Supplemental Information – Serra et al.

Supplemental Experimental Procedures

1. Assessment of PSC-CM viability and metabolic activity after storage

Cell viability, metabolic activity, and metabolism were evaluated at days 0, 1, 3 and 7 of culture post-storage, using the methods described below.

1.1. Fluorescein diacetate-propidium iodide staining. The qualitative assessment of the cell plasma membrane integrity was performed using the enzyme substrate fluorescein diacetate (FDA, Sigma-Aldrich), and the DNA-binding dye propidium iodide (PI, Sigma-Aldrich), as described in the literature [1]. Briefly, cells were incubated with 20 μg/mL FDA and 10 μg/mL PI in Dulbecco's Phosphate Buffered Saline (DPBS, Invitrogen) for 2-3 min and visualized by fluorescence microscopy (DMI 6000, Leica Microsystems GmbH).

1.2. Lactate Dehydrogenase (LDH) activity in the supernatant. The release of intracellular LDH to the supernatant can be correlated with the extent of cell lysis as described by Decker et al [2]. LDH activity from the supernatant of centrifuged (300 g, 5 min) samples was determined spectrophotometrically following (at 340 nm) the rate of oxidation of NADH to NAD^+ coupled with the reduction of pyruvate to lactate as described elsewhere [3].

1.3. Trypan Blue exclusion method. Cells cultured as monolayers were dissociated as described in section 2.2. Cell concentration was estimated by counting cells using a Fuchs–Rosenthal haemocytometer (Brand). Viable cells were determined by using the trypan blue dye (0.1% (v/v) in DPBS) exclusion method. The percentage of cell recovery was estimated taking into account the number of viable cells before cold storage.

1.4. PrestoBlue™ assay. Metabolic activity was determined using the metabolic indicator PrestoBlue (Invitrogen) reagent following manufacturer's recommendation. Briefly, cells were incubated for 1-2 h with culture medium containing 10% (v/v) PrestoBlue reagent. Fluorescence was measured (excitation wavelength: 570 nm, emission wavelength: 585 nm) using a microwell plate fluorescence reader Infinite 200 PRO NanoQuant (TECAN). The active ingredient of PrestoBlue (resazurin) is a nontoxic, non-fluorescence indicator dye that is reduced to red-fluorescent resorufin by metabolically active cells. Fluorescence intensity is proportional to the number of live and metabolic active cells. Thus, PrestoBlue assay provides a quantitative measure of cell viability and metabolic activity. Fluorescence values were normalized to the values obtained with the same cells before cold storage, and are indicated as a percentage of metabolic activity recovery.

1.5. NucView™ 488 and MitoView™ 633 Apoptosis Assay. A qualitative assessment of apoptosis was performed using the Apoptosis Assay Kit NucView™ 488 and MitoView™ 633 (Biotium, Inc., California, USA) following manufacturer's instructions. This kit contains the green fluorescent NucView 488 caspase-3 substrate and the far-red fluorescent MitoView 633 mitochondrial dye for profiling caspase-3 activity and changes in mitochondrial membrane potential, respectively, in intact cells. After incubation with both reagents for 1 h, cells were observed using fluorescence microscopy (DMI 6000, Leica) and representative images were taken.

1.6. Quantification of caspase activity. A quantitative assessment of apoptosis was performed using the Caspase Family Fluorometric Substrate Kit II Plus (Abcam) following manufacturer's instructions. Briefly, the activity of caspases -3/7, -6, -8, -9 and -10 was determined by incubation with the respective AFC conjugated substrates. AFC-based substrates yield fluorescence upon protease cleavage (Ex/Em=400 nm/505 nm). The fold increase in caspase activity was determined by the ratio of the values of fluorescence obtained for the cells subject to cold storage to the values obtained by the cells immediately before storage.

1.7. Metabolite analysis. Glucose (GLC), glutamine (GLN) and lactate (LAC) concentrations in the culture medium were analyzed using an YSI 7100MBS (YSI Incorporated). Specific metabolic rates (qMet., $mol.h^{-1}.cell^{-1}$) were calculated using the equation: qMet. = ΔMet/(Δt ΔXv), where ΔMet (mol/L) is the variation of metabolite concentration during the time period Δt (h), and ΔXv (cell/L) the average of cell concentration during the same time period.

