Stem Cell Reports, Volume *6*

Supplemental Information

Isolation and Mechanical Measurements of Myofibrils from Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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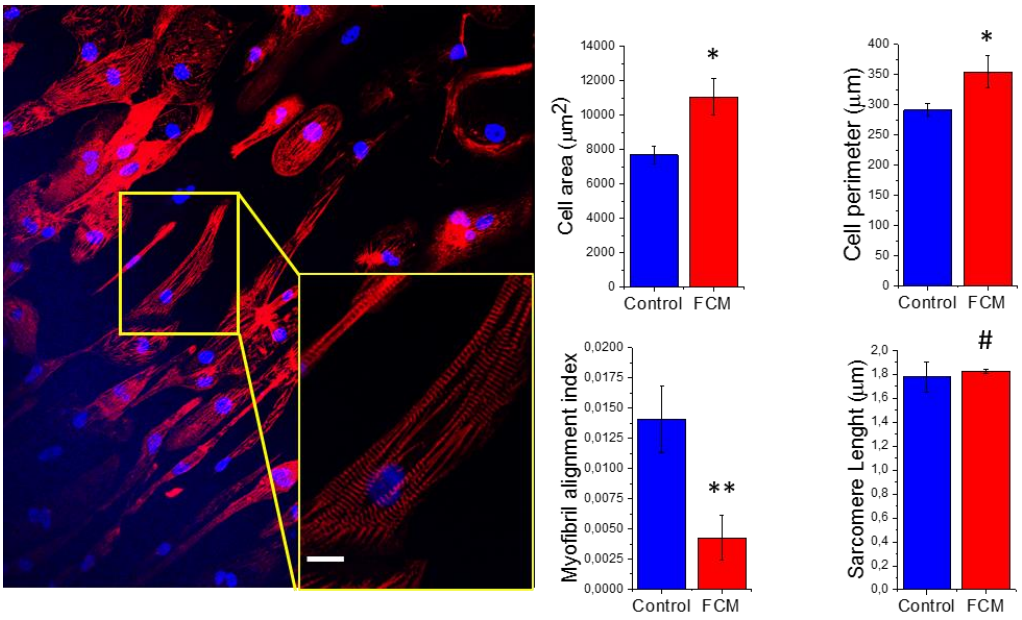
SUPPLEMENT

