SUPPLEMENTAL MATERIAL

Microfluidic Single Cell Analysis of Transplanted Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes Following Acute Myocardial Infarction

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SUPPLEMENTAL METHODS

Pluripotency Marker Analysis. Human iPSC colonies grown in 6-well tissue culture plates (Sigma Aldrich, St. Louis, MO) were fixed in 4% paraformaldehyde for 15 min and washed with PBS. Subsequently, cells were permeabilized with 0.3% Triton X-100 for 20 min and washed with PBS. After blocking for 1 hr at room temperature with 5% BSA, cells were incubated with primary antibody for 1 hr. The primary antibodies used for staining were Oct3/4 (Santa Cruz Biotechnology), Tra-1–60 (Chemicon), Tra-1–81 (Chemicon), and Nanog (Santa Cruz Biotechnology). After washing with PBS, cells were incubated with AlexaFluor-conjugated secondary antibodies (Santa Cruz Biotechnology) for 20 min, in parallel with DAPI. After washing with PBS, immunofluorescence images were acquired using a Leica brightfield microscope.

Immunofluorescence Staining and Confocal Microscopy. Primary antibodies against human cardiac troponin T (Thermo Scientific and Abcam) and sarcomeric alpha-actinin (Sigma) and secondary antibodies (goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594) were used. Cardiomyocyte stainings were done as previously described. Pictures were taken with 10x, 20x, and 40x plan apochromat, and 63x plan apochromat (oil) objectives using a confocal microscope (Carl Zeiss, LSM 510 Meta, Göttingen, Germany) and ZEN software (Carl Zeiss).

Electrophysiological Recordings of iPSC-CMs. Single beating cardiomyocytes were subjected to whole-cell patch-clamp at 36-37°C using an EPC-10 patch-clamp amplifier (HEKA,
Lambrecht, Germany) attached to a RC-26C recording chamber (Warner) and mounted on the stage of an inverted microscope (Nikon, Tokyo, Japan) as previously described\textsuperscript{2}. Data were acquired using PatchMaster software (HEKA, Germany) and digitized at 1.0 kHz. Current-clamp recordings were conducted in Tyrode’s solution.

**Cardiac Magnetic Resonance Imaging (MRI).** For evaluation of myocardial function, mice subjected to MI followed by injection with PBS or iPSC-CMs (n=8 per group) were randomly selected at day 7 and day 35 post-MI. The imaging protocol as well as data analyses were performed as previously described\textsuperscript{3}. Briefly, imaging was performed using a Signa 3.0T Excite HD scanner (GE Healthcare Systems, Milwaukee, Wisconsin) with a Mayo Clinic T/R MRI coil (Mayo Clinic Medical Devices, Rochester, Minnesota). Mice were anesthetized with 1.5% isoflurane with oxygen (1 ml/min) and placed in the prone position for imaging. During the scan, physiologic parameters such as heart rate, respiratory rate, and body temperature were monitored (Small Animal Instruments, Stony Brook, New York). Gradient recalled echo (GRE) and fast spoiled GRE (FSPGR) sequences were used to acquire sequential short-axis slices spaced 1 mm apart from apex to base of the mouse heart. For each sequence, 20 cine frames encompassing 1 cardiac cycle were obtained with the following sequence parameters: TR = 10 ms, TE = 4.6 ms, number of excitations (NEX) = 10, field of view (FOV) = 40 × 49 mm, matrix = 256 × 256, flip angle (FA) = 45°, and slice thickness 1.5 mm. Ejection fraction was calculated using a commercial analysis program (Osirix Version 3.81) that was used to trace the endocardial border of the left ventricle (LV) at end-diastole and end-systole.
Invasive Cardiovascular Hemodynamics. Animals receiving either iPSC-CMs (n=4) or PBS (n=4) following LAD ligation were assayed 5-weeks following surgery. Simultaneous measurements of pressure and volume were obtained using a specialized conductance catheter (Millar Instruments, Houston, Texas) as previously described. Animals were induced and maintained with inhaled isoflurane (1-2%) in 100% oxygen. Mice were intubated and ventilated at a tidal volume of 200 µl at 100 breaths per minute (Harvard Apparatus, Holliston, MA). The internal jugular vein was cannulated with PE-10 tubing and a 10% albumin solution infused at 5 µl/min. The right carotid artery was next cannulated with the pressure-volume catheter which was advanced retrograde across the aortic valve to lie along the long axis of the left ventricle. The temperature of the mice was constantly monitored by a rectal probe and maintained at 37ºC by a self-regulating heating pad (Fine Science Tools, San Francisco, CA). After baseline loops were recorded, occlusion parameters were recorded during and after three 5-second manual occlusions of the inferior vena cava (via transabdominal approach).

