased to 146 ± 4 ms in SMN-deficient cardiomyocytes from 126 ± 4 ms in wild type cells. Reducing SMN levels in cardiomyocytes from control patient iPSCs slowed calcium reuptake similar to SMA patient-derived cardiac cells. Importantly, restoring SMN increased calcium reuptake rate. Taken together, these results indicate that SMN deficiency impairs cardiomyocyte function at least partially through intracellular Ca\(^{2+}\) cycling dysregulation.

Background

Spinal muscular atrophy (SMA) is one of the leading genetic causes of infant mortality. SMA is caused by survival motor neuron (SMN) protein deficiency due to deletions or mutations of the \(\text{SMN1}\) gene. Individuals also carry two or more copies of a paralog gene, \(\text{SMN2}\), which produces suboptimal levels of the SMN protein. The amount of full-length protein produced from \(\text{SMN2}\) is an important determinant of disease severity [1, 2]. SMA patients with more than two copies of \(\text{SMN2}\), as a result of duplication, tend to have less severe forms of the disease. Recently, therapeutic breakthroughs, gene-replacement therapy using viral delivery [3] and altering \(\text{SMN2}\) splicing using antisense oligonucleotides [4], have remarkably improved motor function and survival of SMA patients, but patients remain prone to cardiopulmonary complications leading to opportunistic upper respiratory tract infections [5].

Motor neuron loss and consequent muscle atrophy are the defining features of the SMA phenotype. SMN is ubiquitously expressed in all tissues, where it is known to play a key role in snRNP biogenesis and spliceosome assembly [6–8]. It is unclear why reduced levels of the ubiquitously expressed SMN protein selectively target anterior horn cells of the spinal cord. Since spliceosome
assembly occur in all cells, it is possible that reduced SMN function in other tissues may contribute to the clinical manifestation of the disease.

Indeed, recent observations suggest a role of SMN in tissues outside of the motor neuron with possible contribution to the pathology of SMA [9–14]. Notably, in a study designed to develop an SMA plasma protein biomarker panel to monitor disease progression and therapeutic efficacy, 84 putative markers regressing to motor function were found in patients (n = 108 total; type I = 17; type II = 49; type III = 42) enrolled in the BforSMA study, including several markers of congestive heart failure and cardiovascular dysfunction [15]. Indeed, marked elevation of heart failure markers such as brain natriuretic peptide (BNP), fatty acid-binding protein 3 (FABP3), and creatine kinase (CK) in SMA patients suggests that normal cardiac function may require sufficient functional expression of SMN. Importantly, in vivo assessment of cardiovascular function indicates that SMA mice have impaired cardiac function [16]. Sympathetic denervation of the heart in SMA model mice [16–20] has been suggested as a cause of cardiac pathophysiology, but the mechanisms fully responsible for these cardiac deficits are unknown. Importantly, there have been no direct measurements of cardiomyocyte function in any SMA model so it has been difficult to conclude with certainty whether SMN directly impairs cardiac function.

We sought to determine if SMN deficiency compromises contractile function in ventricular cardiomyocytes isolated from the SMNΔ7 mouse (SMNΔ7;SMN2; Smn−/−) that recapitulates a severe form of SMA with an average life span of 14 days and muscle weakness onset at post-natal day 5 (P5) [21]. In this model, we observe significant elevation of heart failure marker expression as early as P5 and marked impairment of contractile function in isolated cardiomyocytes. Moreover, we observe marked reduction in SERCA2 expression and consequent slowed Ca2+ transient kinetics in cardiomyocytes isolated from SMA mice and generated from SMA patient-derived iPSCs. These results show that SMN deficiency also causes cardiac deficits directly in cardiac myocytes, potentially by altering SERCA2 expression and calcium cycling kinetics, in addition to any autonomic dysfunction.

Materials and methods
Generation of transgenic mice
The generation and phenotypic characterization of the SMN-deficient transgenic SMNΔ7 mouse line (SMNΔ7; SMN2; Smn−/−) has been described previously [22]. Briefly, the SMNΔ7 mouse line is a triple mutant model with a double copy of SMA cDNA lacking exon 7 and two copies of the human SMN2 on an Smn−/− background. SMNΔ7 mouse transgenic and unaffected littermate mice (post-natal day 5 (P5) and 10 (P10), both male and female) were used in the present study. All procedures complied with the standards for the care and use of animal subjects as stated in the Guide or the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1996), and all protocols were approved by the IACUC at the Uniformed Services University of the Health Sciences.