2. Assessment of PSC-CM structure and ultrastructure after hypothermic storage

2.1. Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM)

Monolayers and aggregates of human PSC-CMs were fixed in 2% (v/v) glutaraldehyde in 0.1 M HEPES buffer (pH 7.4) for 20 min, washed in DPBS and stored in 1% (v/v) glutaraldehyde in DPBS until analyses. For TEM analyses, cell aggregates were immersion fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 50 mM HEPES. Subsequently, the samples were washed once in 100 mM HEPES, twice in DPBS, and twice in double-distilled water before postfixation with osmium tetroxide (1% (v/v) in water, 2 h on ice). After several washes in water, the samples were contrasted *en-bloc* with aqueous uranyl acetate (1% (w/v), 2 h on ice), washed several times in distilled water, dehydrated in a graded series of ethanol, infiltrated in epon 812 (epon/ethanol mixtures: 1:3, 1:1, 3:1, 1 h each, pure epon overnight, pure epon 6 h), and embedded in flat embedding molds. Finally, the samples were cured at 65°C in the oven. 2D-Monolayers were fixed with 2% (v/v) glutaraldehyde in 100 mM HEPES

for 1 h, and washed in 100 mM HEPES and distilled water. Postfixation, *en-bloc* contrasting, dehydration, epon infiltration and curing were performed as described above. After curing, the samples were dissected into small blocks and mounted on epon dummy blocks. Ultrathin sections of aggregates and monolayers were cut on a Leica UC6 ultramicrotome using a diamond knife. Sections were collected on formvar-coated slot grids, stained with lead citrate and uranyl acetate as described in the literature [4], and analysed on a FEI Morgagni 268 at 80 kV. Images were taken with an Olympus MegaView III using the iTEM software.

For SEM analyses cell aggregates were immersion fixed in 2% (v/v) glutaraldehyde with 2% (w/v) paraformaldehyde (PFA) in 50 mM HEPES, and washed once in 100 mM HEPES, twice in DPBS, and twice in double-distilled water before post-fixation with osmium tetroxide (1% in water, 2 h on ice). After several washes in distilled water, the samples were dehydrated in a graded series of ethanol, critical-point dried using the Leica CPD 300, mounted to carbon tabs which were glued to 12 mm aluminum stubs, and sputter coated with gold using the Baltec SCD 050 sputter coater. Samples were imaged in a Jeol 7500F cold field emission SEM at 5kV acceleration voltage and 8 mm working distance using the lower secondary electron detector.

3. Evaluation of aggregate size

The aggregate size was determined using an inverted-microscope (DMI 6000, Leica) by measuring two perpendicular diameters of each aggregate. These measures were used to calculate the average diameter of each aggregate.

4. Characterization of PSC-CMs after hypothermic storage

4.1. Flow cytometry. After a 5 min dissociation with TypLE™Select, human PSC-CMs were washed twice with DPBS and a total of $5x10^5$ cells were incubated with each of the antibodies VCAM-1 (CD106-PE, BD Biosciences), SIRPα/β (CD172a/b-PE, BioLegend) and isotype controls IgG1,κ-PE (BD Biosciences) for 1 h at 4°C. Cells were washed twice with DPBS, and analysed in a CyFlow® space (Partec GmbH) instrument, registering 10,000 events/sample.