Analysis of Cardiac Markers by Flow Cytometry. iPSC-CMs were detached using TrypLE and pelleted at 200 g for 3 min at 4°C. Pellets were washed and cells were fixed and permeabilized using BD Cytofix/Cytoperm and BD Perm/Wash kits (BD Biosciences). Cells were incubated with a primary mouse anti-cardiac Troponin T antibody (Thermo Scientific) for 2 hr at 4°C. Afterwards, cells were washed and incubated with a secondary goat anti-mouse IgG (H+L) phycoerythrin-labeled antibody (R&D Systems) for 30 min at 4°C. Cells were washed and resuspended in PBS containing 0.5 mM EDTA and 5% fetal bovine serum (FBS). Subsequently,
samples were analyzed by FACS. Data were evaluated using FlowJo flow cytometry analysis software.

**RNA Sequencing.** RNA was isolated using miRNeasy kit (Qiagen) according to manufacturer’s instructions followed by DNAse treatment using RNase-free DNase kit (Qiagen). In brief, 100 ng of total RNA was converted to cDNA and amplified using NuGen V2 RNA-seq kit (NuGen, San Carlos, CA). Sonication of cDNA was performed to produce an average fragment size of 280 bps and Illumina sequencing adapters were ligated to 500 ng of cDNA using NEBNext® mRNA Library Prep Reagent Set (New England Biolabs, Ipswich, MA). PCR was performed on the adapter ligated cDNA using the following conditions (denaturation 98°C for 30 seconds, following 12 cycles of denaturation 98°C for 10 seconds, annealing 65°C for 30 seconds, and extension 72°C for 30 seconds, ending with an extension at 72°C for 5 min). Libraries were submitted to the Stanford Stem Cell Institute Genome Center for sequencing using Illumina’s HiSeq2000 platform using paired in reads at an average length of 100 bps (2x100).

**In Vitro Analysis of Paracrine Function.** Supernatants from iPSC-CMs (n=4/group) were collected and centrifuged at 10,000 g for 10 min at 4°C and stored at -80°C. RNA was extracted from 1x10^6 cells (n=4/group) via an RNeasy Mini Kit (Qiagen) and quantitative RT-PCR was carried out as described above. Analysis of secreted material was performed using a Luminex-based platform (Affymetrix) as previously described\(^5\). Briefly, bead-conjugated antibodies in a 96-well plate format were used to capture individual cytokines. Plates were incubated at room temperature for 2 hr and at 4°C for 18 hr, washed and subsequently incubated with a biotin-labeled antibody at room temperature for 2 hr. Following incubation with streptavidin-PE for 40
min, samples were washed and subjected to data acquisition with a Luminex 200 instrument with a lower bound of 100 beads per sample per cytokine and mean fluorescence intensity (MFI) was calculated.