RNA extraction and quantification
Tissue (50–100 mg) was homogenized in 1 ml TRIzol reagent (Thermo Fisher, cat 15596018), and total RNA was isolated and converted to cDNA as previously described [23]. Gene-specific Taqman primer and probe sets were purchased from Applied Biosystems: atrial natriuretic peptide (ANP; Nppa, Mm01255748_g1), brain natriuretic peptide (BNP, Nppb, Mm01255770_g1), skeletal muscle actin (Acta1, Mm00808218_g1), SERCA2 (Atp2a2, Mm01201431_m1), hypoxanthine guanine phosphoribosyl transferase (Hprt, Mm01318743_m1), and glyceraldehyde 3-phosphate dehydrogenase (Gapdh, Mm01180221_g1). qRT-PCR reactions were performed in triplicate using the StepOnePlus™ Real-Time PCR System (Applied Biosystems). The following primers were purchased from Integrated DNA Technologies: SERCA2, 5′-TGAGACGCTCAAGTTTGTGG-3′; SERCA2a, 5′-ATGCAGAGGGCTGGTAGATG-3′; SERCA2b, 5′-ACAAACGGCCAGGAAATG-3′; and Gapdh, 5′-GCATGGCCTTCCGTGTTC-3′. The level of each transcript was quantified by the threshold cycle (Ct) method using Gapdh and Hprt as endogenous controls. Values were normalized to the mean of the unaffected group for each gene, which was assigned as 1.

Transcriptome profiling by RNA sequencing
Total RNA was quantified via a fluorescence dye-based methodology (RiboGreen, Thermo Fisher, cat. R11490) on a Spectramax Gemini XPS plate reader (Molecular Devices, Mountain View, CA). RNA integrity was assessed using automated capillary electrophoresis on a Fragment Analyser (Advanced Analytical Technologies, Inc, Santa Clara, CA). Total RNA input of 200 ng was used for library preparation using the TrueSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, cat 20020594). Sequencing libraries were quantified by PCR using KAPA Library Quantification Kit for NGS (Roche, Wilmington, MA, cat KK4854) and assessed for size distribution on a Fragment Analyser. Sequencing libraries were pooled and sequenced on a NextSeq 500 Desktop Sequencer (Illumina) using a NextSeq 500 High Output Kit v2 with paired-end reads at 75 bp length. Raw sequencing data was demuxed using
**Western blots**
Fresh ventricular tissue was homogenized (Polytron) in RIPA lysis buffer (Sigma, cat R0278). Isolated proteins were separated by SDS-PAGE (4–15%, BioRad, cat 456-1084) and transferred onto PVDF membranes (BioRad, cat BR20191004). Blots were probed with the following specific antibodies: anti-SERCA2a antibody (1:1000, Cell Signaling Technology, cat 9580), anti-SMN (1:1000, BD Transduction Laboratories, cat 610647), anti-α-SM actin (1:5000 mybiosource.com, cat MBS477269) anti-BNP (1:1000, Abcam, cat ab236101), and GADPH (1:5000, Abcam, cat ab8245). Bound antibodies were detected with SuperSignal West Dura ECL substrate (Pierce, cat 34075).

**Cell contractility and calcium transient measurements in mouse cardiomyocytes**

Unloaded cell shortening and intracellular calcium (Ca^{2+}) transients were measured in freshly isolated ventricular myocytes, prepared as described previously [24, 25]. Isolated myocytes were transferred into a recording chamber mounted on an Olympus X51 inverted microscope and superfused with normal Tyrode solution (composition in mM: NaCl, 137; KCl, 5.4; NaH_2PO_4, 0.16; glucose, 10; CaCl_2, 1.8; MgCl_2, 0.5; HEPES, 5.0; NaHCO_3, 3.0; pH 7.3–7.4). All experiments were performed at room temperature. Video images were acquired using a Myocam camera (IonOptix). Cells were field stimulated at 1 Hz in all experiments. In experiments aimed at measuring intracellular Ca^{2+}, isolated cells were incubated in Wittenberg Isolation Medium (WIM, composition in mM: NaCl, 116; KCl, 5.3; NaH_2PO_4, 1.2; glucose, 11.6; MgCl_2, 3.7; HEPES, 20; L-glutamine, 2.0; NaHCO_3, 4.4; KH_2PO_4, 1.5; 1X essential vitamins (GIBCO cat 12473-013);1X amino acids (GIBCO cat 11120-052); pH 7.3–7.4) solution containing fluo-3-AM (1 μM) for 30 min at room temperature. Fluo-loaded cells were transferred into the recording chamber and perfused with normal Tyrode solution supplemented with 500 μM probenecid to inhibit dye export. Cells were stimulated to contract at 1 Hz in all cases and fluorescence was captured with a photomultiplier tube (IonOptix). Intracellular Ca^{2+} concentration ([Ca^{2+}]) was estimated using the Maravall equation [26]:

\[
[Ca] = K_d \cdot \left( \frac{F}{F_{max}} \cdot \frac{1}{R_f} \right) 
\]

Where \( K_d \) was assumed to be 600 nM [27] and \( R_f = F_{max}/F_{min} \). \( F_{min} \) and \( F_{max} \) were measured in each cell by incubating the cell in modified Tyrode solutions supplemented with 20 mM 2,3 butadione monoxime, 10 mM A23187 and either 10 mM EGTA, or 100 mM CaCl_2, respectively.