4.2. Immunofluorescence microscopy. Cells were washed with DPBS and fixed in 4% (w/v) PFA and 4% (w/v) sucrose in DPBS solution for 15 min. Afterwards, cells were permeabilized for 10 min in 0.5 M Ammonium chloride (Sigma-Aldrich), 0.25% (v/v) Triton X-100 in DPBS and blocked with 5% (v/v) FBS in DPBS for 1 h, at room temperature. Cells were subsequently incubated overnight at 4°C with primary antibodies diluted in 0.2% (w/v) Fish Skin Gelatin (FSG), 0.1% (w/v) TX-100 in DPBS. The following primary antibodies were used: α-sarcomeric actinin (1:200, Sigma-Aldrich), titin (1:100, Santa Cruz Biotechnology), troponin I (TnI, 1:100, Millipore), troponin T (Tnt, 1:100, Millipore), MYL2 (1:140, ABCAM), MYL7 (H-60, 1:50, Santa

Cruz Biotechnology). Non reporter cell lines were also incubated with F-actin Alexa Fluors 488 dye (Life technologies). Cells were washed in DPBS and secondary antibodies were incubated for 60 min at room temperature in the dark. Secondary antibodies used were: AlexaFluor 594 goat anti-mouse IgG and AlexaFluor 594 goat anti-rabbit IgG (1:500 dilution in 0.2% (w/v) FSG, 0.1% (w/v) TX-100 in DPBS). Cell nuclei were counterstained with Hoechst 33342 nucleic acid dye. Cells were visualized using an inverted fluorescence microscope (DMI 6000, Leica).

4.3. Quantitative RT-PCR. Total RNA was extracted using the High Pure RNA Isolation Kit (Roche), and reverse transcription was performed with High Fidelity cDNA Synthesis Kit (Roche), following manufacturer's instructions. Relative quantification of gene expression was performed using LightCycler 480 Probes Master (Roche) in 20 µL reactions with 50 ng cDNA template and 20x concentrated TaqMan probes. The reactions were performed in 96-well plates using a LightCycler 480 Real-Time PCR System (Roche). Cycle threshold (Ct's) were determined by LightCycler 480 Software. All data was analyzed using the 2-ΔΔCt method for relative gene expression analysis. Changes in gene expression were normalized to the average of RFLP0 and GAPDH gene expression as internal control. The TaqMan probes used for human ISL1, KDR, GATA4, NKX2.5, TNNT2, VCAM1, MYH7, MYL2, MYL7, HCN4, CACNA1C, ACTC1, MYH6, MYH7, SCN5A, RFLP0 and GAPDH genes were HS00158126_m1, HS00911699_m1, Hs00171403_m1, Hs00231763_m1, Hs00165960_m1, Hs01003372_m1, Hs01110632_m1, Hs00166405_m1, Hs00221909_m1, Hs00175760_m1, Hs00167681_m1, Hs00606316_m1, Hs01101425_m1, Hs01110632_m1, Hs00165693_m1, Hs99999902_m1 and Hs99999905_m1, respectively.

5. Assessment of PSC-CM function after hypothermic storage

5.1. **Electrophysiological analyses of mIPSC-CMs.** Single mPSC-CMs were plated on glass cover slips coated with 0.1% gelatin. The patch clamp experiments were performed as described previously [5]. Briefly, cover slips were placed into a recording chamber (37°C) and cells were continuously perfused with extracellular solution. Cell membrane capacitance was determined online using Pulse software (Heka Elektronik). Action potential (AP) recordings of spontaneously beating murine PSC-CMs were performed utilizing the whole-cell current-clamp technique with an EPC-9 amplifier (HEKA Elektronik), and operated through the Pulse acquisition software. Response to hormonal regulation was analyzed by administering 1 µM isoproterenol (Iso, Sigma-Aldrich).

5.2. **Electrophysiological analyses of hIPSC-CMs.** Single hPSC-CMs were plated on 35 mm plastic petri dishes previously coated with Synthemax. These dishes were used as recording chambers mounted on the stage of an inverted microscope. A gravity based superfusion

