

# Long-Term Culture of Patient-Derived Cardiac Organoids Recapitulated Duchenne Muscular Dystrophy Cardiomyopathy and Disease Progression

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2 **Vittoria Marini<sup>1†</sup>, Fabiola Marino<sup>1,2†</sup>, Flaminia Aliberti<sup>1,3</sup>, Nefele Giarratana<sup>1</sup>, Enrico Pozzo<sup>1</sup>,**  
3 **Robin Duelen<sup>1</sup>, Álvaro Cortés Calabuig<sup>4</sup>, Rita Larovere<sup>5</sup>, Tim Vervliet<sup>5</sup>, Daniele Torella<sup>2</sup>, Geert**  
4 **Bultynck<sup>5</sup>, Maurilio Sampaolesi<sup>1,6\*</sup>, Yoke Chin Chai<sup>1\*</sup>**

5 <sup>1</sup>Translational Cardiomyology Laboratory, Stem Cell Institute Leuven, Department of Development  
6 and Regeneration, Herestraat 49, KU Leuven, 3000 Leuven, Belgium.

7 <sup>2</sup>Department of Experimental and Clinical Medicine, Magna Graecia University, Italy.

8 <sup>3</sup>Department of Medical Sciences and Infectious Diseases, Transplant Research Area and Centre for  
9 Inherited Cardiovascular Diseases, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.

10 <sup>4</sup>Genomics Core Leuven, KU Leuven, 3000 Leuven, Belgium.

11 <sup>5</sup>Laboratory of Molecular and Cell Signalling, O&N1, Herestraat 49, bus 802, KU Leuven, 3000  
12 Leuven, Belgium.

13 <sup>6</sup>Department of Anatomy, Histology, Forensic Medicine and Orthopedics, Sapienza University of  
14 Rome, Italy.

15 † These authors have contributed equally to this work and share first authorship

16 **\* Correspondence:**

17 Yoke Chin Chai

18 [yokechin.chai@kuleuven.be](mailto:yokechin.chai@kuleuven.be)

19

20 Maurilio Sampaolesi

21 [maurilio.sampaolesi@kuleuven.be](mailto:maurilio.sampaolesi@kuleuven.be)

22

23 Translational Cardiomyology Lab

24

25 KU Leuven, Herestraat 49 – O&N4 – bus 804

26

27 3000 Leuven, Belgium

28

29 Phone: 0032 16373132

30

31 Fax: 0032 16330294

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35

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**Keywords:** Duchenne muscular dystrophy, cardiomyopathy, cardiac organoids, disease modeling, adipogenesis, fibrosis.

## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

### 39 Abstract

40 Duchenne Muscular Dystrophy (DMD) is an X-linked neuromuscular disease which to-date incurable.  
41 The major cause of death is dilated cardiomyopathy, however the pathogenesis is unclear as existing  
42 cellular and animal models do not fully recapitulate the human disease phenotypes. In this study, we  
43 generated cardiac organoids from patient-derived pluripotent stem cells (DMD-CO) and isogenic-  
44 corrected controls (DMD-Iso-CO) and studied if DMD-related cardiomyopathy and disease  
45 progression occur in the organoids upon long-term culture (up to 93 days). Histological analysis  
46 showed that DMD-CO lacks initial proliferative capacity, displayed a progressive loss of  $\alpha$ -sarcoglycan  
47 localization and high stress in endoplasmic reticulum. Additionally, the cardiomyocyte deteriorated  
48 over time, and fibrosis and adipogenesis were observed in DMD-CO. RNA sequencing analysis  
49 confirmed a distinct transcriptomic profile in DMD-CO which were associated with functional  
50 enrichment in hypertrophy/dilated cardiomyopathy, arrhythmia, adipogenesis and fibrosis pathways.  
51 Moreover, five miRNAs were identified to be crucial in this dysregulated gene network. In conclusion,  
52 we generated patient-derived cardiac organoid model that displayed DMD-related cardiomyopathy and  
53 disease progression phenotypes in long-term culture. We envision the feasibility to develop a more  
54 complex, realistic and reliable *in vitro* 3D human cardiac-mimics to study DMD-related  
55 cardiomyopathies.

56

### 57 1 Introduction

58 Duchenne Muscular Dystrophy (DMD) is one of the most common muscular dystrophies (MD)  
59 which affects 1:5000 live male births (Yiu and Kornberg, 2015). It is a progressive X-linked genetic  
60 disorder caused by mutations within the *DMD* gene, which results in a complete absence of Dystrophin  
61 (DYS) protein expression (Muntoni et al., 2003; Flanigan, 2014; Loboda and Dulak, 2020). Absent of  
62 *DYS* leads to muscle weakness and wasting, owing to the loss of muscle membrane integrity and  
63 susceptibility to stress-induced damages (Lin et al., 2015). In recent years, the use of respiratory assist  
64 device and non-invasive positive pressure ventilation have increased the life expectancy of DMD  
65 patients, nevertheless this has contributed to the rise of previously unknown late-stages DMD  
66 complications, such as dilated cardiomyopathy (DCM) (Kamdar and Garry, 2016; Breuls et al., 2021).

67 DMD-associated DCM is characterized by initial cardiomyocyte degeneration attributed to the  
68 inflammatory response, which leads to the replacement of heart muscle with fat and connective tissue  
69 (i.e. fibrosis of the left-ventricular (LV) myocardial wall) and thus the reduction of cardiac wall  
70 thickness (Finsterer and Stollberger, 2003; Law et al., 2020). Due to the latter, the myocardium  
71 becomes more sensitive to pressure overload causing LV dilatation, cardiac contractility reduction and  
72 ultimately, congestive heart failure (Luk et al., 2009; Fayssol et al., 2010; McNally and Mestroni,  
73 2017). Although DCM represents the major lethal cause of DMD patients, no great research attention  
74 has been directed to DCM – partly due to limited accessibility to human cardiac tissues and the intrinsic  
75 limitation of two-dimensional (2D) cardiomyocyte culture in recapitulating human 3D  
76 physiopathology (Lin et al., 2015; Quattrocchi et al., 2015; Law et al., 2020). Similarly, DMD animal  
77 models (*mdx* mice and canine DMD models) do not fully resemble human DMD features and its disease  
78 progression, mainly due to inter-species variations. It is therefore imperative to develop 3D human  
79 cardiac-mimics of DMD-relevance to bridge this scientific gap (McGreevy et al., 2015; Filippo Buono  
80 et al., 2020; Jensen and Teng, 2020; Zhao et al., 2021).

81 Organoids are *in vitro* self-organize 3D cellular structures derived from either primary tissues or  
82 stem cells [e.g. embryonic (ESCs) or pluripotent stem cells (iPSCs), and primary stem cells]  
83 differentiated into designated functional cell types. They possess organotypic structures including the  
84 cytoarchitecture and the mechanisms involved in the cell behavior and fate within the specific tissue

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85 (Velasco et al., 2020; Heydari et al., 2021; Scalise et al., 2021). The advent of iPSC and CRISPR/Cas  
86 technologies represent a paramount breakthrough for patient-specific model generation, enabling the  
87 development of iPSC-derived cardiomyocyte (CM)-based 3D models and the isogenic controls, which  
88 are widely used to study patient-specific cardiac diseases *in vitro* (Filippo Buono et al., 2020; Richards  
89 et al., 2020). Although cardiac organoids were used for investigating abnormal mechanical and  
90 electromechanical properties of DMD CMs (Caluori et al., 2019; Jelinkova et al., 2020), as to our  
91 knowledge, the organoid technology has not been used to model cardiomyopathies in DMD patients.  
92 Given that, this study focused on the development of 3D cardiac organoids (COs) from DMD patient-  
93 derived iPSC (DMD-CO) and its mutation-corrected isogenic iPSC controls (DMD-Iso-CO), and  
94 studied if these human cardiac-mimics could reproduce DMD-related cardiomyopathy and disease  
95 progression in 3D via long-term culture.

96

## 97 2 Materials and Methods

### 98 2.1 Cell cultures

99 Duchenne Muscular Dystrophy iPSC (DMD-hiPSC) was obtained from DMD patient's fibroblasts  
100 carrying a point mutation in exon 35 (c.4 996C>T; p.Arg1,666X) of the Dystrophin gene that leads to  
101 a premature stop codon (Duelen et al., 2021). Human DMD isogenic control (DMD-Iso iPSC) was  
102 generated through CRISPR/Cas9 gene editing from the *S. pyogenes* system (5'-NGG PAM) as  
103 previously described (Ran et al., 2013; Duelen et al., 2021). Human iPSC lines were cultured feeder-  
104 free on Geltrex LDEV-Free hESC-Qualified Reduced Growth Factor Basement Membrane Matrix and  
105 maintained in Essential 8 Flex Basal Medium (Thermo Fisher Scientific) supplemented with Essential  
106 8 Flex Supplement (50x, Thermo Fisher Scientific) and penicillin–streptomycin (0.1%, Thermo Fisher  
107 Scientific), at 37 °C under normoxic conditions (21% O<sub>2</sub> and 5% CO<sub>2</sub>). Colonies were routinely  
108 passaged non-enzymatically with 0.5 mM EDTA in Phosphate-Buffered Saline (PBS, Thermo Fisher  
109 Scientific). The use of human samples from DMD subjects for experimental purposes and protocols in  
110 the present study was approved by the Ethics Committee of the University Hospitals Leuven  
111 (respectively, S55438 and S65190).

### 112 2.2 Monolayer-based cardiac differentiation of human iPSCs

113 DMD-hiPSC and the isogenic-corrected control lines were differentiated into functional  
114 cardiomyocytes (CMs) according to a monolayer-based cardiac differentiation protocol, as previously  
115 described (Burrige et al., 2014). Briefly, prior to differentiation, the DMD-hiPSC and DMD-Iso-  
116 hiPSC lines were suspended into small colonies and subsequently cultured on Matrigel Growth Factor  
117 Reduced (GFR) Basement Membrane Matrix layer (Corning) in complete Essential 8 Flex Medium at  
118 37 °C under hypoxic conditions (5% O<sub>2</sub> and 5% CO<sub>2</sub>) for three days, in order to obtain the pre-  
119 optimized targeted confluency of 85%. Mesoderm differentiation (day 0) was induced using 6 μM  
120 CHIR99021 (Axon Medchem) for 48 hours in a chemically defined medium consisting of RPMI 1640  
121 (Thermo Fisher Scientific), 500 μg/mL rice-derived recombinant human albumin and 213 μg/mL L-  
122 ascorbic acid 2-phosphate (Sigma-Aldrich). After 24 hours of CHIR99021 stimulation, the cells were  
123 transferred from hypoxia to normoxia. On day 2 of differentiation, iPSC-derived mesodermal cells  
124 were fed with basal medium supplemented with 4 μM IWR-1 (Sigma-Aldrich) for 48 hours, to induce  
125 cardiac progenitor cell differentiation. From day 4 onwards, medium was refreshed every other day  
126 with CM Maintenance Medium (RPMI 1640, rice-derived recombinant human albumin and L-ascorbic  
127 acid 2-phosphate). Contracting CMs appeared at day 8 or 9 of cardiac differentiation.

128

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### 129 **2.3 Agarose microwell culture insert fabrication**

130 A 3% agarose (Invitrogen) gel solution was prepared in PBS. The powder was fully dissolved by  
131 heating in microwave oven and the agarose microwells were fabricated in sterile conditions. In brief,  
132 the heated agarose solution was added into a custom-made 3D printed micropillar molds (in 24-well  
133 plate format). Upon cooling at room temperature for 10 minutes, the agarose were removed from the  
134 molds thus creating 24 culture inserts each consisting of 137 microwells (diameter x height = 500 x  
135 700  $\mu\text{m}$ ). The culture inserts were transferred into a 24-well plate and equilibrated in PBS overnight at  
136 37 °C under normoxia conditions (5% O<sub>2</sub> and 5% CO<sub>2</sub>).

### 137 **2.4 Generation of cardiac organoids**

138 After reaching confluency, the DMD-iPSC and isogenic-corrected control lines were detached using  
139 0.5 mM EDTA at 37 °C and re-suspended in Essential 8™ medium supplemented with Revitacel™  
140 Supplement (dilution 1:100, Thermo Fisher Scientific). After cell count, the hiPSCs were resuspended  
141 in 1 mL of Essential 8 Flex Basal Medium (Thermo Fisher Scientific) and were plated in agarose inserts  
142 at two different cell densities,  $5 \times 10^3$  cells/microwell and  $1 \times 10^4$  cells/microwell respectively. The plates  
143 were centrifuged for 10 min at 1200 rpm to facilitate sedimentation of cells in the microwells. Then,  
144 1 mL of fresh Essential 8 Flex Basal Medium was added to completely cover the microwell area and  
145 incubated at 37 °C under hypoxic conditions (5% O<sub>2</sub> and 5% CO<sub>2</sub>) to promote embryoid bodies (EBs)  
146 formation. The medium was refreshed every day for three days and cardiac differentiation of the EBs  
147 into cardiac organoids (COs) was initiated as described above for the monolayer cardiomyocyte  
148 differentiation protocol. On day 5, the COs were transferred from the agarose molds to an ultra-low  
149 attachment 6-well plate (Costar, Corning) and dynamic culture was carried out using an orbital shaker  
150 at 75 rpm in CM maintenance medium until day 93. The media was changed every two days.  
151 Contracting COs start to appear from day 8 of the differentiation protocol. The samples were collected  
152 on day 10, 14, 28, 56 and 93 for subsequent analysis.