**Single Cell Gene Expression Profiling of Transplanted iPSC-CMs.** Four days following transplantation, explanted hearts injected with iPSC-CMs were harvested from LAD-ligated (n=5) and sham (n=4) animals and subjected to collagenase digestion on a Langendorff apparatus. The resultant cell slurry was FACS sorted and single GFP+ iPSC-CMs were directly sorted into wells containing 10 μl of reaction buffer (CellsDirect kit, Invitrogen). Reverse transcription and human-specific transcript amplification were performed using 1 μl of SuperScript III Reverse Transcriptase / Platinum Taq Mix (Invitrogen) on the thermocycler (ABI Veriti) as follows: 50°C for 15 min, 70°C for 2 min, 94°C for 2 min, 94°C for 15 sec, 60°C for 30 sec, 68°C for 45 sec for 18 cycles, and 68°C for 7 min. We then used a microfluidic platform to conduct single cell qRT-PCR in nanoliter reaction volumes, thereby enabling high throughput processing and enhanced detection sensitivity as previously described. The amplified cDNA was then loaded into Biomark 48.48 Dynamic Array chips using the Nanoflex IFC controller (Fluidigm). Threshold cycle (C_T), which indicates the fractional cycle number at which the amount of amplified target gene reaches a fixed threshold, was extracted for each well using the Biomark Real-Time PCR Analysis software and relative quantification was calculated by the comparative C_T method that was described previously. The data were analyzed using the equation 2^{-ΔΔC_T}, where ΔΔC_T = [C_T of target gene - C_T of housekeeping gene] treated group – [C_T of target gene - C_T of housekeeping gene] untreated control group. For the treated samples, evaluation of 2^{-ΔΔC_T} indicates the fold-change in gene expression, normalized to a housekeeping gene
(GAPDH), relative to the untreated control. All reactions were performed in duplicates or triplicates.

**Quantitative Real-Time RT-PCR.** Gene expression was quantified by real-time PCR. Total RNA was extracted using the RNeasy plus mini kit (Qiagen) from fibroblasts, undifferentiated iPSCs, and differentiated iPSC-CMs at different time points. RNA was reverse transcribed into cDNA with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative polymerase chain reaction was performed in triplicates using TaqMan Assays and Gene Expression Master Mix according to manufacturer’s instructions on a StepONEplus real-time polymerase chain reaction machine (Applied Biosystems). Raw data were analyzed with the StepONE Software v2.2. Cycle threshold (CT) was calculated during the exponential phase at identical threshold values for all runs. Results are shown as $C_T$ and analyzed using an equation that was described in single cell gene expression profiling.

**Laser Capture Microdissection (LCM) of Host Myocardium Surrounding Transplanted Cells.** Murine hearts were removed after perfusion with 20 ml PBS, embedded in optimal cutting temperature, and immediately frozen in liquid nitrogen. Seven thick tissue sections of left ventricle were prepared on polyethylene naphthalate membrane-coated slides (MicroDissect GmbH). For LCM, slides were thawed briefly and air dried 5 min before dissection. Green fluorescence observed under laser microscopy was used as a landmark for microdissection. Specific green fluorescent tissues, as well as normal cardiac tissues without green fluorescence, were independently dissected out by applying Leica LCM Systems (MicroDissect GmbH). The dissected tissues were placed on the caps of microcentrifuge tubes with 5 ul lysis enhanced
buffer. After dissection, tissues were collected by centrifugation at 8,000 g for 5 min. Total RNA extraction and reverse transcription of these samples were performed using a commercial 1-step kit (Invitrogen) (n=4-6 mice per group).

**In Vivo Experiments with Conditioned Medium of iPSC-CMs.** Conditioned medium (CM) was generated as follows: 2x10^6 iPSC-CMs were cultured in routine maintenance medium and incubated in either normoxia or hypoxia for 12 hr. The medium was then collected, filtered through a 0.22 µM filter to clear cell debris, followed by a 50-fold concentration using a Ultracel-100K filter (Amicon, Millipore, USA) according to the manufacturer’s protocol. Control CM was generated in the same way except there were no cells in the plate.