system was used for the exchange of extracellular solution (2–3 mL/min), which had the following composition: 135 mM NaCl, 5 mM KCl, 1.5 mM $MgSO_4$, 0.33 mM NaH_2PO_4, 10 mM HEPES, 1 mM $CaCl_2$, 15 mM D-glucose, adjusted to pH 7.35 with 1 mM NaOH. Cells were voltage-clamped using the whole cell patch-clamp configuration, as described previously [6]. Briefly, the patch pipettes (2–5 MΩ) were pulled from borosilicate glass capillaries (Science Products GmbH, GB150T-8P) and filled with an internal solution containing: 135 mM potassium gluconate, 5 mM KCl, 5 mM NaCl, 10 mM Na1/2HEPES, 1 mM $MgCl_2$, 0.1 mM EGTA, 2 mM Na2ATP, 0.4 mM NaGTP (pH 7.3 adjusted with 1 mM KOH; 305±5 mOsm). The estimated junction potential for the filling and bathing solution combinations mentioned above is -8.9 mV (calculated with JPCalc 2.00, School of Physiology and Pharmacology University of New South Wales). Data were not corrected for the junction potential. Currents were recorded with an Axopatch 200B electrometer (Axon Instruments Inc., USA) and stored on a PC computer using pClamp 6.0.3 software (Axon Instruments Inc., USA) and an analogue digital interface (Digidata 1200; Axon Instruments, USA). Signals were acquired at a sampling rate of 5 kHz and filtered at 2 kHz (-3 dB, four pole Bessel). Electrode and cell membrane capacitances were compensated whenever possible. Cells with significant leak currents were rejected.

The holding potential (VH) was kept at -70mV. Current-voltage relationships were investigated using a set of 10 mV (260 ms) command pulses from -130 to 0 mV. More depolarizing potentials, greater than 0 mV, were not used as in this protocol because patch seals tend to brake. Thus, to avoid depolarization-induced cells damage, we excluded the depolarizing steps above 0 mV from the current-voltage relationships, focusing more on the evaluation of the inward currents. The K^+ outward currents were observable only at potentials greater than ± 40mV (Figure 6.A).

The Ca^{2+} dependence of the outward K^+ currents was assessed using a two-pulse protocol, which consists of a triple set of double depolarizing pulses (Figure 6.B). A prepulse lasting 50 ms to -40, -10 or +20 mV was delivered to trigger Ca^{2+} influx through voltage-activated channels. The prepulse was immediately followed by a second depolarizing pulse to +40 mV for 750 ms to elicit the outward K^+ current.

5.3. **Pharmacological responses of hPSC-CMs.** The effect of pharmacological treatment on the contractile activity of hPSC-CMs was analyzed by administering in the superfusion fluid: 300 pM Nimodipina; 30 mM KCL; 1 µM Verapamil; 0.5 mM Adrenaline. hPSC-CMs were visualized using a Nikon Eclipse Ti-U inverted microscope at room temperature (± 25°C). Bright field video/images were recorded with a FASTCAM MC2 camera (Photron Europe, Limited) controlled with PFV (Photron FASTCAM Viewer) software. A region of interest was drawn

around the contracting structures filmed at 10 fps. Pixel density, used as indirect measurement of contraction, was digitized using ImageJ software (National Institutes of Health [NIH]).

5.4. Detection of Ca^{2+} Transients. Ca^{2+} imaging was performed according to the standard protocol provided in Rhod-3- Calcium Imaging kit (Invitrogen). Aggregates of human PSC-CMs were plated onto eight-well culture plates coated with Synthemax and cultured for 1 week, to promote cell adherence and migration to the plate. Briefly, cells were labelled with Rhod-3 for 1 h at 37°C, washed twice in DPBS, incubated for 1 h with a water-soluble reagent to reduce baseline signal and washed again in DPBS. Cells were imaged live using a spinning disk confocal microscope (Nikon Eclipse Ti-E; System: Andor Revolution XD; Confocal Scanner: Yokogawa CSU-x1) and calcium transients were determined using Micro-Manager 1.4 and ImageJ software.

6. Statistical analysis. All statistical analyses were performed using GraphPad Prism version 5 (GraphPad Software Inc.). Values are represented as mean±standard deviation of independent measurements or assays (at least n=3 replicates were considered). Statistical significance was evaluated using either Student's t test with Welch's correction or one-way analysis of variance (ANOVA). Values of $P<0.05$ were considered statistically significant.