### 153 **2.5 Hematoxylin and Eosin (H&E), Picro-Sirius Red (PSR), and BODIPY stainings**

154 At different time points, the COs were fixed with 4% paraformaldehyde (PFA; Polysciences) for 30  
155 min at room temperature and subsequently embedded in cryogel (Tissue- Tek® O.C.T.™ Compound).  
156 The samples were snap-frozen in liquid nitrogen and stored at -80 °C until cryosectioning. The samples  
157 were sectioned at the thickness of 6  $\mu\text{m}$  using the HM525 NX Cryostat (Thermo Scientific) and stored  
158 at -20 °C prior to analysis. For H&E staining, the cryosections were stained in Harris hematoxylin  
159 solution (Sigma-Aldrich), counterstained in eosin solution (0,1% erithrosin extra bluish Sigma-Aldrich  
160 in 70% ethanol) and mounted with DPX mountant (Sigma) upon dehydration according to routine  
161 protocols. For PSR staining, the cryosections were stained for collagen content using the Vitro View™  
162 Picro-Sirius Red Stain Kit (Cat. No. VB-3017) according to the manufacturer's instructions (Giarratana  
163 et al., 2020). The nuclei were counterstained with Weigert's Hematoxylin Solution and mounted with  
164 DPX mountant (Sigma-Aldrich). Lipid droplets deposition was detected by BODIPY staining. In brief,  
165 the BODIPY™ 493/503 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene  
166 (Invitrogen) powder were dissolved in DMSO at the concentration of 1.3mg/ml. At 1:2500 dilution in  
167 PBS, the cryosections were incubated with the BODIPY solution for 15 min at room temperature and  
168 subsequently mounted with Antifade Mounting Medium with DAPI (VECTASHIELD®). All images  
169 were acquired using Axiocam MRm microscope (Zeiss).

### 170 **2.6 Immunofluorescence staining**

171 After three PBS washes, the cryosections were permeabilized for 1 hour at room temperature using  
172 0.1% Triton X-100 in PBS (Thermo Fisher Scientific). Non-specific antibody binding was blocked by

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173 incubation for 30 min with blocking solution containing 5% normal goat serum (NGS, Dako) at room  
174 temperature followed by overnight incubation at 4 °C with different primary antibodies listed in **Table**  
175 **1**. After washing in phosphate-buffered saline (PBS), the samples were incubated with respective  
176 secondary antibodies using Alexa Fluor 488-, 555-conjugated secondary antibody (4 µg/mL; Thermo  
177 Fisher Scientific). Nuclei were counterstained with Hoechst 33342 (1:1000, Thermo Scientific) for 7  
178 minutes (Santoni de Sio et al., 2008). The sections were mounted with ProLong™ Gold antifade  
179 reagent (Invitrogen) and stored in the dark at 4 °C till imaging. All images were acquired using  
180 Axiocam MRm microscope (Zeiss).

### 181 **2.7 Quantification of beating frequency and surface area of cardiac organoids**

182 To assess the contractile properties of DMD-COs and DMD-iso COs, 3D cardiac organoids were live-  
183 imaged using the Dmi1 Microscope (Leica). The recorded videos were then analyzed to determine  
184 manually the beating frequency by counting the number of spontaneously contracting cardiac  
185 organoids per minute. The cardiac organoids growth area was measured at different time points using  
186 ImageJ software tool.

### 187 **2.8 Intracellular calcium (Ca<sup>2+</sup>) imaging**

188 For Ca<sup>2+</sup> imaging experiments, the DMD-iPSC and DMD-Iso-iPSC monolayers were respectively  
189 plated on 35 mm dishes with four Chamber glass bottom. Following 14 days from cardiac induction,  
190 the DMD-CM and DMD-Iso-CM were incubated with 1µM Fluo-4 AM solubilized in CM  
191 Maintenance Medium. Next, the cells were washed twice with CM Maintenance Medium after which  
192 de-esterification was allowed to occur for 45 min at 37 °C and 5% CO<sub>2</sub>. The Ca<sup>2+</sup> imaging experiments  
193 were performed in pre-warmed (37 °C) modified Krebs-Ringer solution (135 mM NaCl, 6.2 mM KCl,  
194 1.2 mM MgCl<sub>2</sub>, 12 mM HEPES, pH 7.3, 11.5 mM glucose and 2 mM CaCl<sub>2</sub>). Additions were  
195 performed as indicated in *Fig. 1D*: Tetracaine was solubilized in the above modified Krebs-Ringer  
196 solution at 1 mM final concentration; Caffeine was dissolved in the modified Krebs-Ringer solution;  
197 For the KCl stimulus the modified Krebs-Ringer solution was prepared substituting the NaCl for 140  
198 mM KCl. Imaging was performed using a Nikon eclipse Ti2 inverted fluorescence microscope (Nikon)  
199 equipped with excitation filter FF01-378/474/554/635 and dichroic mirror FF01-432/515/595/730 and  
200 emission filter 515/30 all from Semrock. Cooled pR-4000 (Cooled) was used for excitation at 470  
201 nm. Acquisition of the fluorescent signal at 520 nm was performed at 10 Hz using a pco.edge 4.2bi  
202 sCMOS camera (pCO) (Nakamura et al., 2001). For analysis FIJI software was utilized. In each  
203 experiment a region of interest was drawn across spontaneously active cardiomyocytes. The  
204 fluorescence intensities were normalized to F<sub>0</sub>, where the F<sub>0</sub> value was obtained after tetracaine  
205 administration. Area under the curve (AUC) was calculated by multiplying the normalized frequency  
206 for second, in a total of 60 seconds after a 7 frame/sec acquisition (AUC = F/F<sub>0</sub>\*sec).

### 207 **2.9 RNA sequencing and bioinformatics analysis**

208 RNA (>10 µg) extracted from DMD-CO and DMD-Iso-CO on day 56 were verified and processed by  
209 the Genomics Core (KU Leuven – UZ Leuven). As quality control, the RNA concentration was  
210 measured with Nanodrop and quality was checked with Bioanalyzer. The Lexogen QuantSeq 3'  
211 mRNA-Seq library prep kit was used according the manufacturer's protocol with 500 ng input. After  
212 the prep the libraries were measured with Qubit and put on the Fragment analyzer so the libraries can  
213 be pooled equimolar to 2 nM. The pool was then quantified with qPCR and a final pool (2 nM) was  
214 made for single-read sequencing on the HiSeq4000 (Illumina Inc). The settings were 51-8-8. The raw  
215 sequence files generated (.fastq files) underwent quality control analysis using FastQC v0.11.7  
216 (Andrews, 2010). Adapters were filtered with ea-utils fastq-mcf v1.05 (Aronesty, 2011)). Splice-aware  
217 alignment was performed with HiSat2 against the human reference genome hg38 using the default

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218 parameters. Reads mapping to multiple loci in the reference genome were discarded. Resulting BAM  
219 alignment files were handled with Samtools v1.5. (Li et al., 2009). Quantification of reads per gene  
220 was performed with HT-seq Count v2.7.14. Count-based differential expression analysis was done  
221 with R-based (The R Foundation for Statistical Computing, Vienna, Austria) Bioconductor package  
222 DESeq2 (Love et al., 2014). Reported p-values were adjusted for multiple testing with the Benjamini-  
223 Hochberg procedure, which controls false discovery rate (FDR). Gene Ontology (GO) and Biological  
224 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were identified  
225 using g:Profiler (Raudvere et al., 2019). The GO Biological Process 2018 and KEGG 2016 of each  
226 tissue were determined. The significant terms and pathways were selected with the threshold of  
227 adjusted p-value < 0.05. Data has been deposited in the NCBI Gene Expression Omnibus (GEO)  
228 repository under accession code GSE194297.

### 229 **2.10 Generation of protein-protein interaction (PPI) network**

230 The PPI network of differentially upregulated genes in DMD-CO was constructed by feeding a list of  
231 gene symbols and their log<sub>2</sub>fold changes into the NetworkAnalyst platform  
232 (<http://www.networkanalyst.ca/>) using the IMEx interactome database with Steiner Forest Network  
233 (SFN) reduction algorithm. Subsequently, the gene-miRNA interactions (Rotini et al., 2018) for the  
234 selected KEGG pathways were constructed based on the miRTarBase (v8.0) database, and the network  
235 was reduced using the SFN algorithm. The degree of each node was calculated based on its number of  
236 connections to other nodes. In the network, the area of an individual node indicates the degree, and the  
237 color represents the expression. The identified top five miRNAs were mapped out in the KEGG  
238 pathways to show their interactions with the genes of a particular pathway.

### 239 **2.11 Statistical analysis**

240 Data were statistically analyzed using GraphPad Prism. All data were reported as mean ± standard  
241 deviation (SD). Differences between groups were examined for statistical significance using ANOVA,  
242 two-way ANOVA or unpaired T-test. Significance of the differences was indicated as follows: \*p <  
243 0.05, \*\*p < 0.01, \*\*\*p < 0.001, and \*\*\*\*p < 0.0001.

244

## 245 **3 Results**

### 246 **3.1 Characterization of the generated cardiomyocytes monolayers from DMD- and isogenic** 247 **corrected hiPSC lines.**

248 Following the 2D monolayer differentiation protocol, we generated cardiomyocytes (CMs) from both  
249 DMD patient-derived hiPSCs (DMD-CM) and the isogenic control (DMD-Iso-CM) monolayer  
250 cultures (Fig. 1A). These cells started to develop contractile phenotype around day 8 and were  
251 morphologically similar in both conditions. On day 22, RT-qPCR analysis showed that the DMD-CM  
252 expressed significantly lower dystrophin (*DYS*) than the isogenic controls (Fig. 1B), confirming the  
253 restoration of *DYS* expression in the isogenic controls, as described in Duellen R *et al.* The DMD-CM  
254 also expressed significantly lower sarcomeric  $\alpha$ -actinin ( *$\alpha$ ACTN2*), the pacemaker gene *HCN4*, and the  
255 troponin-related genes (*ssTnl*, *cTnC* and *cTnl*) but not the myosin light (*MYL7*, *MYL2*) or heavy chain  
256 (*MYH6*, *MYH7*) genes, than the isogenic controls (Fig. 1C). Next, we established a cell physiological  
257 analysis of 14-day differentiated DMD-CMs and DMD-Iso-CMs. A hallmark of functional CMs is  
258 their ability to generate cytosolic Ca<sup>2+</sup> signals that are driven by ryanodine receptors (RyRs),  
259 intracellular Ca<sup>2+</sup>-release channels residing at the sarcoplasmic reticulum of CMs. Therefore, cytosolic  
260 Ca<sup>2+</sup> imaging was performed in single-cell CMs loaded with Fluo-4. In the presence of extracellular

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261  $\text{Ca}^{2+}$  (1.5 mM  $\text{CaCl}_2$ ), spontaneous  $\text{Ca}^{2+}$  oscillations were observed both DMD-CMs and DMD-Iso-  
262 CMs that could be blocked by tetracaine, an inhibitor of RyR channels. However, spontaneous  $\text{Ca}^{2+}$   
263 oscillations appeared to display a lower frequency with unchanged amplitudes in DMD-CMs compared  
264 to the isogenic controls, indicating a defect in physiological  $\text{Ca}^{2+}$  signalling in dystrophic CMs that is  
265 corrected in the isogenic controls. Moreover, DMD-CMs and DMD-Iso-CMs displayed a comparable  
266  $\text{Ca}^{2+}$  response to Caffeine, a pharmacological activator of RyR channels, and KCl, which provokes  
267 membrane depolarization (Fig. 1D & 1E). These findings validated the dystrophic properties of DMD-  
268 CM and their defects in physiological  $\text{Ca}^{2+}$  signalling, whereby both deficiencies could be reverted in  
269 isogenic-corrected DMD-Iso-CM generated in this study, respectively.

### 270 3.2 Generation of DMD- and DMD-isogenic corrected cardiac organoids (COs).

271 We adapted the cardiomyocyte monolayer differentiation protocol to generate COs by direct  
272 differentiation of the embryoid bodies (EBs) (Fig. 2A). By using the agarose microwell culture inserts,  
273 we could promote self-aggregation of the DMD-hiPSCs and DMD-Iso-hiPSCs into EBs at cell seeding  
274 number of 5000 and 10000 cells per microwell (Fig. 2B). This allowed us to generate 137 EBs per  
275 insert per well of 24 well-plate. On day 5 of cardiomyocyte differentiation, the resulting DMD-CO and  
276 DMD-Iso-CO were transferred to 6-well plate on orbital shaker for dynamic culture in the cardiac  
277 maintenance medium (Fig. 2C). Contractile cardiomyocyte protrusions (Fig. 2C, arrow) and self-  
278 organized cellular structures (Fig. 2C, arrowheads) at the organoid periphery, both with specific spatial  
279 distribution of NKX2.5 and  $\alpha$ ACTN positivity, could be observed. The non-translucent organoid  
280 structure (#) was negative for both NKX2.5 and  $\alpha$ ACTN. Immunofluorescence staining showed  
281 abundant DYS localization in DMD-Iso-CO, which was undetectable in the DMD-CO (Fig. 2D).  
282 Quantification of the organoid surface area over 28 days of dynamic culture showed no significant  
283 differences on the organoid size between the two cell seeding numbers within each cell line, but the  
284 size of DMD-Iso-CO was significantly smaller than DMD-CO on day 14 and 28, respectively (Fig.  
285 2F). The DMD-CO displayed contraction on day 8 ( $\pm$  19 per minute) which decreased over time and  
286 stopped contraction between day 14 and 18 (Fig. 2G). The DMD-Iso-CO displayed contraction on day  
287 12 ( $\pm$  18 per minute) which persisted till day 28.