**Cell lines**
To study the effects of SMN-deficiency on human cardiomyocytes, we utilized iPSCs from a spinal muscular atrophy patient (GM23240; Coriell). These iPSCs were reprogrammed from fibroblasts using lentiviral constructs encoding OCT4, SOX2, NANO, and LIN28. NCRM-1 iPSCs were used as a control line and obtained from XCell Sciences (Novato CA). NCRM-1 iPSCs were obtained from XCell Sciences and reprogrammed by episomal plasmid from CD 34+ human cord blood cells. To grow and maintain iPSC lines, standard 6- well tissue culture plates were coated with growth factor reduced Matrigel (Corning; cat 354277 ) diluted 1:100 in DMEM/F12 (Gibco, cat 11330-032) same day as iPSC plating. Frozen stocks of iPSCs were thawed and plated on Matrigel coated plates in TeSR E8 (StemCell, cat 05990) supplemented with ROCK inhibitor (Y-27632, 10 μM, Tocris, cat 1254). After 24 h, media was replaced with TeSR E8 (without ROCK inhibitor) and culture media was replaced daily until fully confluent. iPSC lines were passaged using 0.5 mM EDTA (Life Technologies, cat 15575-038) in PBS without CaCl_2 and MgCl_2 (Hyclone, Slt30256.01). Cells were maintained at 37 °C, 5% CO2.

**Generation and maintenance of cardiomyocytes**
We began the cardiomyocyte differentiation protocol when cells were 80% confluent using the protocol developed by Feaster et al [28]. Briefly, differentiation was initiated (day 0–2) by replacing TeSR E8 medium with RPMI 1640 medium (Lonza, cat 12-702F) supplemented with B27 (minus insulin, Gibco, cat A1895601) and CHIR99021 (6 μM, LC Laboratories, cat C-6556), a GSK3 inhibitor. On days 3–4, CHIR99021 was removed and replaced with RPMI 1640 medium supplemented with B27 (minus insulin) and IWR-1 (500 μM, Sigma, cat I0761), a Wnt signaling inhibitor. On days 5–9, cells were maintained in RPMI 1640 medium supplemented only with B27 (minus insulin). From days 10–15, a metabolic selection protocol was employed using RPMI 1640 without glucose (Life Technologies, cat 11879) plus B27 without insulin. Following metabolic selection, cells were
maintained in RPMI 1640 supplemented with B27 (Gibco, cat 17504-044) and 1% pen strep (Gibco, cat 10378-016). Beating cardiomyocytes were fed daily until day 20 when functional assays were carried out as described below.

**Transfection**

To increase SMN expression in patient iPSC-derived cardiomyocytes, we transfected cells with SMN-GFP cDNA plasmid (1 μg) using lipofectamine 3000 (Invitrogen, cat 100022052) and P3000 (Invitrogen, cat 100022058) for 48 h according to the manufacturer protocols. Transfection was performed at day 18 of differentiation. Genartion of SMN-GFP plasmid was previously described [29] and a gift from Greg Matera (University of Carolina at Chapel Hill). To reduce SMN expression in control iPSC-derived cardiomyocytes, cells were transfected with 40 pmol of pre-validated siRNA (IDT, cat 37206943) and RNAi Max (Life Technologies, cat 56532) per manufacturer protocol at the 18 day time point. Opti-MEM (Gibco, cat 12605-010) diluent was used in both protocols. Successful transfection was confirmed in western blot analysis.

**Flow cytometry**

SMA and Control patient iPSC-derived cardiomyocytes were dissociated using TrypLE (Gibco, cat 12605-010). Dis-aggregated cells were centrifuged and resuspended in fixation buffer (Biolegend, cat 554655) for 15 min at room temp, washed 3 times in 1X permeabilization wash buffer (Biolegend, cat 421002, and resuspended in permeabilization wash buffer containing FITC conjugated anti-Cardiac Troponin (1:20, Miltenyi Biotec, cat 130-106-689) for 1 h at room temp protected from light. Cells were read in cell staining buffer (Biolegend, cat 420201) on BD Accuri C6 Flow Cytometer CFlow Plus software. Unstained cells were used as a negative control.

**Calcium imaging**

SMA and Control patient iPSC-derived cardiomyocytes wells were washed with PBS and incubated in normal Tyrode’s solution (composition in mM: NaCl, 137 mM; KCl, 5.4 mM; NaH2PO4, 0.16 mM; glucose, 10 mM; CaCl2, 1.8 mM; MgCl2, 0.5 mM; HEPES, 10.0 mM; NaHCO3, 3.0 mM; pH 7.3–7.4) supplemented with cell permeant Fluo-4 AM, fluorescent Calcium indicator (1 μM, Invitrogen, cat F14201) and probenecid (500 μM, Sigma, cat P8761). Cells were incubated for 15 min at 37 °C, 5% CO2. Calcium transients were recorded on a Nikon eclipse Ti2 inverted microscope equipped with a large view CMOS camera on a × 20 objective with NIS Elements AR software (Nikon). Decay at T10, T50, T75, T90, and T100 were analyzed using Clampfit software (Axon).

Reagents

A complete list of reagents, primer sequences with source information can be found in Supplemental Table 2.