References

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Supplemental Figures

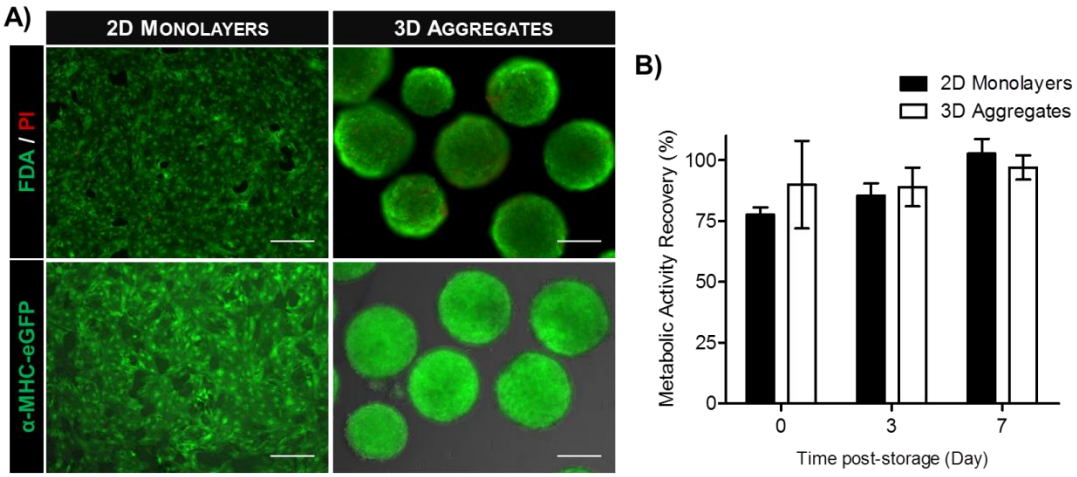

Figure S1 - Hypothermic storage of murine iPSC-CMs. 2D monolayers and 3D aggregates of miPSC-CMs were stored for 7 days at 4°C in HTS solution. Cell recovery post-storage was evaluated for 7 days. Cell viability was assessed by cell staining with FDA (live cells, green) and PI (dead cells, red) - **A upper panel** - and by evaluation of α-MHC-eGFP expression (green) - **A lower level**. Photos were taken at day 1 post-storage. Scale bars: 200 µm. **B)** Evaluation of metabolic activity recovery during 7 days post-storage using the PrestoBlue assay. The fluorescence values of PrestoBlue are presented as a % in relation to the values obtained with the same cells immediately before cold storage.