Supplemental figure legends

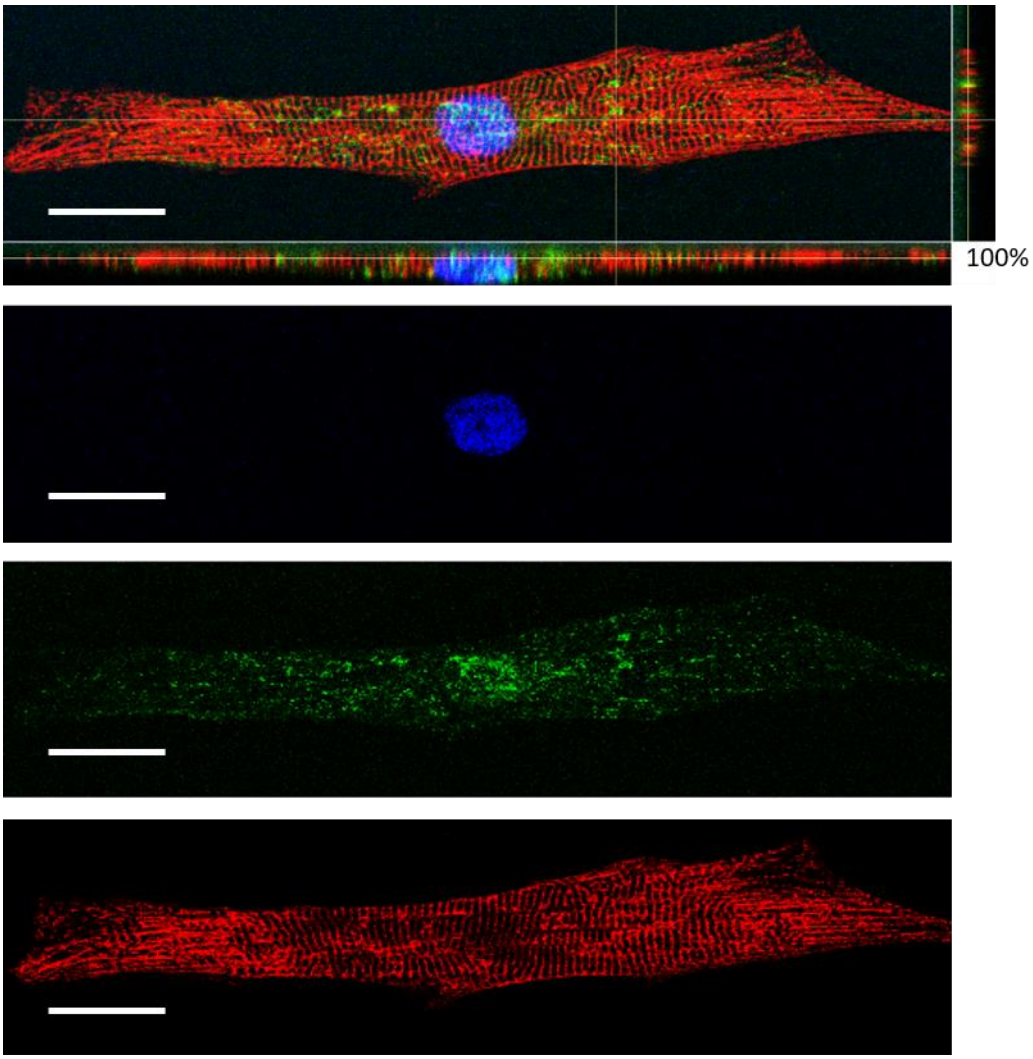
Supplemental Figure 1. Later-stage hiPSC-CMs grown on nanopatterned surfaces showed myofibril disarray, cellular hypertrophy but same resting sarcomere lenght. Confocal images of late-stage FCM-hiPSC-CMs grown nanopatterned surfaces. Cells were stained for Z-bands (α-actinin, red) and for the nuclei (DAPI, blue). In FCM-hiPSC-CMs, Z-bands alignment signs pronounced myofibrillar disarray ($n_{Control}$ = 16; n_{FCM} =5). Cell area, based on myofibril composition, exhibited cellular hypertrophy ($n_{Control}$ = 100; n_{FCM} =34). Resting sarcomere length was 1.8 µm as for Controls, p=0.714 ($n_{Control}$ = 10; n_{FCM} =7). N>3 for each cell line. *p<0.05; **p<0.01; # not significant. Scale bars equal to 20 µm**.**

Supplemental Figure 2. Later-stage hiPSC-CMs grown on nanopatterned surfaces showed myofibril disarray, cellular hypertrophy but still have T-tubule formation. Confocal images of FCM-hiPSC-CMs stained with antibody anti-CAV3 (green), anti-α-actinin (red) and nuclei (DAPI, blue). Side view (100% of magnification) of merged image of representative of FCM-hiPSC-CMs displayed axial tubular elements (green), running perpendicular to Z-bands (red), similar to Control-hiPSC-CMs (Fig.2). Transverse tubules (green) covered the entire cell surface and appear parallel to the Z-lines. Scale bars equal to 20 µm.

Supplemental Table 1. Summary of mechanical and kinetic properties of myofibrils from Control-hiPSC-CM line following maximal and submaximal Ca^{2+} activation. Data from each group of Controls was compared with ANOVA and a Tukey post-hoc test with statistical significance set at p<0.05. No significant differences were found between Controls. For comparisons, $Control_1$ and $Control_2$ data were merged in Table 1. (n) equal to number of myofibrils. N=3 for each condition.