### 288 3.3 Progressive loss of $\alpha$ -sarcoglycan expression in DMD-CO.

289 We performed immunofluorescence staining for  $\alpha$ -sarcoglycan (SCGA), sarcomeric  $\alpha$ -actinin  
290 ( $\alpha$ ACTN), and NKX2.5 on day 10, 14, 28, 56 and 93 in order to assess cardiac differentiation and  
291 contractile protein development within the organoids. The results showed abundant SCGA expression  
292 in DMD-CO on day 10, which became low on day 14 and undetectable from day 28 onwards (Fig. 3A).  
293 Conversely, the SCGA expression in DMD-Iso-CO persisted till day 93. A transient expression of the  
294 early cardiac differentiation marker NKX2.5 was observed up to day 28 in both DMD-CO and DMD-  
295 Iso-CO, which became undetectable on day 56 and 93. Additionally, abundant  $\alpha$ ACTN, a cardiac  
296 contractile protein, was observed in both DMD-CO and DMD-Iso-CO on early time points, which  
297 remained detectable on day 93 (despite at lower expression level) in both conditions (Fig. 3B). There  
298 was no distinguishable difference in the SCGA,  $\alpha$ ACTN and NKX2.5 expression between organoids  
299 generated from the two cell seeding numbers within a cell line. These results demonstrated a  
300 progressive loss of SCGA protein expression in DMD-CO (a member of the dystrophin associated  
301 complex, DAC) as compared to the isogenic controls. Additionally, RT-qPCR analysis showed a  
302 significant upregulation of some gene markers for cardiac contractility in DMD-CO as compared to  
303 DMD-Iso-CO, in particularly from day 56 onwards (Fig. 3C). These include *ACTN1*, *IRX4*, *MYBPC3*,  
304 *MYL2*, *MYOM1*, *TNNC2* and *TPM1*.

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### 306 **3.4 Lack of initial proliferative capacity and high endoplasmic reticulum stress in DMD-CO.**

307 We examined cell proliferation or apoptotic condition within the DMD-CO and DMD-Iso-CO by  
308 immunostaining of the proliferation marker Ki67 and apoptotic marker cleaved caspase 3 (CCASP3).  
309 The results showed low Ki67 staining in DMD-CO but relative higher signal in DMD-Iso-CO on day  
310 10, while the signal become comparable on day 28 and 93 (*Fig. 4A*). Low and comparable CCASP3  
311 staining was observed in both DMD-CO and DMD-Iso-CO at all time points. No significant difference  
312 in Ki67 and CCASP3 staining was observed at both cell seeding densities for both CO conditions (data  
313 not shown). This data suggest that the DMD-CO was lacking an initial proliferative capacity at early  
314 time point and minimal apoptosis occurred in both CO conditions. We then assessed the metabolic  
315 activity within the CO by immunostaining of the glycolytic marker phosphoglycerate kinase 1 (PGK1).  
316 The results showed high and comparable PGK1 staining in both CO conditions at all time points (*Fig.*  
317 *4B*), which was independent of the cell seeding densities (data not shown). This data suggests the  
318 glycolytic condition of immature CO in both CO conditions. The cellular stress was assessed by  
319 immunostaining of two known endoplasmic reticulum (ER) stress markers ARCN1 and GORASP2.  
320 Interestingly, we detected relatively higher level of ARCN1 in DMD-CO than DMD-Iso-CO at all time  
321 points (*Fig. 4C*), whereas GORASP2 increased progressively over the 28 days in DMD-CO,  
322 independent of the cell seeding densities (data not shown) and at higher level than that in DMD-Iso-  
323 CO at all time points (*Fig. 4D*). This finding indicated a high level of ER stress occurred in DMD-CO.

### 324 **3.5 Cardiomyocyte deterioration followed by fibrosis and adipogenesis in DMD-CO after long-** 325 **term culture.**

326 We performed histological examination to assess any cytoarchitecture changes and DMD-related  
327 pathological progression within the COs over 93 days. The DMD-CO displayed normal  
328 cardiomyocyte-like structures similar to that of DMD-Iso-CO on day 10, which deteriorated on day 14  
329 (indicated as “#”) and developed fibrotic-like structure (indicated as “F”) at later time points (*Fig. 5A*;  
330 *H&E staining* on day 56, and *Fig. 5B*; Picro-Sirius red staining for collagen deposition on day 93).  
331 These findings were corroborated by a significant upregulation of gene markers associated with fibrosis  
332 *COL1A2*, *COL3A1* and *FNI* in DMD-CO on day 56 and 93 as compared to DMD-Iso-CO (*Fig. 5C*).  
333 Additionally, H&E staining also revealed adipose tissue formation in DMD-CO on day 28 (*Fig. 5D*),  
334 and this was confirmed by the detection of lipid droplets via BODIPY staining and immunolabelled  
335 PDGFR $\alpha^+$  cells (an adipocyte marker) in DMD-CO on day 28 and 56 (*Fig. 5E*). Interestingly, GDF10  
336 protein (an adipogenesis inhibitor) was also detected near the PDGFR $\alpha^+$  cells in DMD-CO (*Fig. 5F*).  
337 These findings suggest that DMD-CO displayed an initial normal cardiomyocyte phenotype which  
338 deteriorated progressively and exhibited fibrotic and adipogenic phenotypes upon long-term culture,  
339 resembling pathologic events associated with DMD cardiomyopathy.

### 340 **3.6 RNA sequencing revealed functional enrichment of hypertrophy/dilated cardiomyopathy,** 341 **adipogenesis and fibrosis signalings in DMD-CO.**

342 Principle component analysis of the RNA transcriptomic data showed a distinct separation between  
343 DMD-CO and DMD-Iso-CO clusters (PC1: 90%) with low intra-condition variance (PC2: 5%) (*Fig.*  
344 *6A*). Based on the enhanced volcano plot, out of 22371 gene variables, 1518 and 554 genes were  
345 differentially upregulated in DMD-CO and DMD-Iso-CO, respectively (Cut-off:  $\log_2$  fold change = 1.5;  
346  $-\text{Log}_{10}P = 10^{-16}$ ) (*Fig. 6B*). Among the top 30 most differentially upregulated genes in DMD-CO (*Fig.*  
347 *6C*), the expression of *MGP*, *MYL1*, *COL1A2*, *HAPLN1* and *OGN* were the five most significant  
348 upregulated genes in DMD-CO (*Fig. 6B & Table 3*). Based on gProfiler analysis, gene ontologies that  
349 were significantly enriched for molecular function in extracellular matrix regulation (i.e. collagen and  
350 glycosaminoglycan; *GO:MF*), cardiac tissue structure formation (i.e. external encapsulating structure  
351 such as sarcolemma; *GO:MM*), and cardiovascular development (*GO:BP*) could be identified in



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352 DMD-CO (Fig. 6D). Additionally, KEGG pathways associated with protein digestion and absorption,  
353 dilated and hypertrophic cardiomyopathy, ECM-receptor interaction, and cGMP-PKG signalling  
354 pathway (known to positively modulates cardiac contractility, hypertrophy and protects against  
355 apoptosis (Takimoto, 2012)) were significantly enriched in DMD-CO (Fig. 6E (i)). These findings  
356 were corroborated by the analysis on human phenotype ontology, whereby ontology related to  
357 abnormal cardiovascular system physiology, including abnormal left ventricular function, abnormal  
358 endocardium morphology, atrial arrhythmia and fibrillation, supraventricular arrhythmia, myopathy  
359 and cardiac arrest, as well as abnormal adipose tissue morphology and lipodystrophy were significantly  
360 enriched in DMD-CO as compared to DMD-Iso-CO (Fig. 6E (ii)). Moreover, the gProfiler analysis  
361 also identified three top miRNA regulators for the differentially upregulated genes in DMD-CO,  
362 namely *hsa-mir-335-5p*, *hsa-mir-29a-3p* and *hsa-mir-29b-3p*. Altogether, the RNA sequencing data  
363 validated the histological observations described above on cardiomyocyte deterioration, adipogenesis  
364 and fibrosis at the transcriptomic level.

### 365 3.7 Protein-protein interaction (PPI) network analysis of differentially upregulated genes in 366 DMD-CO.

367 PPI analysis of the differentially upregulated genes in DMD-CO revealed a gene network consisted of  
368 2289 nodes and 2288 edges. According to the degree level (d), the top five hub nodes were HNF4A (d  
369 = 257), UBC (d = 108), UBD (d = 66), APP (d = 38) and EGR1 (d = 31) (Fig. 7A). By exploring the  
370 miRNA database (i.e. mirTarBase v8.0), the top three miRNA regulators of this gene network were  
371 *hsa-mir-335-5p*, *hsa-mir-124-3p*, and *hsa-mir-26b-5p*. Together with the *hsa-mir-29b-3p* and *hsa-mir-*  
372 *29a-3p* identified by gProfiler2, we mapped out these miRNAs on the gene-miRNA regulatory  
373 networks for the selected KEGG pathways relevant to the DMD-CO phenotypes: (1) Hypertrophy  
374 cardiomyopathy, (2) Dilated cardiomyopathy, (3) Arrhythmogenic right ventricular cardiomyopathy  
375 (ARVC), (4) PPAR signalling pathway (for adipogenesis), and (5) PI3K-Akt signalling pathway (for  
376 cardiac fibrosis (Qin et al., 2021)). The results showed that hypertrophy and dilated cardiomyopathy  
377 networks shared the same gene set (50 nodes, 49 edges), miRNA interactions (Fig. 7B) and 16 genes  
378 similarity with the ARVC network (Fig. 7C). Except *hsa-mir-124-3p*, the other four top miRNAs were  
379 mapped in these three networks, respectively. The PPAR signalling gene-miRNA network consisted  
380 of 33 nodes and 43 edges (Fig. 7D). In addition to *hsa-mir-26b-5p* and *hsa-mir-335-5p*, the *hsa-mir-*  
381 *124-3p* was mapped in the network and found interacts with the gene ACSL5 and ACADL. The *has-*  
382 *mir-29b-3p*, *hsa-mir-26b-5p* and *hsa-mir-335-5p* were the main miRNA regulators in the PI3K-Akt  
383 signalling (147 nodes, 146 edges), which interact with one of the two hub genes CCND2 (Fig. 7E).

384

## 385 4 Discussion

386 There is currently no cure for DMD patients. They are solely treated symptomatically via  
387 palliative therapies in combination with cardio-respiratory supporting devices in case of cardio-  
388 pulmonary complications – a major lethal cause in DMD patients. As DMD-related cardiomyopathy  
389 often manifested as hypertrophic or dilated heart due to cardiomyocyte deterioration followed by  
390 fibrosis and adipose tissue formation, novel therapeutic modality should be developed to prevent these  
391 pathological events from taking places in the heart. For this, gaining in-depth understanding on the  
392 human disease mechanisms is necessary. Unfortunately, limited accessibility to patient biopsy/autopsy  
393 and the inferiority of *in vitro* 2D cellular and animal models in fully recapitulating the human disease  
394 phenotype have precluded this scientific endeavour. Therefore, we anticipated that it is imperative to  
395 develop *in vitro* 3D human cardiac-mimics of DMD-relevance to bridge this scientific gap.

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396 In this study, we generated DMD-CO that displayed a lack in proliferative capacity and a  
397 progressive deterioration of cardiomyocytes in early culture stage, followed by adipose tissue and  
398 fibrous tissue formation at later culture stage. These are encouraging findings showing the potential of  
399 these human cardiac-mimics as novel *in vitro* 3D cellular models for studying DMD cardiomyopathy.  
400 We attempted to quantify the immunofluorescence signals for the different analysis including the  
401 adipose tissue and fibrosis areas. However, the results were affected by the heterogeneity in the  
402 cytoarchitectures, as the organoids were derived from hiPSC-EBs that might have undergone  
403 inhomogeneous mesodermal induction and cardiac-lineage commitment due to diffusion variation of  
404 the chemical inducers in 3D space under an uncontrolled static culture-driven condition. Nonetheless,  
405 comparing to pre-differentiated cardiomyocyte spheroids, cardiac organoids derived from hiPSC-EBs  
406 have the advantage of possibly containing other non-cardiogenic cells (as seen during heart  
407 development) that could contribute to the adipogenesis and fibrosis phenotypes upon cardiomyocyte  
408 deterioration. Noteworthy that these pathological events were not observed in the DMD-Iso-CO  
409 controls. Herein, adding endothelial cells to the EBs to generate 3D vascularized human cardiac models  
410 would also be highly valuable to study the cardiomyocyte-endothelial interplays in relation to DMD  
411 pathogenesis.

412 The advent of hiPSC technology represents a paramount breakthrough for patient-specific  
413 model generation that can better mimic the individual phenotype. By using the isogenic-corrected  
414 controls (instead of healthy wild-type controls), we could compare the results at minimal genetic  
415 background variability. The reasons of the development of adipocytes and fibrous tissues are still  
416 unclear and further experiments have to be performed to elucidate the causes. As reported in literature,  
417 dystrophic myocardium, due to the  $Ca^{2+}$  overload, is characterized by cell death and inflammatory  
418 response, which result not only in myocyte hypertrophy, atrophy/necrosis, fibrosis, but also in the  
419 replacement of heart muscle by connective tissue and fat (Flanigan, 2014). In addition, DMD-CO  
420 showed stable  $\alpha$ ACTN localization while SCGA became minimal present from day 14. These results  
421 confirmed the formation of cardiac tissue within the organoids, whereby the formed sarcoglycan  
422 complex possibly deteriorated within the DMD-COs over time due to its intrinsic DMD pathological  
423 phenotypes. It's known by the literature that iPSC-derived CMs, are qualitatively and quantitatively  
424 immature, resembling fetal hearts, where the majority of the ATP is produced by glycolysis. After birth  
425 the CMs metabolism switches to the oxidative phosphorylation to fulfil the energy demand of the  
426 contracting myocardium (Allen et al., 2016). In fact, the glycolytic marker PGK1 was strongly  
427 expressed in both DMD-CO and DMD-Iso-CO. The endoplasmic reticulum stress marker GORASP2  
428 increased over time in DMD-CO, while ARCN1 was more prominent in DMD-CO, but they weren't  
429 co-localized with NKX2.5, suggesting other cell type than differentiating cardiomyocytes experienced  
430 high ER stress within the generated DMD-CO and DMD-Iso-CO. Moreover, we argued that the  
431 presence of GDF10 near the PDGFR<sup>+</sup> adipocytes could be a feedback regulation mechanism to inhibit  
432 pathological formation of adipose tissues in the DMD-CO, as GDF10 was not detected in DMD-Iso-  
433 CO where adipogenesis did not occur.