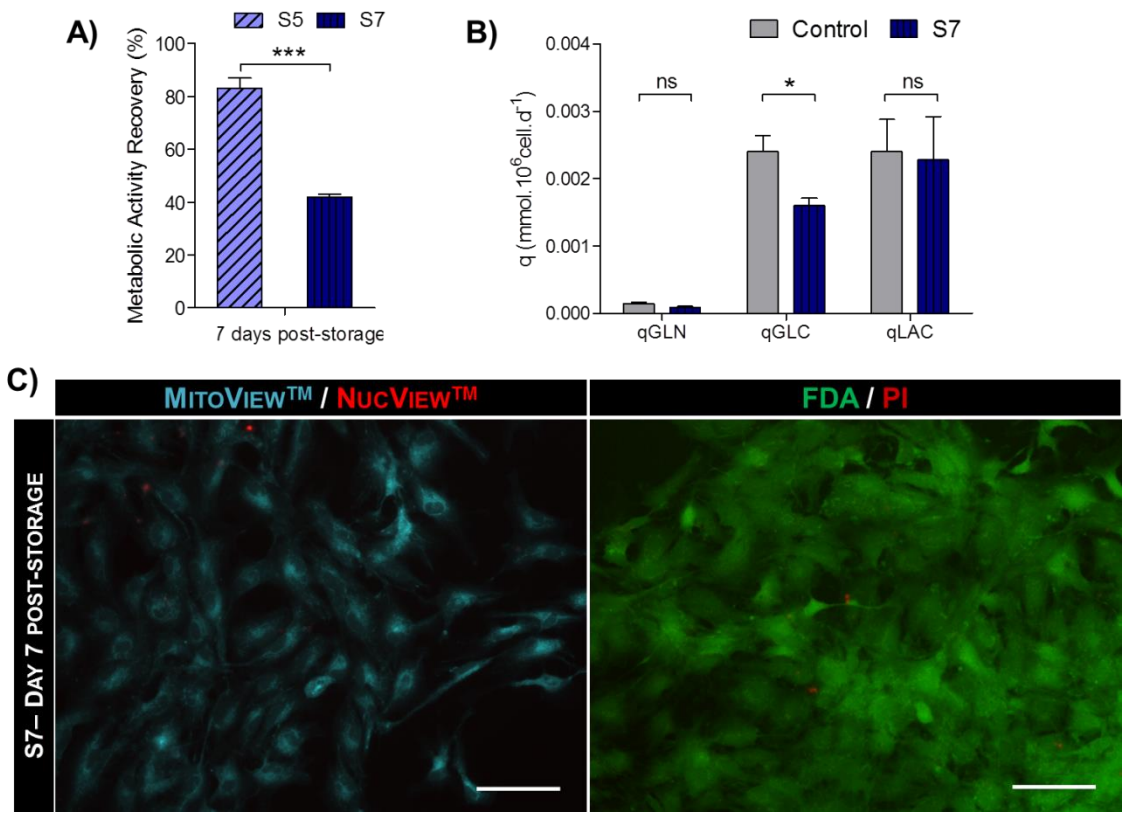

Figure S2 - Hypothermic preservation of hESC-CM as 2D monolayers. Cells were stored for 5 (S5) and 7 (S7) days at 4°C in HTS solution. Cell recovery was evaluated for 7 days post-storage. **A-B)** Metabolic performance of hESC-CMs post-storage. **A)** Evaluation of metabolic activity recovery using the PrestoBlue assay. **B)** Mean specific rates of glutamine (qGLN) and glucose (qGLC) consumption and lactate production (qLAC) during one week in culture post-storage. Control refers to cells not subjected to hypothermic storage. Data are presented as mean ± SD of five measurements. $P<0.05$ (*), $P<0.001$ (***), Not-significant (ns), determined by unpaired t test. **C)** Viability analysis of hESC-CMs stored for 5 days at 4°C, assessed at day 7 post-storage, using the MitoView™ (functional mitochondria, cyan) and NucView™ (apoptotic cells, red) – **right panel** and FDA (live cells, green) and PI (dead cells, red) – **left panel**. Scale bars: 100µm.