A total volume of 80 µl of CM (control CM, normoxic CM, or hypoxic CM; n=6/group) was injected in three different sites at the infarct border zone 15 min after ligation. The mice in each group (n=6/group) were also given an additional intraperitoneal injection of 80 µl of CM on days 2 and 4 after infarction. Infarct size was measured as described above on day 28 post-LAD ligation.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure I. Schematic description of surgical models and treatment groups. Adult female NOD/SCID mice were subjected to either sham surgery or LAD-induced myocardial infarction followed by injection with PBS (control) or 2x10^6 iPSC-CMs stably expressing firefly luciferase and GFP respectively. Cell fate and cardiac function were assessed by bioluminescence imaging (BLI), magnetic resonance imaging (MRI), and pressure-volume (PV) loops. Harvested tissues were analyzed *ex vivo* by FACS, RT-PCR, Luminex cytokine profiling and laser capture microdissection (LCM).

Supplemental Figure II. Pluripotency characterization of undifferentiated iPSCs. (A) Teratoma formation was confirmed by identification of cell types of endodermal, mesodermal, and ectodermal origin on histological samples obtained at 8 weeks post-transplant. (B) *In vivo* proliferation of human undifferentiated iPSCs following hindlimb transplantation in immunodeficient mice. (C) BLI signal quantification of panel A.

Supplemental Figure III. Transduction of undifferentiated iPSCs. Undifferentiated iPSCs generated from human fibroblasts and grown on Matrigel were transduced with a lentiviral construct with a constitutive human MSCV promoter driving expression of Fluc and a constitutive human EF1 promoter driving eGFP, with a Puromycin cassette. (A) Representative brightfield image of an iPSC colony (passage 20) with 10x magnification (upper row) and 40x magnification (bottom). (B) Corresponding green fluorescence image demonstrating strong GFP+
expression of human iPSCs after transduction; 10x magnification (upper row) and 40x magnification (bottom).

**Supplemental Figure IV.** Reduced apoptotic cell death in the peri-infarct area after iPSC-CM transplantation. **(A)** Representative TUNEL-staining for cell apoptosis (green) and DAPI (blue) for nuclear staining in transverse sections of animals treated with either PBS or iPSC-CMs at day 4 after MI. **(B)** Quantification of panel A (N=5, *p<0.05 by Mann-Whitney’s rank sum test).

**Supplemental Figure V.** Reduced infarct size in mice subjected to MI supplemented with conditioned medium from iPSC-CMs (N=6, *p<0.05 by one way ANOVA followed by Tukey’s post hoc test). There was no discernible difference between conditioned medium harvested from iPSC-CMs grown in normoxic and hypoxic conditions.

**Supplemental Table I.** A summary of key cardiac AP parameters for iPSC-CMs. All iPSC-CMs were analyzed around day 30-40 of cardiac differentiation. Note that only 1 nodal-like iPSC-CM was detected in this study. Data were presented as average ± SEM.
REFERENCES


Supplemental Figure I

Groups:
1. Sham with no occlusion
2. MI with PBS
3. MI with 2x10^6 iPSC-CM injection
A

Muscle (mesoderm)  Glandular structures (endoderm)  Neural epithelium (ectoderm)

B

C

Supplemental Figure II
Supplemental Figure IV
Supplemental Figure V

Infarct Size (%)

Control             Norm                Hyp

CM                   CM

*
<table>
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<tr>
<th>Cell Type</th>
<th>Cells (%)</th>
<th>BPM (beat/min)</th>
<th>Vm (mV)</th>
<th>Overshoot (mV)</th>
<th>APA (mV)</th>
<th>APD50 (ms)</th>
<th>APD70 (ms)</th>
<th>APD90 (ms)</th>
<th>APD90/APD50</th>
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<td>Nodal-like</td>
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<td>Atrial-like</td>
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<td>411.3±28.2</td>
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<td>13.5±1.6</td>
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- BPM: Beating per minute
- Vm: Membrane resting potential
- APA: Action potential amplitude
- APD50, APD70, APD90: Action potential durations at 50%, 70%, and 90% repolarization
- Vmax: Upstroke velocity