Functional abnormalities in induced Pluripotent Stem Cell-derived cardiomyocytes generated from titin-mutated patients with dilated cardiomyopathy

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Working

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ABSTRACT

Aims: Dilated cardiomyopathy (DCM), a myocardial disorder that can result in progressive heart failure and arrhythmias, is defined by ventricular chamber enlargement and dilatation, and systolic dysfunction. Despite extensive research, the pathological mechanisms of DCM are unclear mainly due to numerous mutations in different gene families resulting in the same outcome - decreased ventricular function. Titin (TTN) - a giant protein, expressed in cardiac and skeletal muscles, is an important part a fundamental part of the sarcomere, and thus TTN mutations are the most common cause of adult DCM. To decipher the basis for the cardiac pathology in titin-mutated patients, we investigated the hypothesis that induced Pluripotent Stem Cell (iPSC)-derived cardiomyocytes (iPSC-CM) generated from patients, recapitulate the disease phenotype. The hypothesis was tested by 3 Aims: (1) Investigate key features of the excitation-contraction-coupling machinery; (2) Investigate the responsiveness to positive inotropic interventions; (3) Investigate the proteome profile of the AuP cardiomyocytes using mass-spectrometry (MS). Methods and Results: iPSC were generated from the patients' skin fibroblasts. The major findings were: (1) Sarcomeric organization analysis in mutated iPSC-CM showed defects in assembly and maintenance of sarcomeric structure. (2) Mutated iPSC-CM exhibited diminished inotropic and lusitropic responses to β-adrenergic stimulation with isoproterenol, increased [Ca2+]out and angiotensin-II. Additionally, mutated iPSC-CM displayed prolonged recovery in response to caffeine. These findings may result from defective or lack of interactions of the sarcomeric components with titin through its kinase domain which is absent in the mutated cells. Conclusions: These findings show that the mutated cardiomyocytes from DCM patients recapitulate abnormalities of the inherited cardiomyopathies, expressed as blunted inotropic response.

EXTERNAL LINK

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PROTOCOL STATUS

Working

Generation of patient-specific induced pluripotent stem cells

1 Skin biopsies were acquired according to approval #3611 issued by the Helsinki Committee for experiments on human subjects at the Rambam Health Care Campus, Haifa, Israel. The biopsy [human dermal fibroblasts (HDF)] was obtained from a 28-year-old DCM patient (denoted IsP) from an Israeli family carrying an adenine insertion mutation, causing a frame shift and resulting in a stop codon and protein truncation after 19,628 amino acids [10]. Additional skin (HDF24) and hair (KTN3 and KTI [13, 14]) biopsies were obtained from healthy subjects as control. The dermal fibroblasts generated from the skin biopsies were reprogrammed as previously described [12] using the STEMCCA Cassette (a single lentiviral vector containing the four factors: Oct4, Sox2, Klf4 and c-Myc) and induced Pluripotent Stem Cells (iPSC) were generated (IsP DCM clones 23.2 and 23.10). These iPSC clones were spontaneously differentiated into functional cardiomyocytes as previously described [12, 14, 15].

Karyotype analysis

2 Karyotype analysis was performed using standard G-banding chromosome analysis by the cytogenetic laboratory according to standard procedures.

Teratoma formation

3 To evaluate the iPSC differentiation capacity in vivo, 5-day-old iPSC colonies from one 6-well plate were detached using 1 mg/ml type IV collagenase, washed 3 times in PBS and then injected into thigh muscle of severe combined immunodeficient (SCID) mice. Teratomas were observed 8-12 weeks after injection, and images were obtained from formalin-fixed (4%) and paraffin-embedded teratoma sections stained with hematoxylin and eosin (H&E) [16–18].