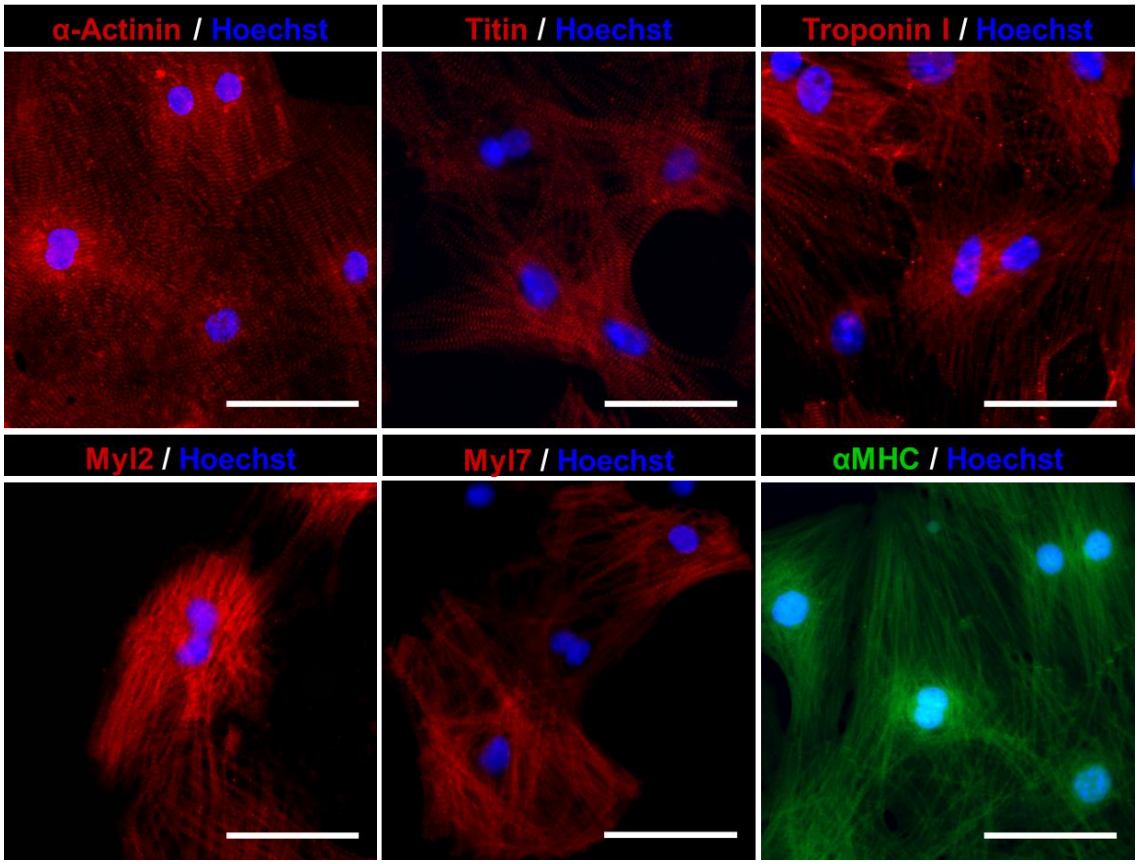

Figure S3 - Immunofluorescence labelling of miPSC-CMs after hypothermic storage as 2D monolayers. Cells were stained using the CM-specific, sarcomeric α-actinin, titin, troponin I, Myl2 and Myl7, antibodies (red). Expression of eGFP driven by the cardiac promoter α-MHC (green) was also detected. Nuclei were labelled with Hoeschst 33432 (blue). Scale bars: 50 μm.

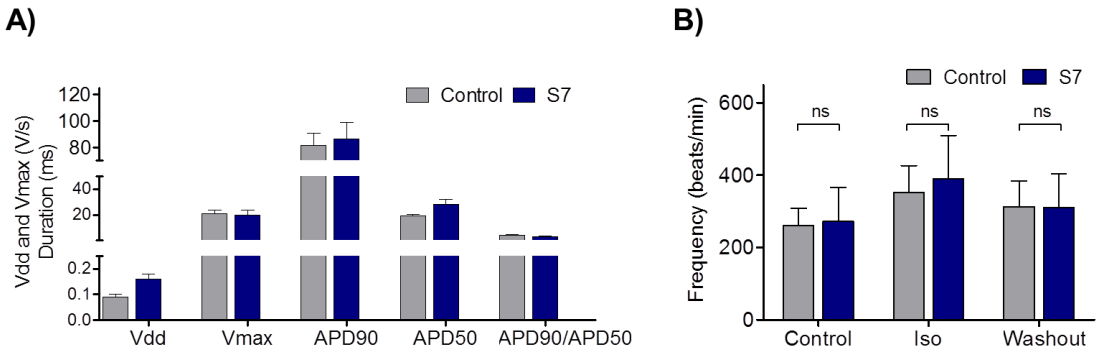

Figure S4 – Functional characterization of miPSC-CMs subjected to hypothermic storage for 7 days. Cell functionality was evaluated in cells stored for 7 days (S7, blue bars) and non-stored miPSC-CMs (control, grey bars) cultured as 3D aggregates by the detection of CM-specific action potentials (AP, **A**) and typical CM response to drugs (**B**). **A)** Action potential properties of miPSC-CMs: Vdd, velocity of diastolic depolarization; Vmax, the maximum rate of rise of the action potential upstroke; APD90, action potential duration at 90% of repolarization; APD50, action potential duration at 50% of repolarization. **B)** Effect of 1 μM Isoproterenol (Iso) on beating frequency of miPSC-CMs. Data are presented as mean ± SD of six (A-B) measurements. Not-significant (ns) determined by unpaired t test.