Data analysis

All data were analyzed using IonWizard, Clampfit, and Microsoft Excel software and, except where noted, results are presented as means ± SEM (standard error of the mean). In all cases, p < 0.05 was considered significant. Statistical tests used and resultant p values are given in the Figure legends. Statistical analysis was performed in GraphPad Prism 7.

Results

**Heart failure markers are elevated in SMN-deficient mice**

There is growing evidence suggesting that peripheral tissues, including the heart, may be affected by the loss of SMN function in SMA patients. Heart failure is associated with the reactivation of fetal genes, including atrial natriuretic peptide (ANP; Nppa), brain natriuretic peptide (BNP; Nppb), and skeletal α-actin (Acta1), associated with structural and functional remodeling of the heart [30]. The activation of ANP and BNP in particular has been shown to correlate well with the clinical severity and prognosis of heart failure [31–33]. ANP and BNP mRNA expression was markedly increased in whole heart tissue from SMN deficient mice modeling a severe form of the disease [22] at both post-natal day 5 (P5; early in the disease progression) and post-natal day 10 (P10; closer to end-stage) when compared to unaffected littermates (Fig. 1: Supplemental Figure 1). Similarly, we found increased expression of skeletal α-actin at P5. Together, these results imply that mechanical function of the heart may be altered early in the disease progression of this severe SMA mouse model.

**Diastolic function is impaired in SMN-deficient mouse cardiomyocytes**

To directly test cardiac function, we compared unloaded sarcomere shortening in paced (1 Hz) ventricular cardiomyocytes isolated from unaffected and SMN-deficient mice at P12–P13 (Fig. 2). The mean amplitude of sarcomere shortening and fractional shortening were similar in myocytes from control and SMN deficient littermates. While the extent of sarcomere shortening was unaffected by SMN deficiency, the rate of contraction and relaxation were both markedly slowed in SMN deficient cells. Both time-to-peak shortening (131 ± 5 vs.105 ± 3 ms, *p < 0.01, t test) and time-to-50% re-lengthening (97 ± 5 vs. 68 ± 2 ms, *p < 0.01, t test) were markedly increased in SMN-deficient cells compared with cells from unaffected littermates. We also noted a significant reduction of resting (diastolic) sarcomere length in SMA...
myocytes (1.60 ± 0.02 μm vs. 1.73 ± 0.02 μm in control, n = 36 and 37, respectively,*p < 0.01, t test). Thus, as predicted by the elevated heart failure markers, cardiomyocyte function, particularly during diastole, is compromised in SMN-deficient mice.

**Ca²⁺ handling gene expression is altered in SMN-deficient mouse heart**

To gain an understanding of how SMN deficiency might affect cardiac function, we performed transcriptome expression profiling of whole heart tissue from SMA model mice at P10. Differential expression analysis identified 637 transcripts that were upregulated and 541 transcripts that were downregulated in heart tissue of SMA mice as compared to unaffected littermates (Fig. 3a; Supplemental Table 1). Subsequent analysis of the set of 1178 differentially expressed transcripts yielded significantly enriched gene ontologies for biological functions in regulation of muscle cell and fiber development (3.8-fold enrichment, p value = 3.9E−04). Additionally, specific key genes involved in muscle function and calcium-ion handling (1.8-fold enrichment, p value 1.6E−04) are lower (Atp2a2, Casq1, CaBP1, S100a10, Rcan1, and Hrc) and higher in transcript level (Scin,
**Efhd2**, and **Myom2** in heart tissue of SMA mice as compared to unaffected mice (Fig. 3b). Transcript expression of all identified calcium handling-related genes were significantly different prior to disease end-stage, suggesting that SMN-deficiency alters the cardiac transcriptome during disease progression.

**SERCA2 expression is reduced in SMN-deficient cardiomyocytes**

It is notable that **Atp2a2**, the gene encoding the sarcoplasmic reticulum (SR) Ca\(^{2+}\) ATPase or SERCA2, is found among the major Ca\(^{2+}\) handling related genes altered in the SMN-deficient heart. Impaired contractile function of the failing heart is often associated with dysregulation of intracellular Ca\(^{2+}\) cycling and reduced expression of SERCA2 protein [34–36]. As predicted from transcriptome data, we found that SERCA2 protein expression is reduced in SMN-deficient cardiomyocytes as early as P5 (Fig. 4). There are two major splice variants of SERCA2—SERCA2a and SERCA2b [37]. SERCA2a is considered the muscle specific isoform expressed in both cardiac muscle and slow twitch skeletal muscle, while the SERCA2b isoform is ubiquitously expressed. Given the role of SMN in regulating spliceosome assembly and function, we examined the relative expression of the two major splice variants in SMN-deficient and control hearts. Interestingly, there appears to be an isoform switch, with reduced SERCA2a and increased SERCA2b mRNA expression, but overall there is a significant reduction of SERCA2 protein regardless of the isoform.