Genotyping

4 GenotypingTo confirm that the mutation is preserved in the iPSC clones, sequence analysis was performed on the titin gene both in the patients-derived iPSC and fibroblasts by performing PCR using primers which delimit the mutation area in the mutated gene. The PCR was performed on genomic DNA produced from the patients' fibroblasts and iPSC using Promega DNA purification kit, with the primers: F-5'-TATTGCCTGGGTTAAGCCGC-3' and R-5'-AGCTCCTGTTGTTAGTCCGC-3'.

Immunofluorescence staining

5 Immunofluorescence staining was performed according to standard protocols using the following antibodies: Alexa Fluor 555 donkey anti-rabbit (1:100; Life Technologies, Eugene, OR, USA), Alexa Fluor 488 donkey anti mouse (1:100; Life Technologies, Eugene, OR, USA), cy5 donkey anti goat (1:100; Invitrogene, Eugene, Oregon, USA), OCT3/4 (1:100; Millipore, Santa Cruz, CA, USA), SSEA4 (1:100; Millipore, Temecula, CA, USA), TRA1-60 (1:100; Millipore, Temecula, CA, California), Nanog (1:50; R&D, Minneapolis, MN, USA), DAPI (1:500; Sigma Aldrich, St. Louis, MO, USA).

Action potential recordings

6 For action potentials (AP) recordings, spontaneously contracting areas of EBs (control data was obtained from experiments on KTI and KTN3 clones used in Ben-Ari et al [19]) were mechanically and/or enzymatically dispersed (collagenase II 1 mg/ml; Worthington, Lakewood, New Jersey, USA, http://www.worthington-biochem.com). Small clusters were then plated on gelatin-coated glass coverslips (13 mm diameter) in 24-well plates.The coverslips were incubated at 37°C, and a recovery period of at least two days was allowed before the electrophysiological experiment was performed [13]. In all experiments, the coverslips were perfused at 37°C with an external solution containing (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl, 1 MgCl, 10 glucose and 10 HEPESTitrated to pH 7.4 with NaOH. The patch pipette solution contained (mM): 120 KCl, 1 MgCl, 3 Mg-ATP, 10 HEPES, and 10 EGTA titrated to pH 7.2 with KOH and adjusted at 290 mOsm with saccharose (all materials were purchased from Sigma-Aldrich). Axopatch 200B, Digidata 1322 and pClamp10 (Molecular Devices, Sunnyvale, CA) were used for data amplification, acquisition and analysis. Signals were digitized at 10 kHz and filtered at 2 kHz. Microelectrodes with resistances of 4-7 MΩ were pulled from borosilicate glass capillaries (Harvard Apparatus, Holliston, USA). Analysis was preformed using MATLAB software (MathWorks, Natick, MA, USA). Corrected AP duration (APD) was calculated by the Bazett's correction

Extracellular electrograms recorded from spontaneously contracting EBs and analysis of Beat Rate Variability (BRV)

7 Extracellular electrograms were recorded from spontaneously contracting 30-60 day-old EBs using the Micro-Electrode-Array (MEA) apparatus (Multi Channels Systems, Reutlingen, Germany) routinely used in our lab [14, 21]. The MEA set-up consists of a 50×50-mm glass substrate, in the center of which is embedded a 1.4×1.4 mm matrix of 60 titanium-nitride electrodes. The electrode diameter is 30 µm and inter-electrode distance is 200 µm. Spontaneously contracting EBs were mechanically dissected and adhered onto the MEA dish and their electrical activity was recorded by the MEA data acquisition software at sampling rate of 1000 Hz which was down-sampled to 200 Hz. During the recording, the cultures were kept in a bath-like conformation of a glass cylinder (glued to the center of the MEA plane) filled with 500 µL of culture medium saturated with a gas mixture consisting of 5% CO2+ 95% air. The temperature was kept at 37°C using a heating element and a temperature controller. For BRV analysis, all recordings were analyzed to detect peaks of the activation spikes, from which inter-beat intervals (denoted 'IBI') were calculated using MATLAB software (MathWorks, Natick, MA, USA). To generate Poincaré plots, each R-R interval (IBI n+1) is plotted against its predecessor (IBI n), creating a scattered mass of points in a two-dimensional array. Quantitative analysis of the plot is performed by fitting an ellipse to the group of points, with its center coinciding with the centroid of the ellipse (the point of the average IBI), and adjusting two perpendicular lines traversing the centroid. The longitudinal line designated SD2, represents long-term variability of the data (reflecting the standard deviation of the IBIs. The perpendicular line designated SD1 represents short-term beat-to-beat variability [21].