Supplemental Figure 1



Supplemental Figure 2

Supplemental Table 1

Myofibril type	Tension	k_{ACT}	k_{REL}, slow	t_{REL}, slow	k_{REL}, fast
pCa 4.0	**mN/mm^2**	**s^{-1}**	**s^{-1}**	**ms**	**s^{-1}**
Control$_1$	19.4±3.4 (14)	0.65±0.07 (12)	0.28±0.04 (13)	202±10 (15)	2.36±0.3 (14)
Control$_2$	15.2±3.3 (12)	0.47±0.05 (11)	0.31±0.06 (12)	181±38 (14)	2.15±0.1 (11)
p value	0.68	0.07	0.71	0.16	0.41
pCa 5.8					
Control$_1$	10.1±2 (13)	0.40±0.06 (13)	0.28±0.03 (9)	154±14 (11)	1.98±0.4 (13)
Control$_2$	6.2±1.8 (9)	0.37±0.11 (9)	0.44±0.07 (8)	154±6 (10)	1.87±0.2 (9)
p value	0.19	0.84	0.06	0.99	0.15

Supplemental experimental procedures

Human pluripotent stem cell culture and cardiac differentiation

For cardiac differentiation we applied an established monolayer directed differentiation protocol(Palpant et al., 2015). In brief, hiPSC are grown in serum-free conditions until a confluent monolayer of cells seeded onto Matrigel-coated (Fisher) wells appears as compact at 37°C, 5% CO_2. One day before cardiac differentiation induction, the Wnt signaling pathway agonist Chiron 99021 (1µM) was applied. On day 0, the directed differentiation is started by adding Activin A (100 ng/ml, R&D) for 18 hours. On day 1, medium was supplemented with bone morphogenetic protein-4 (BMP4, 5 ng/ml, R&D) and Chiron 99021 until day 3 when replaced with Wnt antagonist Xav 939 (1µM). From day 0 to day 7 the base media is composed of RPMI supplemented with B27 minus insulin (Invitrogen). From day 7 onwards, cultures were fed every other day with RPMI medium with B27 supplement (Invitrogen). Spontaneous beating monolayers are generally noticeable from day 8 from Activin A induction onwards. On day 20 post differentiation, single cells are obtained from beating monolayers for further cardiac maturation. Monolayers more than 60% positive for cardiac troponin T by flow cytometry were used for subsequent experiments.

Fabrication and cell maturation onto a nanopatterned substratum

A nanopatterned silicon master was used as template for a polyurethane acrylate (PUA) mold used to pattern a polyurethane (PU)-based polymer via UV-assisted CFL. The PUA master mold used for our purpose was designed with parallel grooves and ridges of 800 nm wide and 500nm deep. PU pre-polymer is drop dispensed onto a glass coverslip (18mm diameter, Fisher) and the PUA mold is placed on top. For photopolymerization, the mold was cured via UV radiation (wavelength of 250-400nm) and then peeled off leaving behind an ANFS for cell culture. For the maturation process, single cells were replated at low density (10,000 cells/cm^2) onto fibronectin coated nanopatterned surfaces (5µg/cm^2) and long-term cultured (80 days for myofibril isolation, 80-100 for structural analysis, average 88.1±2.9 days). For mechanical studies and structural analysis, only FCM-CMs were frozen beforehand at day 50 onto nanopatterned surfaces and then matured for other 30 days with the same procedure after thawing. For proteomic analysis, single Control- and FCM-CMs were matured onto flat surfaces and frozen at day 50. After thawing, cells were matured onto nanopatterned surfaces for additional 30 days. Cultures were monitored daily for cell spreading and physiological hypertrophy.