434 We also observed a defect in physiological RyR-driven  $Ca^{2+}$  signals in DMD-CMs compared to  
435 isogenic-corrected controls. This further underpins the validity of our model since RyR dysfunction  
436 has also been implicated in dystrophic skeletal muscle cells (Andersson et al., 2012). In this work,  
437 dystrophic skeletal muscle was linked with leaky (skeletal muscle-type) RyR1 channels due to its  
438 oxidation. Hence, our work suggests that also the functional properties (cardiac muscle-type) RyR2  
439 channels may be affected in DMDs, thereby contributing to cardiac pathophysiology.

440 Through RNA sequencing analysis, we demonstrated that the DMD-CO generated on day 56  
441 were valuable 3D cellular models to gain insight into the disease mechanism of DMD-associated  
442 hypertrophic/dilated cardiomyopathy, as well as adipogenesis and fibrosis. We focused on mapping  
443 out the functionally enriched pathways based on the differentially upregulated genes in DMD-CO as  
444 compared to DMD-Iso-CO, as well as their main miRNA regulators. Among the top five hub genes

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445 identified in the protein-protein interaction network, only HNF4A ( $\log_2FC = 1.89$ ,  $p < 2.92e^{-5}$ ), UBD  
446 ( $\log_2FC = 2.69$ ,  $p < 7.37e^{-5}$ ) and EGR1 ( $\log_2FC = 1.47$ ,  $p < 5.31e^{-12}$ ) were significantly and differentially  
447 upregulated in DMD-CO. Despite HNF4A could be linked to cardiac differentiation and heart  
448 development (Duelen et al., 2017), we could not find in literature the association of these three hub  
449 genes with the development of cardiomyopathy, adipogenesis and fibrosis. We turned into looking at  
450 the identified miRNA regulators. The *hsa-mir-335-5p* was reported as a regulator of cardiac  
451 differentiation by upregulating cardiac mesoderm and cardiac progenitor commitments, potentially  
452 mediated through the activation of WNT and TGF $\beta$  pathways (Kay et al., 2019). In contrast, the  
453 upregulation of *hsa-mir-335-5p* was seen in fibrotic lung model (Honeyman et al., 2013). Additionally,  
454 a study showed that the *hsa-mir-29a-3p* and *hsa-mir-29b-3p* levels in cardiac tissue from patients with  
455 congenital heart disease was significantly increased, and the injection of *miR-29b-3p* into zebrafish  
456 embryos induced higher mortality and developmental disorders including cardiac malformation and  
457 dysfunction, as well as inhibition of cardiomyocyte proliferation by targeting NOTCH2 (Yang et al.,  
458 2020). Interestingly, delivery of *miR-29a-3p* has a beneficial effect in myocardial injury (Ren et al.,  
459 2021) and cardiac hypertrophy (Xie et al., 2020). Similarly, the *hsa-mir-26a/b-5p* was highly expressed  
460 in cardiac hypertrophy (Tang et al., 2020) and promoted myocardial infarction-induced cell death (Jung  
461 et al., 2021), yet overexpression of *miR-26a/b* attenuated cardiac fibrosis (Tang et al., 2017; Wang et  
462 al., 2019) and alleviated cardiac hypertrophy and dysfunction (Shi et al., 2021). Lastly, the *hsa-mir-  
463 124-3p* was reported to promote cardiac fibroblast activation and proliferation (Zhu et al., 2021), and  
464 its inhibition protects against acute myocardial infarction by suppressing cardiomyocyte apoptosis (Hu  
465 et al., 2019). Based on the duality effects of these miRNAs, the potential of these miRNAs as  
466 therapeutic targets for DMD-related cardiomyopathy need to be assessed carefully. Furthermore, the  
467 identified PI3K/Akt signaling pathway enriched in DMD-CO is interesting, as accumulating evidences  
468 showed that it plays a role in regulating the occurrence, progression and pathological cardiac fibrosis  
469 (Qin et al., 2021) and hypertrophy (Aoyagi and Matsui, 2011).

470 In conclusion, we demonstrated the development of 3D human cardiac-mimics with DMD-  
471 relevances as these models reproduce *in vitro*, even if partially, the DMD-related cardiomyopathy (i.e.  
472 cardiomyocytes stress and deterioration) and disease progression (i.e. adipogenesis and fibrosis) in 3D  
473 space via long-term culture. Additionally, by studying the transcriptomic dysregulations in DMD-CO  
474 versus the isogenic controls via RNA sequencing and *in silico* analysis, we have identified five  
475 miRNAs that were significantly and differentially expressed in late DMD-CO which could be  
476 associated with the functionally enriched hypertrophy and dilated cardiomyopathy, fibrosis and  
477 adipogenesis signaling pathways. These findings are encouraging and prompting us to investigate in  
478 future the potential of these miRNAs as therapeutic targets to inhibit the aberrant functional  
479 enrichments in DMD-CO. In turn, this will enable us to further validate DMD-CO as reliable *in vitro*  
480 3D human cardiac models for DMD-related disease modelling, drug discovery and regenerative  
481 medicine.

482

### 483 Data Availability Statement

484 The RNA sequencing datasets generated/analyzed for this study can be found in the NCBI Gene  
485 Expression Omnibus (GEO) repository with accession code GSE194297.

486

### 487 Author Contributions

488 VM and FM performed all experiments. RL and TV performed calcium imaging experiment. VM, FM,  
489 NG, EP, ACC, TV, FA and YCC analyzed the data. VM, FM, RD, TV, GB, DT, MS and YCC designed  
490 the experiment, wrote and/or revised the manuscript.

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### 491 **Funding**

492 This work was supported by FWO (#G066821N), INTERREG – Euregio Meuse-Rhine (GYM -  
493 Generate your muscle 2020-EMR116), and KU Leuven C1-3DMuSyC (C14/17/111) sustaining also  
494 YCC. The authors gratefully acknowledge Sylvia Sauvage for technical assistance, Christina Vochten  
495 and Vicky Raets for the administrative assistance. RD is supported by KU Leuven Rondoufonds voor  
496 Duchenne Onderzoek (EQQ-FODUCH-O2010) and KU Leuven grant (C24/18/103).

497

### 498 **Conflict of Interest**

499 The authors declare that the research was conducted in the absence of any commercial or financial  
500 relationships that could be construed as a potential conflict of interest.

501

### 502 **References**

- 503 Allen, D.G., Whitehead, N.P., and Froehner, S.C. (2016). Absence of Dystrophin Disrupts Skeletal  
504 Muscle Signaling: Roles of Ca<sup>2+</sup>, Reactive Oxygen Species, and Nitric Oxide in the  
505 Development of Muscular Dystrophy. *Physiol Rev* 96(1), 253-305. doi:  
506 10.1152/physrev.00007.2015.
- 507 Andersson D.C., Meli A.C., Reiken S., Betzenhauser M.J., Umanskaya A., Shiomi T., et al. (2012)  
508 Leaky ryanodine receptors in  $\beta$ -sarcoglycan deficient mice: a potential common defect in  
509 muscular dystrophy. *Skelet Muscle*. 2(1): 9. doi: 10.1186/2044-5040-2-9. PMID: 22640601.
- 510 Andrews, S. (2010). FastQC: A Quality Control Tool for High Throughput Sequence Data [Online].  
511 . Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.
- 512 Aoyagi, T., and Matsui, T. (2011). Phosphoinositide-3 kinase signaling in cardiac hypertrophy and  
513 heart failure. *Curr Pharm Des* 17(18), 1818-1824. doi: 10.2174/138161211796390976.
- 514 Aronesty, E. (2011). ea-utils : "Command-line tools for processing biological sequencing data  
515 <https://github.com/ExpressionAnalysis/ea-utils>
- 516 Breuls, N., Giarratana, N., Yedigaryan, L., Garrido, G.M., Carai, P., Heymans, S., et al. (2021).  
517 Valproic acid stimulates myogenesis in pluripotent stem cell-derived mesodermal progenitors  
518 in a NOTCH-dependent manner. *Cell Death Dis* 12(7), 677. doi: 10.1038/s41419-021-03936-  
519 w.
- 520 Burridge, P.W., Matsa, E., Shukla, P., Lin, Z.C., Churko, J.M., Ebert, A.D., et al. (2014). Chemically  
521 defined generation of human cardiomyocytes. *Nat Methods* 11(8), 855-860. doi:  
522 10.1038/nmeth.2999.
- 523 Caluori, G., Pribyl, J., Pesl, M., Jelinkova, S., Rotrekl, V., Skladal, P., et al. (2019). Non-invasive  
524 electromechanical cell-based biosensors for improved investigation of 3D cardiac models.  
525 *Biosens Bioelectron* 124-125, 129-135. doi: 10.1016/j.bios.2018.10.021.
- 526 Duelen, R., Costamagna, D., Gilbert, G., De Waele, L., Goemans, N., Desloovere, K., et al. (2021).  
527 Human iPSC-Based Model Reveals NOX4 as Therapeutic Target in 1 Duchenne  
528 Cardiomyopathy. *bioRxiv*. doi: <https://doi.org/10.1101/2021.09.13.460090>.
- 529 Duelen, R., Gilbert, G., Patel, A., de Schaetzen, N., De Waele, L., Roderick, L., et al. (2017). Activin  
530 A Modulates CRIPTO-1/HNF4alpha(+) Cells to Guide Cardiac Differentiation from Human  
531 Embryonic Stem Cells. *Stem Cells Int* 2017, 4651238. doi: 10.1155/2017/4651238.

## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

- 532 Fayssoil, A., Nardi, O., Orlikowski, D., and Annane, D. (2010). Cardiomyopathy in Duchenne  
533 muscular dystrophy: pathogenesis and therapeutics. *Heart Fail Rev* 15(1), 103-107. doi:  
534 10.1007/s10741-009-9156-8.
- 535 Filippo Buono, M., von Boehmer, L., Strang, J., Hoerstrup, S.P., Emmert, M.Y., and Nugraha, B.  
536 (2020). Human Cardiac Organoids for Modeling Genetic Cardiomyopathy. *Cells* 9(7). doi:  
537 10.3390/cells9071733.
- 538 Finsterer, J., and Stollberger, C. (2003). The heart in human dystrophinopathies. *Cardiology* 99(1), 1-  
539 19. doi: 10.1159/000068446.
- 540 Flanigan, K.M. (2014). Duchenne and Becker muscular dystrophies. *Neurol Clin* 32(3), 671-688, viii.  
541 doi: 10.1016/j.ncl.2014.05.002.
- 542 Giarratana, N., Conti, F., La Rovere, R., Gijbsers, R., Carai, P., Duelen, R., et al. (2020). MICAL2 is  
543 essential for myogenic lineage commitment. *Cell Death Dis* 11(8), 654. doi: 10.1038/s41419-  
544 020-02886-z.
- 545 Heydari, Z., Moeinvaziri, F., Agarwal, T., Pooyan, P., Shpichka, A., Maiti, T.K., et al. (2021).  
546 Organoids: a novel modality in disease modeling. *Biodes Manuf*, 1-28. doi: 10.1007/s42242-  
547 021-00150-7.
- 548 Honeyman, L., Bazett, M., Tomko, T.G., and Haston, C.K. (2013). MicroRNA profiling implicates  
549 the insulin-like growth factor pathway in bleomycin-induced pulmonary fibrosis in mice.  
550 *Fibrogenesis Tissue Repair* 6(1), 16. doi: 10.1186/1755-1536-6-16.
- 551 Hu, G., Ma, L., Dong, F., Hu, X., Liu, S., and SUn, H. (2019). Inhibition of microRNA-124-3p  
552 protects against acute myocardial infarction by suppressing the apoptosis of cardiomyocytes.  
553 *Mol Med Rep* 20(4), 3379-3387.
- 554 Jelinkova, S., Vilotic, A., Pribyl, J., Aimond, F., Salykin, A., Acimovic, I., et al. (2020). DMD  
555 Pluripotent Stem Cell Derived Cardiac Cells Recapitulate in vitro Human Cardiac  
556 Pathophysiology. *Front Bioeng Biotechnol* 8, 535. doi: 10.3389/fbioe.2020.00535.
- 557 Jensen, C., and Teng, Y. (2020). Is It Time to Start Transitioning From 2D to 3D Cell Culture?.  
558 *Frontiers in molecular biosciences* 33(7). doi: <https://doi.org/10.3389/fmolb.2020.00033>.
- 559 Jung, S.E., Kim, S.W., Jeong, S., Moon, H., Choi, W.S., Lim, S., et al. (2021). MicroRNA-26a/b-5p  
560 promotes myocardial infarction-induced cell death by downregulating cytochrome c oxidase  
561 5a. *Exp Mol Med* 53(9), 1332-1343. doi: 10.1038/s12276-021-00665-0.
- 562 Kamdar, F., and Garry, D.J. (2016). Dystrophin-Deficient Cardiomyopathy. *J Am Coll Cardiol*  
563 67(21), 2533-2546. doi: 10.1016/j.jacc.2016.02.081.
- 564 Kay, M., Soltani, B.M., Aghdaei, F.H., Ansari, H., and Baharvand, H. (2019). Hsa-miR-335 regulates  
565 cardiac mesoderm and progenitor cell differentiation. *Stem Cell Res Ther* 10(1), 191. doi:  
566 10.1186/s13287-019-1249-2.
- 567 Law, M.L., Cohen, H., Martin, A.A., Angulski, A.B.B., and Metzger, J.M. (2020). Dysregulation of  
568 Calcium Handling in Duchenne Muscular Dystrophy-Associated Dilated Cardiomyopathy:  
569 Mechanisms and Experimental Therapeutic Strategies. *J Clin Med* 9(2). doi:  
570 10.3390/jcm9020520.
- 571 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence  
572 Alignment/Map format and SAMtools. *Bioinformatics* 25(16), 2078-2079. doi:  
573 10.1093/bioinformatics/btp352.

## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

- 574 Lin, B., Li, Y., Han, L., Kaplan, A.D., Ao, Y., Kalra, S., et al. (2015). Modeling and study of the  
575 mechanism of dilated cardiomyopathy using induced pluripotent stem cells derived from  
576 individuals with Duchenne muscular dystrophy. *Dis Model Mech* 8(5), 457-466. doi:  
577 10.1242/dmm.019505.
- 578 Loboda, A., and Dulak, J. (2020). Muscle and cardiac therapeutic strategies for Duchenne muscular  
579 dystrophy: past, present, and future. *Pharmacol Rep* 72(5), 1227-1263. doi: 10.1007/s43440-  
580 020-00134-x.
- 581 Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion  
582 for RNA-seq data with DESeq2. *Genome Biol* 15(12), 550. doi: 10.1186/s13059-014-0550-8.
- 583 Luk, A., Ahn, E., Soor, G.S., and Butany, J. (2009). Dilated cardiomyopathy: a review. *J Clin Pathol*  
584 62(3), 219-225. doi: 10.1136/jcp.2008.060731.
- 585 McGreevy, J.W., Hakim, C.H., McIntosh, M.A., and Duan, D. (2015). Animal models of Duchenne  
586 muscular dystrophy: from basic mechanisms to gene therapy. *Dis Model Mech* 8(3), 195-213.  
587 doi: 10.1242/dmm.018424.
- 588 McNally, E.M., and Mestroni, L. (2017). Dilated Cardiomyopathy: Genetic Determinants and  
589 Mechanisms. *Circ Res* 121(7), 731-748. doi: 10.1161/CIRCRESAHA.116.309396.
- 590 Muntoni, F., Torelli, S., and Ferlini, A. (2003). Dystrophin and mutations: one gene, several proteins,  
591 multiple phenotypes. *Lancet Neurol* 2(12), 731-740. doi: 10.1016/s1474-4422(03)00585-4.
- 592 Nakamura TY, Iwata Y, Sampaolesi M, Hanada H, Saito N, Artman M, et al. (2001) Stretch-  
593 activated cation channels in skeletal muscle myotubes from sarcoglycan-deficient hamsters.  
594 *Am J Physiol Cell Physiol* 281(2): C690-C699. doi: 10.1152/ajpcell.2001.281.2.C690.
- 595 Qin, W., Cao, L., and Massey, I.Y. (2021). Role of PI3K/Akt signaling pathway in cardiac fibrosis.  
596 *Mol Cell Biochem* 476(11), 4045-4059. doi: 10.1007/s11010-021-04219-w.
- 597 Quattrocelli M., Swinnen M., Giacomazzi G., Camps J., Barthélemy I., Ceccarelli G., et al. (2015)  
598 Mesodermal iPSC-derived progenitor cells functionally regenerate cardiac and skeletal  
599 muscle. *J Clin Invest* 125(12): 4463-4482. doi: 10.1172/JCI82735.
- 600 Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome  
601 engineering using the CRISPR-Cas9 system. *Nat Protoc* 8(11), 2281-2308. doi:  
602 10.1038/nprot.2013.143.
- 603 Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., et al. (2019). g:Profiler: a  
604 web server for functional enrichment analysis and conversions of gene lists (2019 update).  
605 *Nucleic Acids Res* 47(W1), W191-W198. doi: 10.1093/nar/gkz369.
- 606 Ren, S., Pan, L., Yang, L., Niu, Z., Wang, L., Feng, H., et al. (2021). miR-29a-3p transferred by  
607 mesenchymal stem cells-derived extracellular vesicles protects against myocardial injury after  
608 severe acute pancreatitis. *Life Sci* 272, 119189. doi: 10.1016/j.lfs.2021.119189.
- 609 Richards, D.J., Li, Y., Kerr, C.M., Yao, J., Beeson, G.C., Coyle, R.C., et al. (2020). Human cardiac  
610 organoids for the modelling of myocardial infarction and drug cardiotoxicity. *Nat Biomed*  
611 *Eng* 4(4), 446-462. doi: 10.1038/s41551-020-0539-4.
- 612 Rotini A, Martínez-Sarrà E, Pozzo E, Sampaolesi M. (2018). Interactions between microRNAs and  
613 long non-coding RNAs in cardiac development and repair. *Pharmacol Res* 127: 58-66. doi:  
614 10.1016/j.phrs.2017.05.029.

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- 615 Santoni de Sio FR, Gritti A, Cascio P, Neri M, Sampaolesi M, Galli C, et al. (2008). Lentiviral vector  
616 gene transfer is limited by the proteasome at postentry steps in various types of stem cells.  
617 *Stem Cells* 26(8): 2142-2152. doi: 10.1634/stemcells.2007-0705.
- 618 Scalise, M., Marino, F., Salerno, L., Cianflone, E., Molinaro, C., Salerno, N., et al. (2021). From  
619 Spheroids to Organoids: The Next Generation of Model Systems of Human Cardiac  
620 Regeneration in a Dish. *Int J Mol Sci* 22(24). doi: 10.3390/ijms222413180.
- 621 Shi, H., Li, H., Zhang, F., Xue, H., Zhang, Y., and Han, Q. (2021). MiR-26a-5p alleviates cardiac  
622 hypertrophy and dysfunction via targeting ADAM17. *Cell Biol Int* 45(11), 2357-2367. doi:  
623 10.1002/cbin.11685.
- 624 Takimoto, E. (2012). Cyclic GMP-dependent signaling in cardiac myocytes. *Circ J* 76(8), 1819-  
625 1825. doi: 10.1253/circj.cj-12-0664.
- 626 Tang, C.M., Zhang, M., Huang, L., Hu, Z.Q., Zhu, J.N., Xiao, Z., et al. (2017). CircRNA\_000203  
627 enhances the expression of fibrosis-associated genes by derepressing targets of miR-26b-5p,  
628 Col1a2 and CTGF, in cardiac fibroblasts. *Sci Rep* 7, 40342. doi: 10.1038/srep40342.
- 629 Tang, L., Xie, J., Yu, X., and Zheng, Y. (2020). MiR-26a-5p inhibits GSK3beta expression and  
630 promotes cardiac hypertrophy in vitro. *PeerJ* 8, e10371. doi: 10.7717/peerj.10371.
- 631 Velasco, V., Shariati, S.A., and Esfandyarpour, R. (2020). Microtechnology-based methods for  
632 organoid models. *Microsyst Nanoeng* 6, 76. doi: 10.1038/s41378-020-00185-3.
- 633 Wang, B., Zhang, A., Wang, H., Klein, J.D., Tan, L., Wang, Z.M., et al. (2019). miR-26a Limits  
634 Muscle Wasting and Cardiac Fibrosis through Exosome-Mediated microRNA Transfer in  
635 Chronic Kidney Disease. *Theranostics* 9(7), 1864-1877. doi: 10.7150/thno.29579.
- 636 Xie, Y., Hu, J., Zhang, X., Li, C., Zuo, Y., Xie, S., et al. (2020). Neuropeptide Y Induces  
637 Cardiomyocyte Hypertrophy via Attenuating miR-29a-3p in Neonatal Rat Cardiomyocytes.  
638 *Protein Pept Lett* 27(9), 878-887. doi: 10.2174/0929866527666200416144459.
- 639 Yang, Q., Wu, F., Mi, Y., Wang, F., Cai, K., Yang, X., et al. (2020). Aberrant expression of miR-  
640 29b-3p influences heart development and cardiomyocyte proliferation by targeting NOTCH2.  
641 *Cell Prolif* 53(3), e12764. doi: 10.1111/cpr.12764.
- 642 Yiu, E.M., and Kornberg, A.J. (2015). Duchenne muscular dystrophy. *J Paediatr Child Health* 51(8),  
643 759-764. doi: 10.1111/jpc.12868.
- 644 Zhao, D., Lei, W., and Hu, S. (2021). Cardiac organoid - a promising perspective of preclinical  
645 model. *Stem Cell Res Ther* 12(1), 272. doi: 10.1186/s13287-021-02340-7.
- 646 Zhu, P., Li, H., Zhang, A., Li, Z., Zhang, Y., Ren, M., et al. (2021). MicroRNAs sequencing of  
647 plasma exosomes derived from patients with atrial fibrillation: miR-124-3p promotes cardiac  
648 fibroblast activation and proliferation by regulating AXIN1. *J Physiol Biochem*. doi:  
649 10.1007/s13105-021-00842-9.

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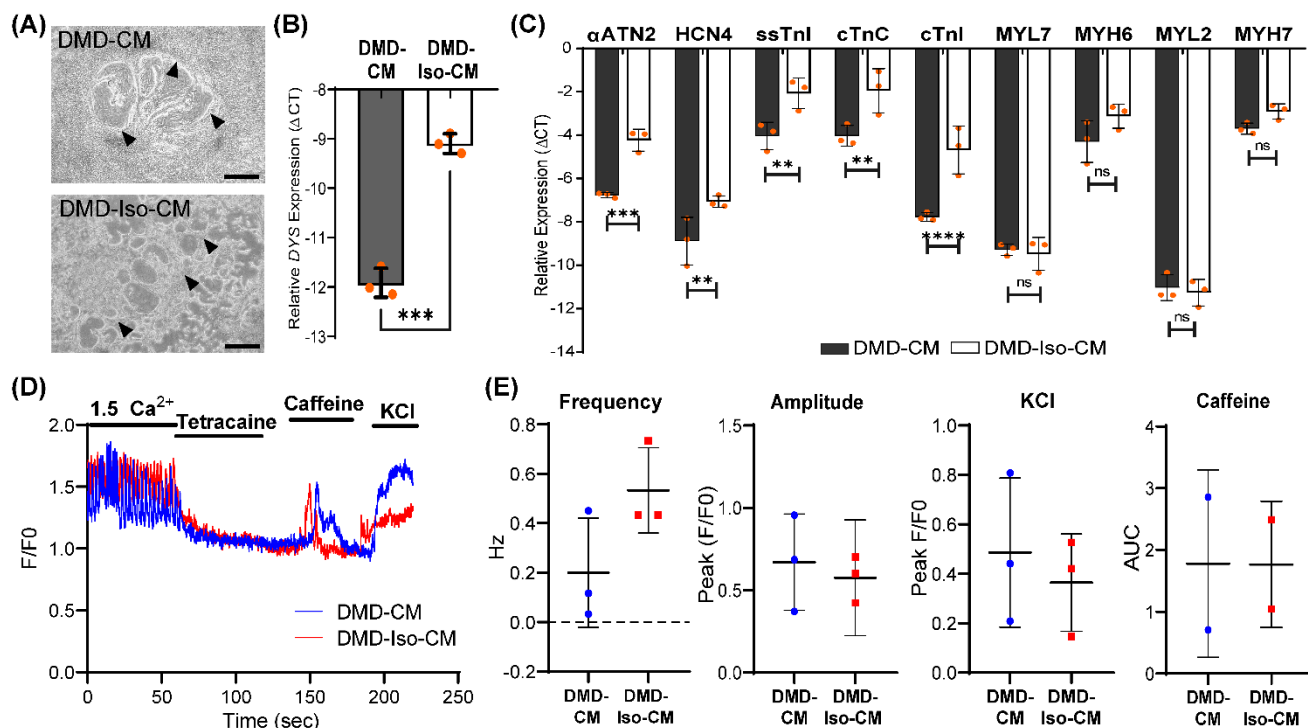
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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