Measurements of intracellular Ca2+ transients and contractions

8 Intracellular Ca2+([Ca2+]i) transients and contractions were recorded from small contracting 40-70-day old embryoid bodies (EBs) by

means of fura-2 fluorescence and video edge detector, respectively, using the IonOptix Calcium and Contractility system (Westwood, MA, USA) as previously described [15,22,23]. In brief,spontaneously contracting EBs were mechanically dissected and adhered onto 18 mm diameter gelatin-coated glass slides. Subsequently, fura-2-stained (2.5 µM) contracting areas were transferred to a chamber mounted on the stage of an inverted microscope and perfused at a rate of 1–1.5 ml/min Tyrode's solution at 370C. The Tyrode's solution contains (mmol/l): 140 NaCl, 5.4 KCl, 1 MgCl, 2 sodium pyrovate, 1 CaCl, 10 HEPES, 10 glucose (pH 7.4 adjusted with NaOH). The EBs were paced at 0.5-2.5 Hz which corresponded to a frequency 20-50% higher than the spontaneous beating rate. The acquisition rate of both the [Ca2+]i transients and contractions was 100 points/sec. Analysis was performed using the IonOptix designated system. To characterize the [Ca2+]i transients amplitude, the differences between maximal (systolic) and minimal (diastolic) ratio were calculated in 20 successive transients and averaged (Ramp). Similarly, the contraction amplitude (Lamp) was calculated from the average differences between minimal and maximal video cursor positions of 20 successive contractions. In addition, the maximal rates of [Ca2+]i rise (+d[Ca2+]i/dt) and decay (-d[Ca2+]i/dt Relax), and maximal rates of contraction (dL/dt Contraction)) and relaxation (dL/dt Relaxation)) were calculated and averaged over 20 [Ca2+]i transients and contractions, respectively. The data from the Israeli iPSC-derived cardiomyocytes (iPSC-CM) are pulled from two clones.

Immunocytological analysis in iPSC-CM

9 iPSC-CM were fixed in 3.7% (vol/vol) formaldehyde and subjected to immunostaining as previously reported [11]. Sarcomere structure was visualized using primary antibodies against cardiac troponin T (mouse monoclonal clone 13-11, Lab Vision, 1:500) and α-actinin (mouse monoclonal clone EA-53, Sigma-Aldrich, 1:300). Alexa-Fluor-488, and -594 conjugated secondary antibodies specific to the appropriate species were used (Life Technologies, 1:500). Nuclei were detected with 1 µg/ml Hoechst 33528. Microscopy was performed using imaging systems (DMI6000-AF6000), filter cubes and software from Leica microsystems. Images were assigned with pseudo-colors. Morphological analyses were performed by investigators blinded to the genotype of the cells.