Immunocytochemistry

Long-term cultured cells onto nanopatterned surfaces were washed with PBS and in succession fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature. For Z-bands staining, cells were blocked in 1.5% normal goat serum with Triton X-100 0.1% for 30 minutes and then incubated with anti-α-actinin primary antibody 1:800 (A7811, Sigma) at 4°C overnight. After PBS rinses, samples were stained with Alexa Fluor 594 goat anti-mouse secondary antibody 1:100 (A-11005, Life Technologies) for 1 hour at room temperature. For caveolin-3 (CAV3), cells were incubated with 1.5% normal goat serum before labeling with anti-CAV3 primary antibody 1:500 (AV09021, Sigma) and the day after with Alexa Fluor 488 goat anti-rabbit 1:200 (A-11008, Life Technologies). Each staining with secondary antibody was incubated for 1 hour and later PBS washed prior to nuclei staining with DAPI (Vectashield). Nanopatterned coverslides were then flipped over a normal miscroscope slide for confocal images (Nikon A1R confocal microscope).

Imaging and morphological analysis

Images captured by confocal microscope were analyzed for average cell area, cell perimeter and sarcomere length (SL) in Fijii (ImageJ) Software using standard analysis plugin. Myofibril alignment resulted from Z-bands density and bidimensional organization was analyzed using an algorithm based on the Fast Fourier Transform (FFT) similarly to previously described method(Ferrantini et al., 2014). Single cells were discernible from each other for the quantification of myofibril alignment. The periodicity of Z-bands along the entire cell surface were reflected in the peak components of the power spectrum profile. The area of the first order of spatial frequency components centered at 0.55±0.05 corresponding to the measured resting SL was normalized for the entire area under the power spectrum curve. The resulted value is as much greater as the myofibril alignment index of each single cell.

Transmission electron microscopy

Single hiPSC-CMs onto nanopatterned surfaces were fixed in 4% glutaraldehyde in sodium cacodylate buffer overnight at 4°C, washed and then post fixed in 2% Osmium Tetroxide for 1 hour on ice. Following Osmium, samples were washed in water and block stained in 2% Uranyl Acetate, washed again and dehydrated through a series of ethanol. Finally, they were infiltrated and embedded in Epon Araldite. Ultrathin (70-80 nm) axial sections of single cells were contrasted with lead citrate prior to imaging with a JEOL JEM 1200EXII at 80 kV.

Myofibril protein analysis

Sarcomeric protein composition was studied using frozen remains of myofibril preparations. Myofibril sediments were centrifuged at 10,000 RPM and supernatant taken off the top. Protein suspension was then solubilized in SDS-loading buffer, sonicated 3*30seconds on ice and then boiled for 4 min. Samples were separated on 10% or 12% SDS-PAGE gels using a Mini-PROTEAN® Tetra Cell (Bio-Rad Laboratories). The SDS-PAGE gel was transferred onto a PVDF membrane using the Mini-PROTEAN® Tetra Cell wet transfer system (Bio-Rad Laboratories). The primary antibodies used were as follows; TNI 1:2500 (sc-15368, Santa Cruz), CTNT 1:2500 (C-19, sc8121, Santa Cruz), α-ACTIN 1:5000 (A1203, Santa Cruz) and β-MHC (1:5000), Secondary antibody 1:10,000. β-MHC expression was tested to provide evidence that the presence of slow β-MHC isoform may be responsible for similarities in kinetics of myofibrils from hiPSC-CMs, fetal human and adult human cells (Fig. 3D), while expression of the embryonic SSTNI or cTNI isoforms can be used as markers for maturation.

Isolation of myofibrils

A cell length of >50 µm in most of the culture was used as parameter for evaluate myofibril elongation state. Myofibrils were isolated using two different approaches. In the *skinning in suspension* approach, cells were fed including Rho Kinase (ROCK) inhibitor (1:1000) and incubated for 1 hour. After PBS rinse, cells were incubated again in Versene for 1 hour. Detached cells were gently resuspended into rigor solution (KCl 100 mM, $MgCl_2$ 2 mM, EGTA 1 mM, Tris-HCl 50 mM, pH 7.0) supplemented with protease inhibitor cocktail 5 µl/ml and DTT 2 µl/ml (Sigma) and 1% Triton X for 5 minutes on ice after rapid vortexing for the skinning process. Samples were spun at 3000 rpm for other 5 minutes and the pellet was resuspended into fresh supplemented rigor solution without Triton X. Two consecutive rinses were performed as described in the previous step. In the *skinning on plate* approach, cells were PBS washed before direct treatment with skinning solution for 5 minutes on ice. Supernatant containing cells under skinning was collected and processed as described

for the first approach. Myofibril suspensions were stored at 4°C for mechanical experiments and used for 1-2 days.