### 654 FIGURES AND FIGURE LEGENDS

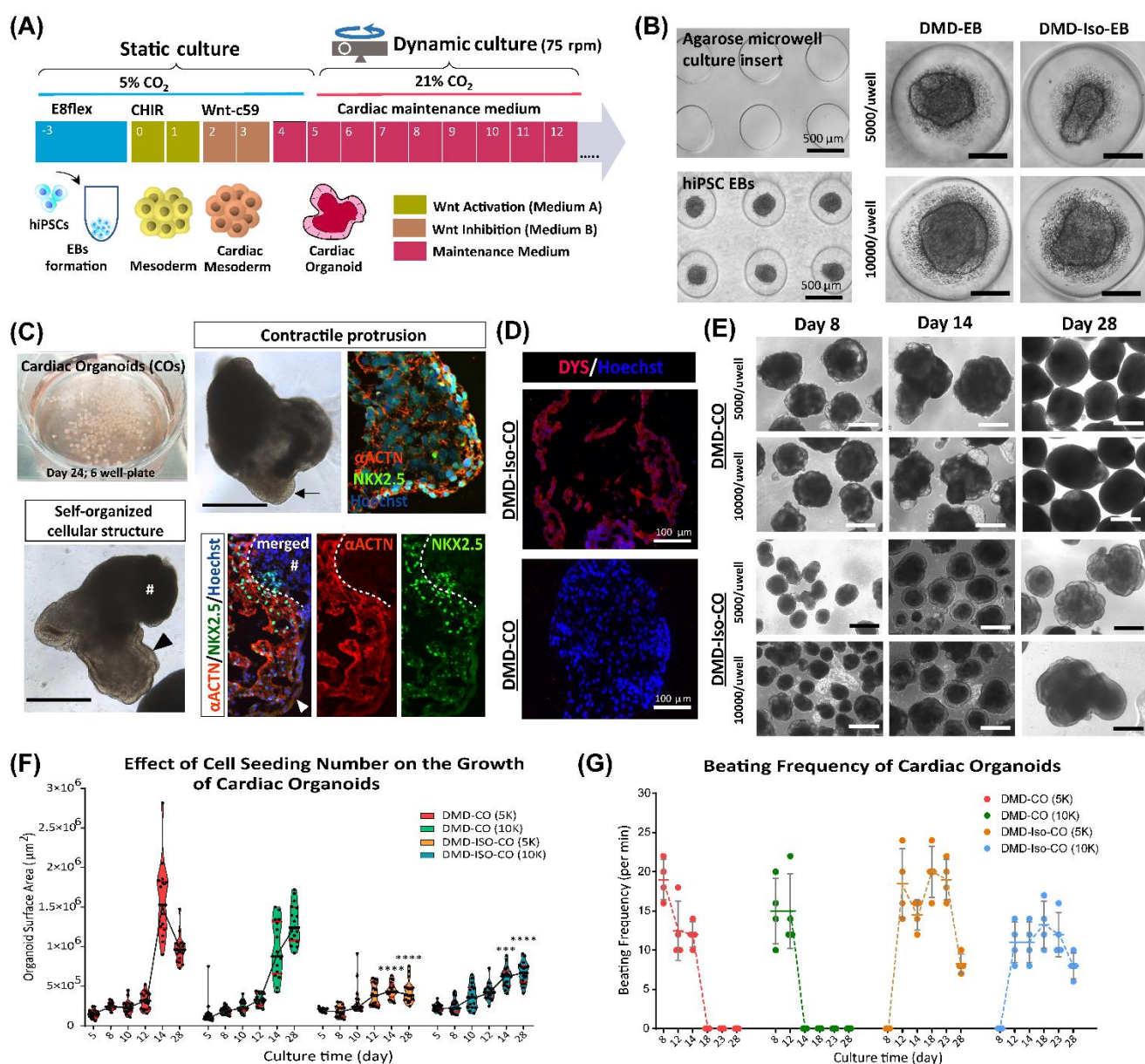


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656 **Figure 1:** (A) Representative 2D culture morphology of differentiated cardiomyocytes from DMD patient-  
 657 derived hiPSCs (DMD-CM) and the isogenic controls (DMD-Iso-CM) on day 22. Arrowheads indicated the  
 658 contractile filaments. Scale bar = 200  $\mu\text{m}$ . (B & C) Comparison of the expression of *dystrophin* (*DYS*) and key  
 659 cardiac gene markers between DMD-CM and DMD-Iso-CM 2D cultures on day 22. Data shown are mean  $\pm$   
 660 s.d. (n = 3). Unpaired student t-test (two-tailed): \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, n.s = not significant.  
 661 (D & E) Intracellular  $\text{Ca}^{2+}$  imaging of DMD-CM and DMD-Iso-CM 2D cultures on day 14 showing higher  
 662 frequency (not the amplitude) of spontaneous  $\text{Ca}^{2+}$  oscillation in DMD-CM than the isogenic controls, and  
 663 comparable KCl and Caffeine responses in both conditions. tetracaine was used to validate that  $\text{Ca}^{2+}$  oscillations  
 664 were driven by RyR channels.  
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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression



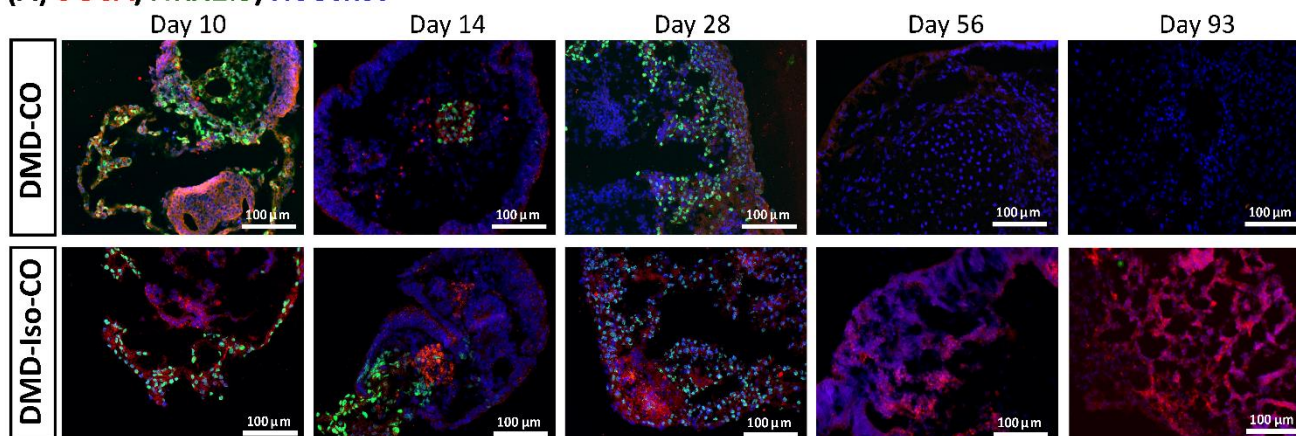
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667 **Figure 2: Generation of DMD-CO and DMD-Iso-CO from patient-derived hiPSC.** (A) Schematic showing  
 668 the culture protocol to generate DMD-CO and DMD-Iso-CO from patient-derived hiPSC embryoid bodies  
 669 (EBs). (B) Brightfield images of the microwells of the agarose insert and the formed EBs. (C) EBs pooled from  
 670 two agarose inserts inside a well of 6-well plate for dynamic culture, and the morphology of COs at high  
 671 magnification showing contractile cardiomyocyte protrusion (arrow) and distinct self-organized cellular  
 672 structure at the organoid periphery (arrow head) both with specific spatial distribution of NKX2.5 and αACTN  
 673 positivity. Non-translucent organoid structure (#) was negative for NKX2.5 and αACTN. (D)  
 674 Immunofluorescence staining showing the expression of DYS in DMD-Iso-CO, which was undetectable in  
 675 DMD-CO. Nuclei were counterstained with Hoechst. (E) Representative images of CO morphology and the  
 676 changes of organoid size (generated at two cell seeding numbers) over 28 days of dynamic culture. (F & G)  
 677 Effect of cell seeding numbers on the growth (n = 17) and the beating frequency (n = 4) of DMD-CO or DMD-  
 678 Iso-CO over 28 days. DMD-CO versus DMD-Iso-CO at 5K (5000 cells/microwell) or 10K (10,000  
 679 cells/microwell); unpaired student t-test (two-tailed): \*\*\*p<0.001, \*\*\*\*p<0.0001. CO = Cardiac organoid;  
 680 DMD-CO and DMD-Iso-CO = cardiac organoids from DMD patient-derived hiPSC and isogenic corrected  
 681 hiPSC, respectively. Scale bar = 1 mm or as stated in the figure.

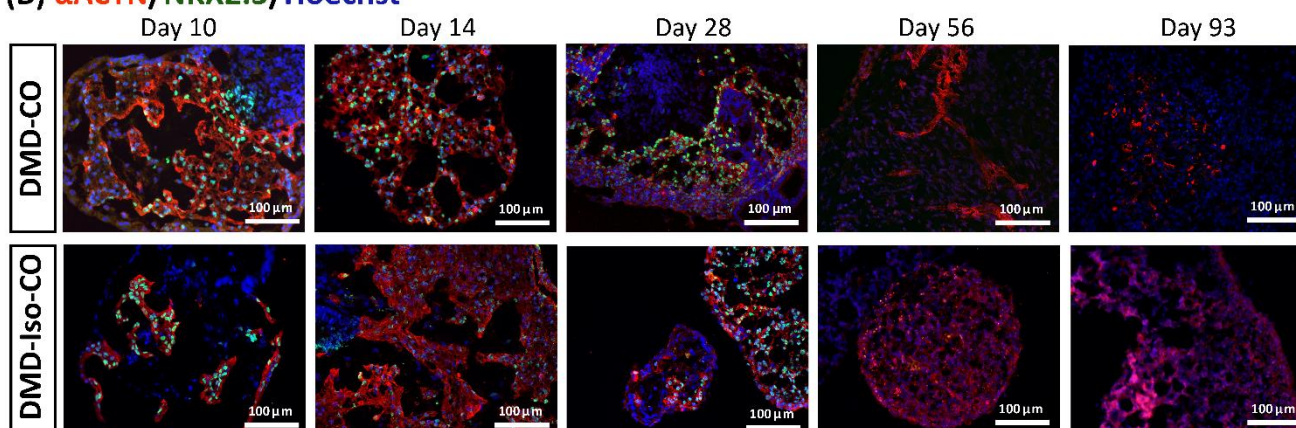
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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

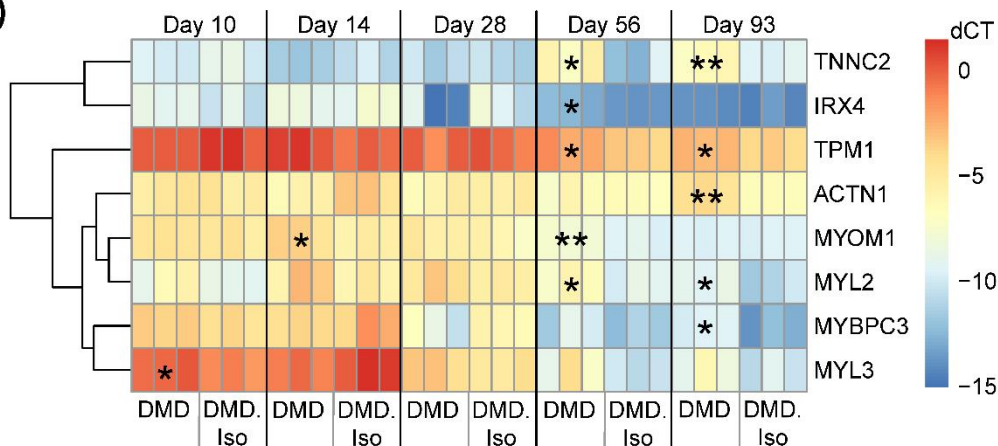
### (A) *SGCA*/*NKX2.5*/Hoechst



### (B) $\alpha$ ACTN/*NKX2.5*/Hoechst



### (C)



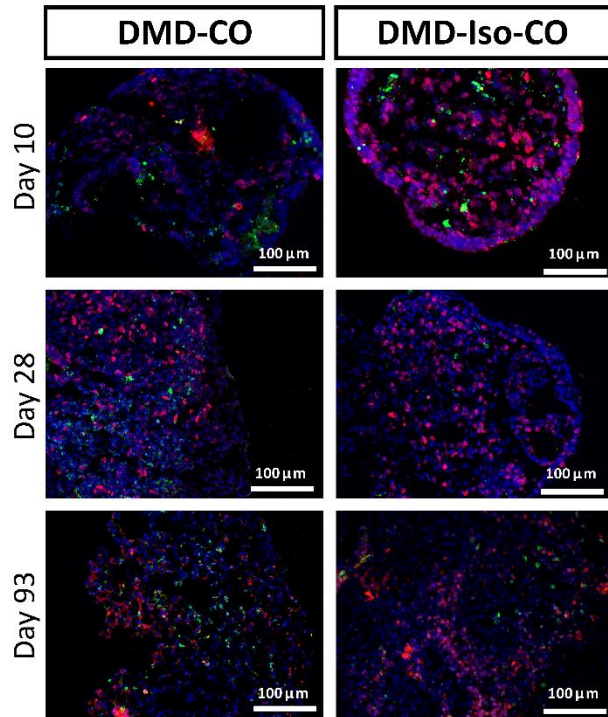
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684 **Figure 3: Assessment of cardiac differentiation and contractile proteins development in DMD-CO and**  
 685 **DMD-Iso-CO over 93 days of dynamic culture.** Representative immunofluorescence images for: (A)  
 686  $\alpha$ -sarcoglycan (*SGCA*)/*NKX2.5*, and (B) sarcomeric  $\alpha$ -actinin ( $\alpha$ ACTN)/*NKX2.5* co-staining in DMD-CO and  
 687 DMD-Iso-CO, respectively. Nuclei were counterstained with Hoechst. (C) RT-qPCR analysis of representative  
 688 gene markers expression for cardiac contractility in DMD-CO and DMD-Iso-CO. Data shown are mean  $\pm$  s.d.  
 689 (n = 3, each pooled from ~10 organoids). Unpaired student t-test (two-tailed): \*p<0.05, \*\*p<0.01.