**Ca\(^{2+}\) reuptake kinetics are slowed in SMN-deficient mouse cardiomyocytes**

SERCA2 is the major determinant of Ca\(^{2+}\) sequestration from the cytoplasm in murine cardiomyocytes [38–40]; therefore, it is predicted that SMN-deficient cells exhibit slowed removal of cytosolic Ca\(^{2+}\) following a triggered release. To test this, we estimated [Ca\(^{2+}\)]\(_i\), during triggered Ca\(^{2+}\) transients in fluo-3-AM loaded ventricular myocytes isolated from unaffected and SMN-deficient littermates at P12–P13 (Fig. 5). Although resting [Ca\(^{2+}\)]\(_i\), tended to be elevated prior to stimulation, there were no significant differences in diastolic or peak systolic [Ca\(^{2+}\)]\(_i\), between control and SMA cardiomyocytes. Consistent with reduced expression of SERCA2, however, there was marked slowing of Ca\(^{2+}\) removal from the cytoplasm with \(T_{50}\) significantly (*\(p < 0.01\), t-test) increased in SMN cardiomyocytes (146 ± 4 ms) compared with...
control cells (126 ± 4 ms). These data are consistent with the conclusion that SMN-deficiency is associated with reduced SERCA2 expression, causing slowed \( \text{Ca}^{2+} \) reuptake and impaired cardiomyocyte mechanical function.

**SMN deficiency slows \( \text{Ca}^{2+} \) reuptake kinetics and reduces SERCA2a expression in human iPSC-derived cardiomyocytes**

Human-induced pluripotent stem cells (iPSCs) have emerged as a powerful system to model human cardiac disease and study \( \text{Ca}^{2+} \) handling [41–44]. To confirm that our findings are not restricted to the mouse model, we assessed \( \text{Ca}^{2+} \) transients in spontaneously contracting clusters of cardiomyocytes generated from patient-derived (SMA) and unaffected control iPSCs. As expected, SMN protein expression was markedly reduced in SMA patient-derived cells. In agreement with data obtained from mouse cardiomyocytes, there was also marked slowing of \( \text{Ca}^{2+} \) reuptake kinetics in SMA cells with \( T_{50} \) (556 ± 49 ms) increased compared with control (348 ± 21 ms) (Fig. 6a). Moreover, transfection of
patient-derived cells with SMN to increase SMN levels, increased both SERCA2 protein expression and Ca\(^{2+}\) re-uptake rate (Fig. 6b). Conversely, using siRNA to reduce SMN expression in control cells, reduced both SERCA2 expression and the T\(_{50}\) for Ca\(^{2+}\) reuptake (Fig. 6c; Supplemental Figure 2). However, we did not observe the isoform switch from SERCA2a to SERCA2b, as observed in the mouse tissue, in the SMN-deficient iPSC-derived cardiomyocytes. Taken together, these results demonstrate that SMN regulates SERCA2 expression and intracellular Ca\(^{2+}\) cycling kinetics in cardiomyocytes that may impair cardiac function and lead to elevation of heart failure markers, as observed in mice (Fig. 1) and patients with SMA [15].

Discussion
The major pathological feature of SMA is neuromuscular degeneration; however, there have been reports that cardiovascular function is impaired in both SMA patients and mouse models of the disease [16, 17, 45–47]. The data in the present study demonstrate increased expression of heart failure molecular markers, reduced expression of SERCA2, and impairment of cardiomyocyte contraction in a severe mouse model of SMA. Moreover, these findings are reversibly recapitulated in cardiomyocytes generated from patient-derived iPSCs, indicating that SMN deficiency likely causes a similar phenotype in human cardiac muscle as well. Taken together, these results add support to the conclusion that cardiovascular function is compromised by loss of SMN function and indicate that dysregulation of SERCA2 expression and Ca\(^{2+}\) homeostasis lies at the center of cardiac pathophysiology.

Heart function and failure in SMA
Efforts to treat SMA are largely focused on preventing or reversing neuromuscular degeneration. We showed previously that daily injections of the HDAC inhibitor, trichostatin A, improves motor function and modestly extend the lifespan of SMA mice [48]. Interestingly, however, these mice ultimately die from cardiovascular causes [48]. Here, we show that BNP and skeletal α-actin are elevated as early as P5 in SMA mice, prior to the development of profound neuromuscular degeneration. Moreover, even though cell shortening and Ca\(^{2+}\) transient amplitude are preserved in SMN-deficient cells,
we show that the rate of relaxation and diastolic sarco-
merere length are markedly reduced in SMA cardiomyo-
cytes, suggesting that SMN deficiency may be associated
with diastolic heart failure or heart failure with preserved
ejection fraction [49]. It is interesting to note, that SMA
patients often require artificial ventilation as a result of
lung congestion and respiratory distress even though the
diaphragm muscle is relatively spared from degeneration
[50]. Although speculative, heart failure resulting from
SMN deficiency as evidenced here may contribute to the
respiratory pathology in SMA patients in addition to
neuromuscular degeneration. Nevertheless, the present
findings support the conclusion that cardiac function is
compromised in SMN deficient animals, even though
the major pathological feature is neuromuscular
degeneration.