Mass Spectrometry sample preparation and data processing

10 iPSC-CM were resuspended in a lysis buffer containing 6M guanidinium chloride (GCl), boiled for 5 minutes and subsequently reduced with 10 mM tris (2-carboxyethyl) phosphine (TCEP) and alkylated with 40 mM 2-chloro-N,N-diethylacetamide. Samples were diluted (10% acetonitrile, 25 mM Tris pH 8.5), first 1:3 Lysate: Buffer for LysC digestion (25°C 3 hours), then 1:10 for Trypsin digestion. Samples were incubated at 37°C overnight under continuous shaking. The digestion was blocked by acidifying the sample with TFA (1% total). Peptides were de-salted using SDB-RPS StageTips as described [24]. Mass-spectrometry (MS) analysis was performed in triplicates using a nanoflow uHPLC system (Easy1000 nLC,) coupled via a nanoelectrospray ion source to a Q Exactive mass spectrometer (all from Thermo Fisher Scientific). Peptides were separated on a 50 cm long column with 75 µm inner diameter, packed in-house with ReproSil-Pur C18-AQ 1.9 µm resin (Dr. Maisch GmbH). Column temperature was kept at 50 °C. Peptide separation was carried out by loading the peptides in buffer A (0.1% (v/v) formic acid) and eluting them in 120 or 240 minutes with a nonlinear gradient of 5-60% buffer B (0.1% (v/v) formic acid, 80% (v/v) acetonitrile) at a flow rate of 250 nl/min. MS analysis of peptides was performed in a data-dependent acquisition mode, with survey scans (300-1700 m/z, maximum ion injection times 60 ms) acquired at a resolution of 60,000 followed by higher-energy collisional dissociation (HCD) based fragmentation of up to 15 most abundant precursor ions. The MS/MS scans were acquired at a resolution of 15,000 (maximum ion injection times 60 ms). Repeated sequencing of peptides was minimized by setting a dynamic exclusion of 20 s.
Raw MS files were processed with MaxQuant (version. 1.6.1.3) [25]. The false discovery rate (FDR) cut-off was set to 1% for protein and peptide spectrum matches. Peptides were required to have a minimum length of 7 amino acids and a maximum mass of 4600 Da. Peak list files were searched against the UniprotKB Homo sapiens database, based on the 2018_02 release, combined with 245 common contaminants by the integrated Andromeda search engine [26]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the partner repository with the dataset identifier PXD010513. Data analysis, statistics and annotation enrichment analysis was performed with the Perseus software package, version 1.5.4.2 [27]. Protein Groups were filtered for at least two valid values in at least one group of triplicates. Differentially expressed proteins were identified by t-test at a permutation-based FDR cut-off of 0.05, 250 randomizations and S0=0.5, which was used to determine the curves of the volcano plot. Pathway enrichment analysis was performed using Fisher exact test with a Benjamini-Hochberg FDR cutoff of 0.02. GOCC, GOBP, GOMF, CORUM, Uniprot Keywords and KEGG pathway annotations were used for the analysis. Bar plots were generated of significant protein of interest was performed by differential enrichment analysis of proteomic data, DEP [28].

Transmission electron microscopy

11 Transmission electron microscopy (TEM) was performed on 31- and 56-day-old (post-plating) iPSC-CM from IsP and AuP DCM patients (n=2 each for two clones) and from healthy controls (n=2). The samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, post fixed in potassium ferrocyanide reduced osmium, and further processed for epoxy resin (Agar100) embedded as previously reported [29]. The ultra-thin sections were cut with a diamond knife at 60 nm thicknesses using an EM UC7 Leica ultramicrotome (Leica Microsystems, Wetzlar, Germany) and double stained with 1% uranyl acetate and Reynolds lead citrate. TEM was performed using a Morgagni 286 transmission electron microscope (FEI Company, Eindhoven, The Netherlands) at 80 kV. Digital electron micrographs were recorded with a MegaView III CD and iTEM-SIS software (Olympus, Soft Imaging System GmbH, Münster, Germany). Digital electron micrographs were recorded with a MegaView III CD. From each sample, two separate EBs from each batch were examined under electron microscope. Ten CMs of each EBs were photographed. The measurements were performed to assess the length of sarcomeres and the size of the Z-bands (sarcomeres width). The measurements were done using iTEM-SIS software (Fig. 4-5, on-line Supplement) (Olympus, Soft Imaging System GmbH, Münster, Germany) and exported as Excel

documents.

Statistical analysis

12 Results are expressed as Mean±SEM and represent mean percentage of change (unless indicated otherwise). Data were analyzed with Sigmastat (Systat Software Inc., Chicago, Illinois) and Prism 5.0 (GraphPad Software, San Diego, California). P<0.05 was considered significant.