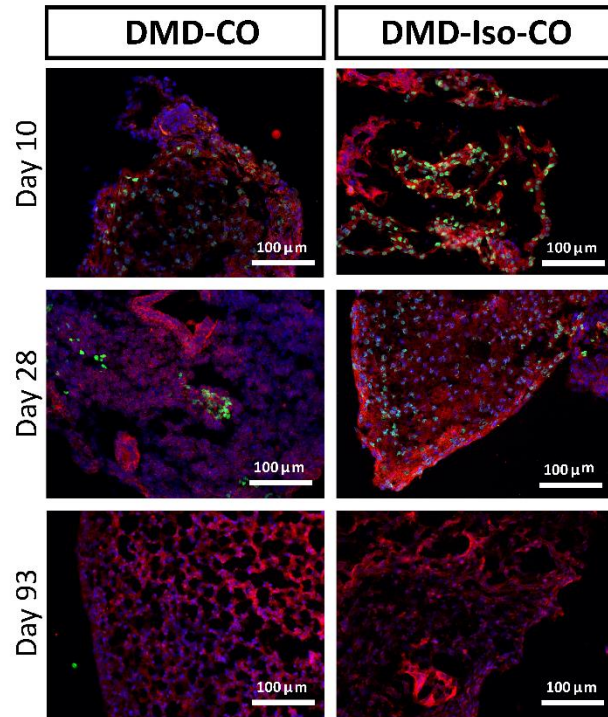
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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

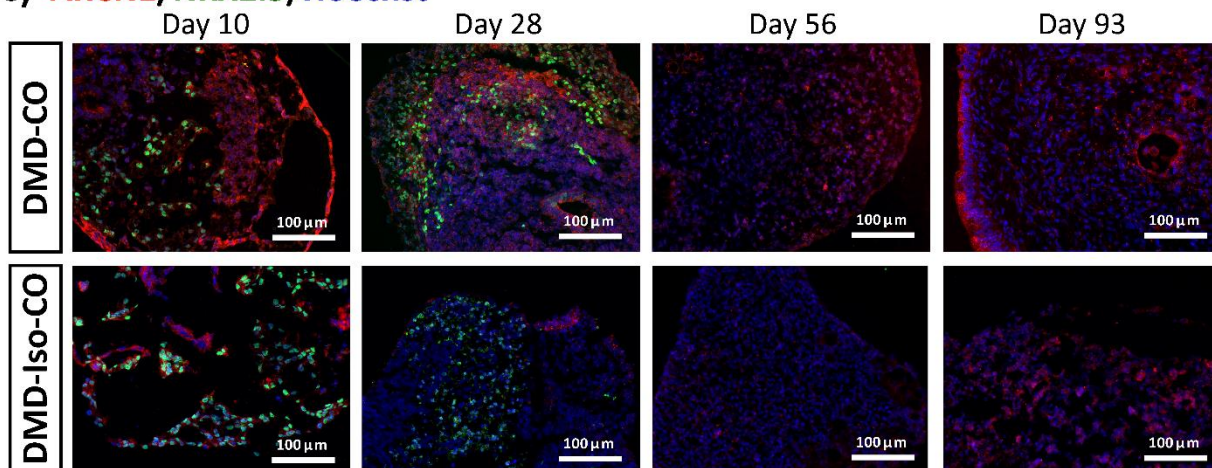
### (A) Ki67/CCASP3/Hoechst



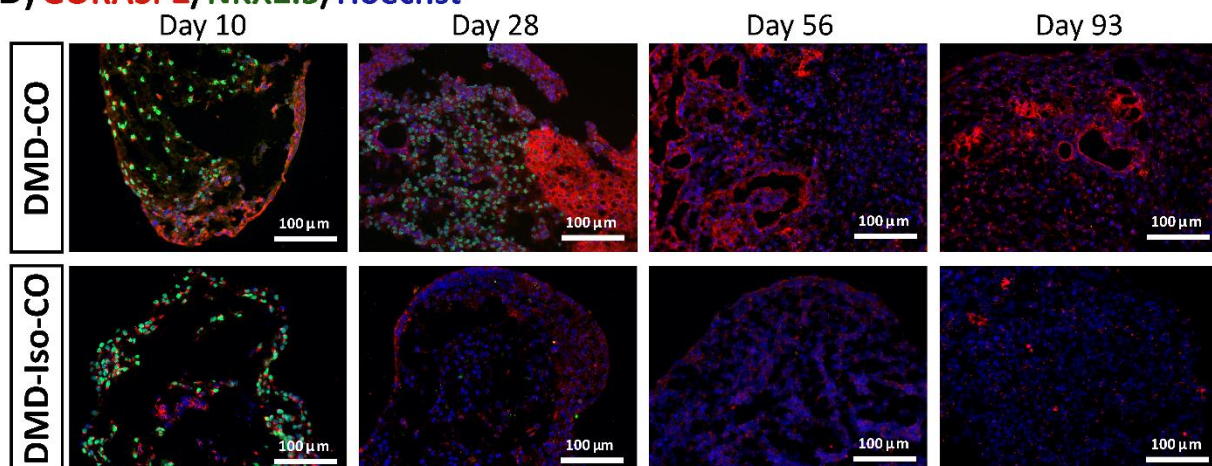
### (B) PGK1/NKX2.5/Hoechst



### (C) ARCN1/NKX2.5/Hoechst



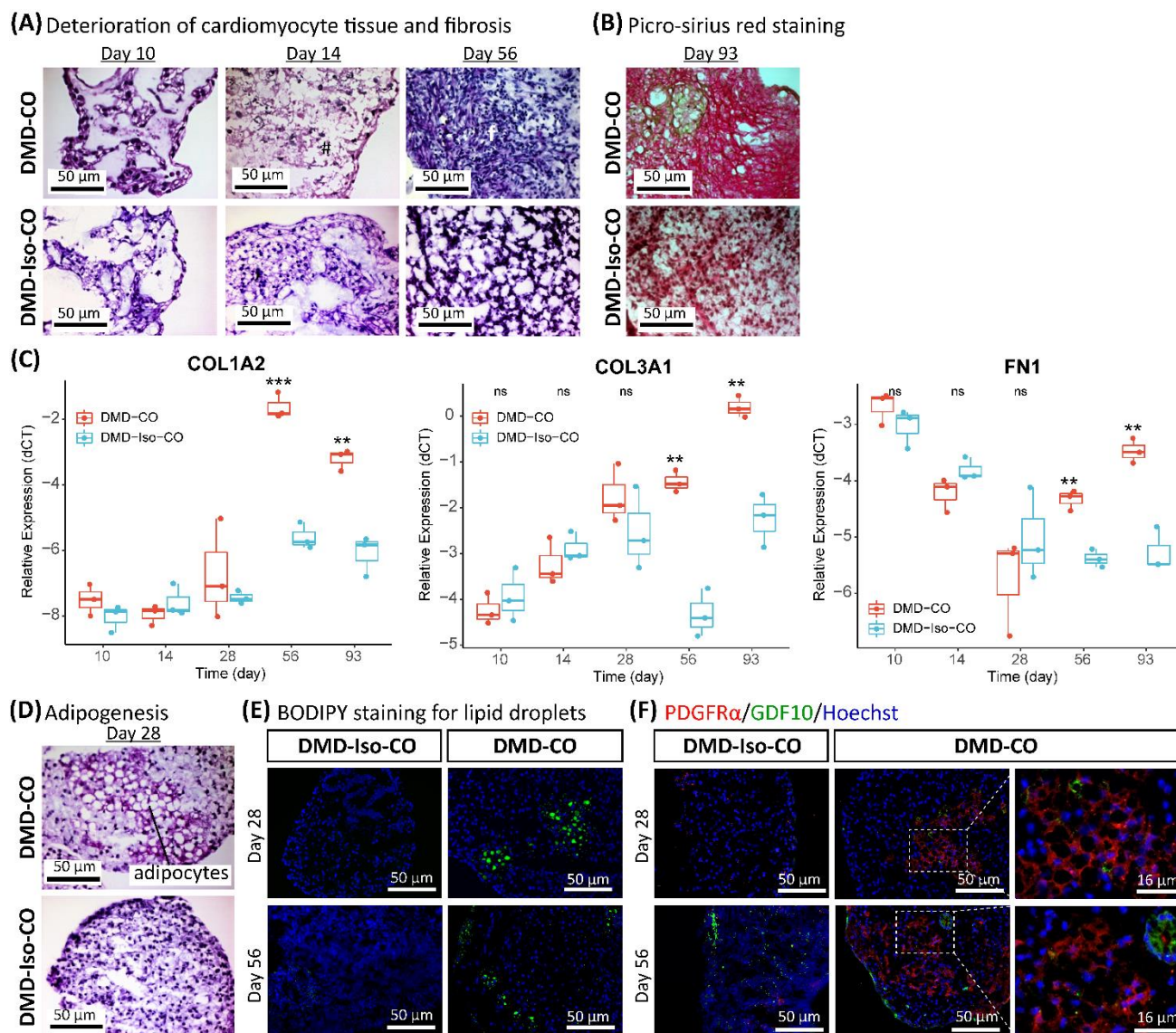
### (D) GORASP2/NKX2.5/Hoechst



## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

692 **Figure 4: Assessment of cell proliferation, apoptosis and ER stress in DMD-CO and DMD-Iso-CO over**  
 693 **93 days of dynamic culture.** Representative immunofluorescence images for: (A) Ki67/CCASP3, (B)  
 694 PGK1/NKX2.5, (C) ARCN1/NKX2.5 and (D) GORASP2/NKX2.5 on day 10, 14 28, 56 and 93. Nuclei were  
 695 counterstained with Hoechst.

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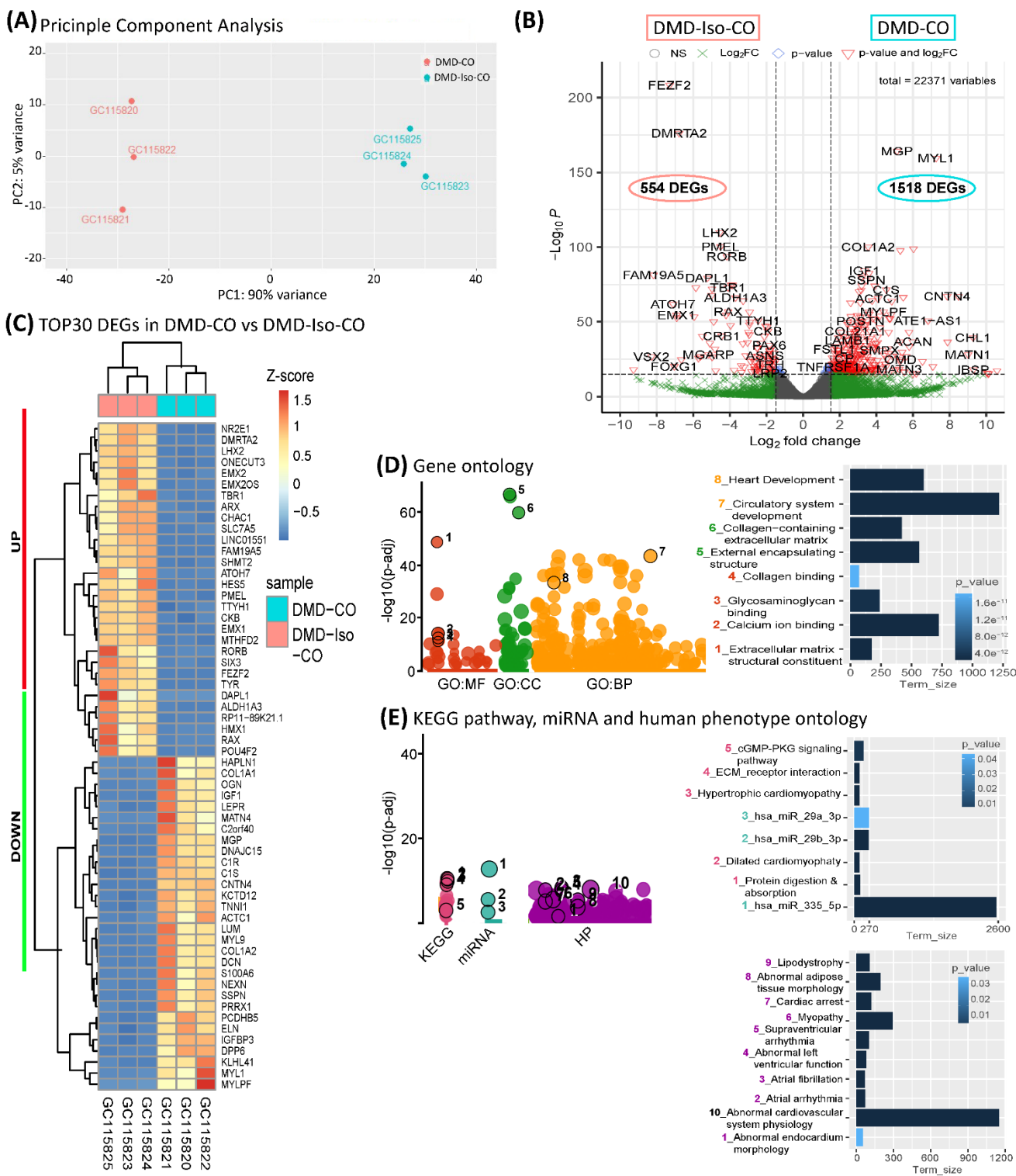


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698 **Figure 5: Assessment of fibrosis and adipogenesis in DMD-CO and DMD-Iso-CO over 93 days of dynamic**  
 699 **culture.** (A) H&E staining showing deterioration of cardiomyocyte tissue on day 14 and fibrosis on day 56 in  
 700 DMD-CO. (B) Picro-Sirius red staining showing abundant collagen deposition in DMD-CO on day 93. (C) RT-  
 701 qPCR analysis of representative fibrosis gene markers showing a significant upregulation of *COL1A2*, *COL3A1*  
 702 and *FNI* expression in DMD-CO on day 56 and 93 as compared to DMD-Iso-CO. Data shown are mean  $\pm$  s.d.  
 703 (n = 3, each pooled from ~10 organoids). Unpaired student t-test (two-tailed): \*\*p<0.01, \*\*\*p<0.001. (D, E, F)  
 704 Adipogenesis in DMD-CO as indicated by the formation of adipocytes with cytoplasmic vacuoles (H&E  
 705 staining), lipid droplet deposition (BODIPY staining), and PDGFR $\alpha$  positivity on day 28 and 56. The  
 706 adipogenesis inhibitor GDF10 was also detected near the PDGFR $\alpha$ <sup>+</sup> cells in DMD-CO. Nuclei were  
 707 counterstained with Hoechst.