**Effects of SMN deficiency appear to be cell autonomous**
Because neurodegeneration is central to SMA pathology,
it is reasonable to consider the influence of innervation
of the heart as a cause of pathology. Indeed, several
studies suggest that sympathetic denervation and
sympatho-vagal imbalance are evident in SMA patients
and mouse models of the disease [16–18, 51]. Bradycar-
dia and slowed conduction has been reported in pa-
tients, consistent with sympathetic denervation of the
heart [19, 20]. We cannot rule out that sympathetic de-
nervation contributes to cardiovascular pathology in
SMA; however, here we show clear evidence of impaired
cardiac function with slowed intracellular Ca$$^{2+}$$ dynamics
and reduced SERCA2 expression in a model of severe,
type I SMA. It is likely that the degree of cardiac in-
volve ment varies with disease severity and a study in pa-
tients with the less severe type II/III SMA variant
reported no evidence of cardiac dysfunction [45]. Im-
portantly, the reduction in SERCA2 expression and
slowing of Ca$$^{2+}$$ reuptake was recapitulated and revers-
brle in cardiomyocytes generated from SMA patient-
derived iPSCs. When coupled with the observation that
evidence of heart failure emerges as early as P5 in the
mouse, this result provides strong evidence that the
impairment of cardiomyocyte function is a result of cell
autonomous loss of SMN function, and not secondary to
neu rodegeneration.

**Altered Ca$$^{2+}$$ handling as a common disease mechanism
in SMA**
It remains unclear why SMN deficiency so potently tar-
gets motor neurons for degeneration. The principal find-
ing in this study is that SMN deficiency causes a decrease
in the expression of SERCA2 protein in both
SMA mouse heart and cardiomyocytes generated from
patient-derived iPSCs. SMN is known to play a role in
snRNP biogenesis and spliceosome assembly [6–8]. It is
not known how SMN deficiency inhibits SERCA2
biosynthesis, but we did observe an apparent isoform
switch, with reduced SERCA2a and increased SERCA2b
mRNA expression albeit with a significant reduction in
Defects in [Ca\textsuperscript{2+}]\textsubscript{i} handling are recapitulated in cardiomyocytes derived from SMA patient iPSCs. 

**a** Representative [Ca\textsuperscript{2+}]\textsubscript{i} transients measured in iPSC-derived cardiomyocytes engineered from unaffected control (NCRM1) and SMA patient (GM23240) iPSCs illustrating the slowed removal of cytoplasmic Ca\textsuperscript{2+}. 

**b** Expression of SERCA2a expression is increased (western blot, left) and T\textsubscript{50} is decreased (right) in SMA patient iPSC-derived cardiomyocytes when SMN protein is restored. Cells were transfected on day 18 of differentiation and recordings were made on day 20. 

**c** Similarly, expression of SERCA2a expression is reduced (western blot, left) and T\textsubscript{50} is increased (right) in unaffected control iPSC-derived cardiomyocytes when SMN protein is reduced. Cells were transfected on day 18 of differentiation and recordings were made on day 20. 

Statistical analysis: p values shown for one-way ANOVA, Tukey’s post hoc test, n = 7
total SERCA2 protein regardless of the isoform. One possibility is that disruption of alternative splicing mechanisms in cardiomyocytes leads to reduced SERCA2 expression. However, the SERCA2 alternative splicing was not observed in the human cardiomyocytes when SMN was knocked down even though total SERCA2 expression was reduced. This suggests that alternative splicing cannot fully account for the reduced SERCA2 expression in SMN-deficient cells.

Interestingly, SMN deficiency also affects intracellular Ca\(^{2+}\) handling in motor nerve terminals. Similar to the observations in the present study, Ca\(^{2+}\) removal from the cytoplasm is significantly impaired in motor neuron terminals following repeated stimulation [52]. Elevated basal Ca\(^{2+}\) has also been observed in SMN-deficient iPSC-derived astrocytes [53], and there is also a report showing reduced SERCA1a protein in hindlimb muscles of SMN-deficient mice [54]. These findings coupled with the data in the present study suggest that dysregulation of Ca\(^{2+}\) may be a common disease mechanism in SMA.

### Conclusion
Collectively, these results show for the first time that SMN deficiency alters intracellular Ca\(^{2+}\) signaling and cardiac excitation-contraction coupling in a model of SMA. These findings are recapitulated in SMA patient iPSC-derived cardiomyocytes suggesting that this is a cell autonomous outcome of SMN deficiency and suggest that dysregulation of intracellular Ca\(^{2+}\) may be a common pathological mechanism in the disease. Finally, while neuromuscular degeneration remains the hallmark feature of the disease, impaired heart function may be a contributing factor in disease progression that will require monitoring in light of new therapies that are improving motor function and extending survival.

### Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13395-020-00232-7.

### Additional file 1: Supplemental Figure 1
Elevated expression of ANP in SMA model mice. qRT-PCR analysis of ANP mRNA expression. Expression levels (mean ± SEM) in SMN-deficient (SMA) heart tissue are represented relative to unaffected controls at each time point. Statistical analysis; p values shown for one-way ANOVA, Tukey’s post hoc.