Solutions for mechanical measurements

Experimental solutions for myofibril mechanics were calculated as previously described(Brandt et al., 1998). Experimental solutions were composed as follows (in mM): phosphocreatine 10, EGTA 10 (ratio of CaEGTA/EGTA set to obtain Ca^{2+} values between 10^{-9} and 10^{-4} M), MOPS 10, free Mg^{2+} 1, (Na^{+} + K^{+}) 155, MgATP 5, and propionate and sulphate to adjust the final solution ionic strength to 0.2 M. Both solutions were adjusted to pH 7.0 with NaOH.

Myofibril mechanics

Myofibril were mounted in a custom built apparatus and measured in the mechanical properties as previously described(Colomo et al., 1998; Moussavi-Harami et al., 2015; Racca et al., 2015; Tesi et al., 1999). Single or bundles of few myofibrils were mounted between the tip of two needles micro-forged from borosiliacate glass capillary tubes (OD 1.0 mm, ID 0.5 mm, Sutter Instruments, Novato, CA, USA) (Fig. 3A right). One of the two glass microneedles that held the myofibrils was relatively stiff and was mounted on the lever arm of a piezo-controller motor (PZT Servo controller, LVPZT amplifier; Physik Instrumente, Irvine, CA, USA). In these experimental conditions, the compliance of the force transducer microneedle was 44.15±8.5 nN μm^{-1}. The flexibility of the arm of the needle was calibrated with a given force by increasing the frequency of a galvanometer and measuring its deflection under 40x lens as previously described(Colomo et al., 1998). The tip of the force transducer was blackened with ink in order to increase the sensitivity of the photosensor. During the experiment, the force transducer was positioned over a photodiode system which detect the displacement of the needle that correlates to the force development of the myofibril(Cecchi et al., 1993). The flow of relaxing and the activating solution streamed by gravity from a double-barrelled borosilicate theta glass pipette (Capillary glass tubing OD 2.0 mm, ID 1.4 mm, SEP 0.2 mm, modified in house to OD of 0.55 mm, Warner Instruments, Hamden, CT, USA) and was controlled by a computerized motor for switching the solution over the preparation in about 10 ms (SF-77B Perfusion Fast step, Warner Instruments Corporation, Hamden, CT, USA). The initial SL of the attached myofibril was set at 2.34 µm that corresponded about 15% above the slack length(Tesi et al., 1999). The time course of activation and relaxation kinetics were collected constantly at 15°C(Kreutziger et al., 2011)(Fig. 4A). Maximal (pCa 4.0) and submaximal (pCa 5.8) isometric tension generation was calculated from the force amplitude and normalized for the cross sectional area of the myofibril. The rate of tension generation (k_{ACT}) follows Ca^{2+} application and was estimated from monoexponential fits. The rate of the slow phase of relaxation (slow k_{REL}) is calculated from the slope of a regression line fitted to the tension trace and normalized to the entire amplitude of the tension relaxation trace. The duration of slow phase (slow t_{REL}) was estimated from the onset of the linear slope to the beginning of the shoulder leading to the fast phase of relaxation. The relaxation rate for the fast phase (fast k_{REL}) was measured from the monoexponential decay fitted to the data. The time to generate or reduce half-maximal force ($t_{1/2}$) was used for calculate k_{ACT} and fast k_{REL} and converted to a rate $\tau = \ln(2)/t_{1/2}$.

Supplemental references

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