### Additional file 2: Supplemental Figure 2
Serca2 levels reduced in cardiomyocytes derived from SMN-deficient iPSCs. Expression of SERCA2a expression was determined in control and SMN-deficient iPSC-derived cardiomyocytes. Cells were transfected with siRNA targeting SMN day 18 of differentiation and qRT-PCR performed 48 hours after transfection. Values represent mean ± SEM.

### Additional file 3: Table 1 Transcriptome analysis. Table 2 Reagents

### Abbreviations
SMA: Spinal muscular atrophy; SMN: Survival motor neuron; SERCA: Sarco/endoplasmic reticulum Ca\(^{2+}\)–ATPase; iPSC: Induced pluripotent stem cell; BNP: Brain natriuretic peptide; Acta1: Skeletal α-actin; P: Post-natal

### Disclaimer
The views expressed are those of the authors and do not reflect the official policy or position of the Uniformed Services University of the Health Sciences, the Department of the Defense, or the United States Government.

### Authors’ contributions
GK, REM, INM, and NF conducted and analyzed biochemical experiments. GK, JS, and JTS performed cell isolation, contractility and Ca\(^{2+}\) imaging experiments. CLD and CD performed RNA sequencing and transcriptome analysis. MLD provided iPSCs and expertise in stem cell culture. TPF designed experiments, analyzed contractility and imaging data, prepared figures, and wrote the manuscript. BGB designed experiments, analyzed expression data, prepared figures, and wrote the manuscript.

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### Availability of data and materials
All data generated or analyzed during this study are included in this published article.

### Ethics approval and consent to participate
All procedures were approved by the Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, and were performed in accord with the National Research Council’s Guide for the Care and Use of Laboratory Animals (2011).

### Consent for publication
Not applicable.

### Competing interests
The authors declare they have no competing interests.

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### References
1. Feldkotter M, Schwarzer V, Wirth R, Winker TF, Wirth B. Quantitative analyses of SN1 and SN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. Am J Hum Genet. 2002;70(2):358–68.
2. Lefebvre S, Bucllet P, Liu Q, Bertrand S, Clermont O, Dunnich A, Dreyfuss G, Melki J. Correlation between severity and SMN protein level in spinal muscular atrophy. Nat Genet. 1997;16(3):265–9.
3. Mendell Jr, H-Zaidy S, Shell R, Arnold WQ, Rodino-Klapac LR, Prior TW, Lowes L, Alafano L, Berry K, Church K, et al. Single-dose gene-replacement therapy for spinal muscular atrophy. N Engl J Med. 2017;377(18):1713–22.
4. Finkel RS, Mercuri E, Darras BT, Connolly AM, Kuntz NL, Kischner J, Chiriboga CA, Saito K, Servais I, Tizzano E, et al. Nusinersen versus sham control in infantile-onset spinal muscular atrophy. N Engl J Med. 2017;377(18):1723–32.
5. Ali G, Gleich F, Carroll WD, Alexander J, Clayson S, Kushretha R, Willis T, Samuels M. Healthcare utilisation in children with SMA type I treated with nusinersen: a single centre retrospective review. BMJ Paediatr Open. 2019; 3(1):e000572.
6. Meister G, Buhler D, Pillai R, Lottspeich F, Fischer U. A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. Nat Cell Biol. 2001;3(11):945–9.
7. Meister G, Eggert C, Fischer U. SMN-mediated assembly of RNP’s: a complex story. Trends Cell Biol. 2002;12(10):472–8.
8. Pellizzoni L, Yong J, Dreyfuss G. Essential role for the SMN complex in the specificity of snRNP assembly. Science. 2002;298(5599):1775–9.
9. Hua Y, Liu YH, Sahashi K, Rigo F, Bennett CF, Kramer AR. Motor neuron cell-
nonautonomous rescue of spinal muscular atrophy phenotypes in mild and
severe transgenic mouse models. Genes Dev. 2015;29(3):288–97.

10. Bricken RV, Martinez T, Leikina E, Duguez S, Partridge TA, Chernomordik LV,
Fischbeck KH, Sumner CJ, Burnett RG. Survival motor neuron protein
deficiency impairs myotomy formation by altering myogenic gene
expression and focal adhesion dynamics. Hum Mol Genet. 2014;23(18):
4745–57.

11. Boyer JG, Deguise MO, Murray LM, Yazdani A, De Repentigny Y, Boudreau-
Lavriere C, Kothary R. Myogenic program dysregulation is contributory to
disease pathogenesis in spinal muscular atrophy. Hum Mol Genet. 2014;
23(10):4249–59.

12. Deguise MO, De Repentigny Y, McFall E, Auclair N, Sad S, Kothary R.
Immune dysregulation may contribute to disease pathogenesis in
spinal muscular atrophy mice. Hum Mol Genet. 2017;26(4):801–19.

13. Khairallah MT, Astroski J, Custer SK, Androphy EJ, Franklin CL, Lorson CL.
SMN deficiency negatively impacts red pulp macrophages and spleen
development in mouse models of spinal muscular atrophy. Hum Mol Genet.
2017;26(5):932–41.

14. Thomson AK, Somers E, Powis RA, Shorrock HK, Murphy K, Swoboda KJ,
Gillingwater TH, Parson SH. Survival of motor neuron protein is required
for normal postnatal development of the spine. J Anat. 2017;230(2):
337–46.

15. Kobayashi DT, Shi J, Stephen L, Ballard KL, Shi J, Stephen L, Ballard KL,
Dewey R, Mapes J, Chung B, Kobayashi DT, Shi J, Stephen L, Ballard KL,
Dewey R, Mapes J, Chung B. Structural and functional analysis of the
centromeric survival motor neuron (SMN2) gene, extends survival in
Spinal Muscular Atrophy: A Case-Control Study. J Child Neurol. 2018;
33(7):487–96.

16. Bevan AK, Hutchinson KR, Fouot KD, Braun L, McCarron D, Schneider L,
Ward JG, Petsuka JC, Lucchesi PA, Burghes AH, et al. Early heart failure in
the SMNDelta2 model of spinal muscular atrophy and correction by
postnatal scaa4-SMN delivery. Hum Mol Genet. 2011;20(10):1985–95.

17. Heier CR, Satta R, Lutz C, DiDonato CJ. Arrhythmia and cardiac defects are a
feature of spinal muscular atrophy model mice. Hum Mol Genet. 2010;
19(20):3906–18.

18. Shababi M, Habibi J, Yang HT, Vale SM, Sewell WA, Lorson CL. The Smn-independent
expression and focal adhesion dynamics. Hum Mol Genet. 2014;
23(12):2920–3.

19. Falsaperla R, Vitaliti G, Collotta AD, Fiorillo C, Pulvirenti A, Alaimo S, Romano
M, Pulvirenti A, Alaimo S, Romano M, Pulvirenti A, Alaimo S, Romano M.
Cardiac pathology in spinal muscular atrophy: a systematic review.
J Neuropathol Exp Neurol. 2014;73(6):559–74.

20. Fischbeck KH, Sumner CJ. The role of histone acetylation in SMN gene expression. Hum
Mol Genet. 2017;26(4):801–19.

21. Liu H, Yazdani A, Murray LM, Beauvais A, Kothary R. The Smn-independent
defects contribute to the pathology of spinal muscular atrophy model mice. J Mol Cell
Cardiol. 2015;85(0):79–88.

22. Le TT, Pham LT, Butchbach ME, Zhang HL, Monani UR, Coovert DD,
Pol WL. Cardiac pathology in spinal muscular atrophy: a systematic review.
J Neuropathol Exp Neurol. 2014;73(6):559–74.

23. Shpargel KB, Matera AG. Gemin proteins are required for efficient assembly of
Sm-class ribonucleoproteins. Proc Natl Acad Sci U S A. 2005;102(48):
17372–7.

24. Frey N, Olsson EN. Cardiac hypertrophy: the good, the bad, and the ugly.
Annu Rev Physiol. 2003;65(1):45–79.

25. Kogler H, Schott P, Toischer K, Mutting H, Van PN, Kohlhaas M, Gereb C,
Kaisser A, Domeier E, Teucher N, et al. Relevance of brain natriuretic peptide in preload-dependent regulation of cardiac sarcoplasmic reticulum
Ca2+ ATPase expression. Circulation. 2006;113(23):2724–32.

26. Saito Y, Nakao K, Arii H, Nishimura K, Okumura K, Obata K, Takemura G,
Fujwara H, Sugawara A, Yamada T, et al. Augmented expression of atrial natriuretic polypeptide gene in ventricle of human failing heart. J Clin
Invest. 1989;83(3):308–15.

27. Shababi M, Habibi J, Ma L, Glascock JJ, Sowers JR, Lorson CL. Partial
phospholamban knockout mouse: relaxation and endogenous CaMKII
phosphorylation. Am J Physiol. 2002;286(4):H1361–69.

28. Fischbeck KH, Sumner CJ, Burnett BG. Survival motor neuron protein
function is required for nonautonomous rescue of spinal muscular atrophy
phenotypes in mild and severe transgenic mouse models. Genes Dev.
2015;29(3):288–97.
52. Ruiz R, Casañas JJ, Torres-Benito L, Cano R, Tabares L. Altered Intracellular Ca2+ Homeostasis in Nerve Terminals of Severe Spinal Muscular Atrophy Mice. J Neuroscience. 2010;30(3):849–57.
53. McGivern JV, Pattucci TN, Nord JA, Barabas ME, Stucky CL, Ebert AD. Spinal muscular atrophy astrocytes exhibit abnormal calcium regulation and reduced growth factor production. Glia. 2013;61(9):1418–28.
54. Boyer JS, Murray LM, Scott K, De Repentigny Y, Renaud JM, Kothary R. Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. Skelet Muscle. 2013;3(1):24.

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