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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression



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710 **Figure 6: RNA sequencing analysis of DMD-CO and DMD-Iso-CO via DESeq2 method.** (A) Principal  
 711 component analysis (PCA) showing distinct separation of the DMD-CO and DMD-Iso-CO clusters (PC1: 90%)  
 712 with low intra-condition variance (PC2: 5%) in both conditions, respectively. (B) Enhanced volcano plot  
 713 showing the differentially expressed genes (DEGs) in DMD-CO versus DMD-Iso-CO. Cut-off  $\log_2$  fold change  
 714 = 1.5; Cut-off  $-\log_{10}P = 10^{-16}$ . (C) Heatmap showing the TOP30 DEGs in DMD-CO versus DMD-Iso-CO. (D,  
 715 E) Functional enrichment analysis of the differentially upregulated genes in DMD-CO versus DMD-Iso-CO

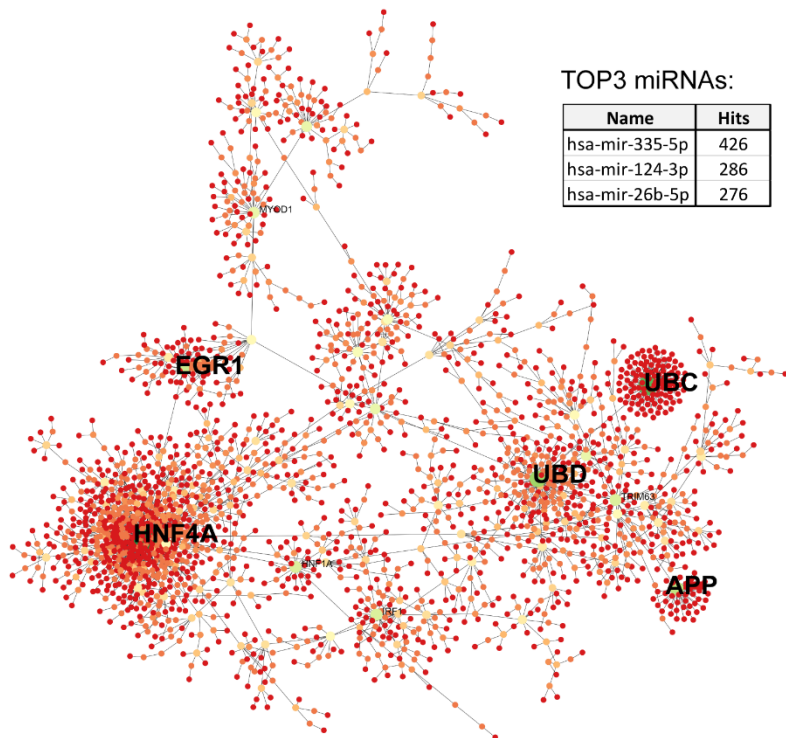
## **Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression**

716 using gProfiler2 for Gene ontology, KEGG pathway, miRNA and human phenotype ontology. (All data shown:  
717 n = 3, each sample was a pooled of ~ 10 organoids).

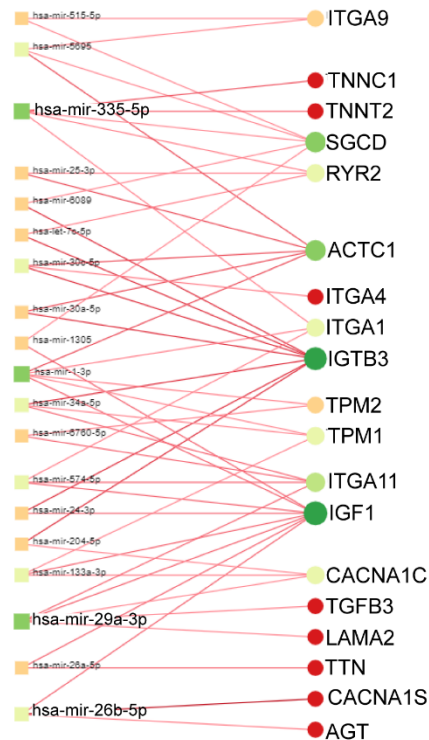
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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

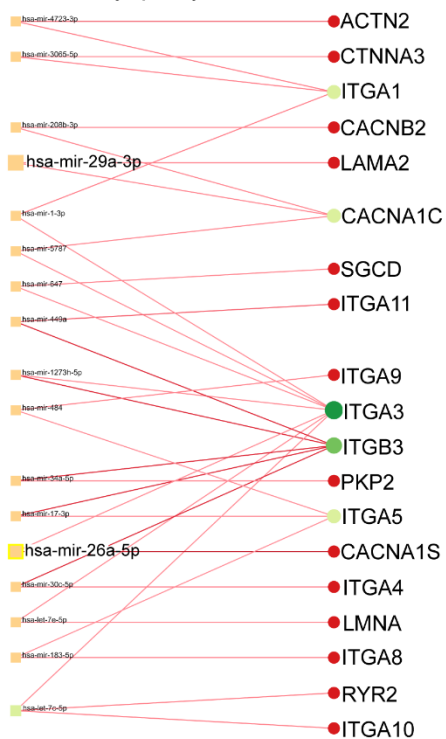
### (A) Generic protein-protein interaction network



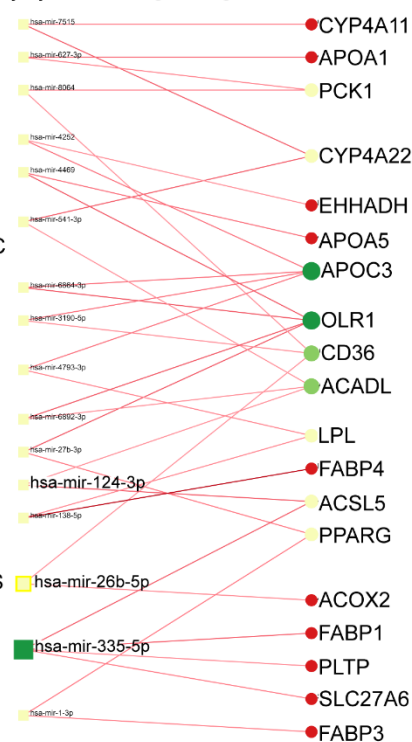
### (B) Hypertrophy/Dilated Cardiomyopathy



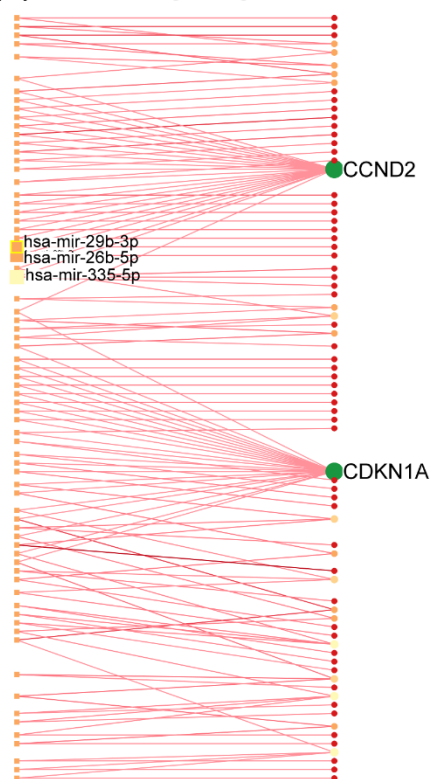
### (C) Arrhythmogenic right ventricular cardiomyopathy



### (D) PPAR Signaling



### (E) PI3K-Akt Signaling



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720 **Figure 7: Protein-protein interaction network analysis based on differentially upregulated genes in DMD-**  
 721 **CO using Network Analyst platform. (A) Top five main hub genes (HNF4A, UBC, UBD, APP and EGR1)**  
 722 **were identified based on the degree levels. (B–D) The gene-miRNA networks for hypertrophy/dilated**

## **Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression**

723 cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, PPAR and PI3K-Akt signalling pathways,  
724 respectively. The identified top three miRNAs by PPI and top two miRNAs by gProfiler2 analysis were mapped  
725 in each gene-miRNA network to indicate the genes they interact with.

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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

### 751 Tables

752 **Table 1:** List of antibodies dilutions used for immunofluorescence analysis.

Antibodies	Dilution
$\alpha$ -Actinin (mouse, Abcam)	1:250
$\alpha$ -Sarcoglycan (mouse, Novacastra)	1:250
NKX2.5 (Rabbit, Bioke)	1:250
PGK1 (mouse, Santa Cruz)	1:250
GORASP2 (mouse, Proteintech)	1:1000
ARCNI (mouse, Santa Cruz)	1:250
CCASP3 (Rabbit, Bioke/ Cell Signaling Technology)	1:400
Ki67 (mouse, BD Pharmigen)	1:300
DYS1 (mouse, Leica)	1:50
DYS3 (mouse, Leica)	1:50

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754 **Table 2:** List of primer used for gene expression analysis.

Gene	Primer sequence	Gene	Primer sequence
<i>SsTnl</i>	FW: CCCAGCTCCACGAGGACTGAACA RV: TTTGCCGGAGGCAGTGATCTTGG	MYL3	FW: TCACACCTGAGCAGATTGAAGA RV: GCTGGAGCATAGGCAGGAAAAG
<i>CTnc</i>	FW: TGCTGCAGGCTACAGGCGAG RV: TCGATGCGGCCGTCGTTGTT	MYL2	FW: TTGGGCGAGTGAACGTGAAAA RV: CCGAACGTAATCAGCCTTCAG
<i>CTnl</i>	FW: GGAACCTCGCCTGCACCAG RV: GCGCGGTAGTTGGAGGAGCG	TPM1	FW: TTGAGAGTCGAGCCAAAAAAG RV: CATATTTGCGGTCGGCATCTT
<i><math>\alpha</math>ACTN2</i>	FW: CTCAAAGCTTAACAAGGATGACC RV: GTGGTAGAAGCAAGAGACGTA	MYOM1	FW: GAGTCGATATGGGATGCACAC RV: TCCTTTAACATTCATCGCCGAG
MYL2	FW: TTGGGCGAGTGAACGTGAAAA RV: CCGAACGTAATCAGCCTTCAG	MYBPC3	FW: AGCGGGTGGAGTTTGGAGT RV: GCGATGCTCTGGTACACCTC
MYH7	FW: ACTGCCGAGACCGAGTATG RV: GCGATCCTTGAGGTTGTAGAGC	TNNC2	FW: TGATGGTGCGCCAGATGAAAAG RV: TGCATTCTGTGCGAAGATGCG
MYH6	FW: GCCCTTTGACATTCGCACTG RV: CGGGACAAAATCTTGGCTTTGA	IRX4	FW: GGCTCCCAGTCTTGTATGG RV: TAGACCGGGCAGTAGACCC
MYL7	FW: ACATCATCCCCACGGAGAAGAGA RV: ATTGGAACATGGCCTCTGGATGGA	MPK11	FW: CTGAACAACATCGTCAAGTGCC RV: CATAGCCGGTCATCTCCTCG
<i>HCN4</i>	FW: GAACAGGAGAGGGTCAAGTCG RV: CATTGAAGACAATCCAGGGTGT	FN1	FW: CGGTGGCTGTCAAGTCAAAG RV: AAACCTCGGCTTCCTCCATAA
CASP3	FW: GAAATTGTGGAATTGATGCGTGA RV: CTACAACGATCCCCTCTGAAAAA	COL3A1	FW: TTGAAGGAGGATGTTCCCATCT RV: ACAGACACATATTTGGCATGGTT
CASP8	FW: GGAGTTGTGTGGGGTAATGAC RV: TTCCTGTCCCTAATGCTGTGA	COL1A2	FW: GGCCCTCAAGGTTTCCAAGG RV: CACCCTGTGGTCCAACAACCTC
CASP9	FW: CTCAGACCAGAGATTCGCAAAC RV: GCATTTCCCCTCAAACCTCTCAA	HMGR	FW: TGATTGACCTTTCCAGAGCAAAG RV: CTAAAATTGCCATTCACGAGC
XIAP	FW: AATAGTGCCACGCAGTCTACA RV: CAGATGGCTGTCTAAGGCAA	F2R	FW: CCACCTTAGATCCCCTGTCAT RV: GTGGGAGGCTGACTACAAACA
FOXO3	FW: CGGACAAACGGCTCACTCT RV: GGACCCGATGAATCGACTAT	CALM1	FW: TTGACTTCCCCGAATTTTGTACT RV: GGAATGCCCTACGGATTCTT
P63/TRP63	FW: GGACCAGCAGATTCAGAACGG RV: AGGACACGTCGAAACTGTGC	NPPA	FW: CAACGCAGACCTGATGGATTT RV: AGCCCCGCTTCTTCATTC
MAPK14/P38	FW: TCAGTCCATCATTATGCGAAA RV: AACGTCCACAGACCAATCAC	HDAC2	FW: GAGCTGTGAAGTTAAACCGACA RV: ACCGTCAATACAGATCTGTGTG
MAPK8/JNK	FW: TGTGTGGAATCAAGCACCTTC RV: AGGCGTCATCATAAAAACCTGTTT	GAPDH	FW: TCAAGAAGGTGGTGAAGCAGG RV: ACCAGGAAATGAGCTTGACAAA
DIABLO	FW: CGCGCAGCGTAACTTCATTC RV: CCAAAGCCAATCGTCACAGTTTT	Dp427m	FW: ATGCTTTGGTGGGAAGAAGT RV: GGGCATGAACTCTTGTGGAT
ACTNI	FW: CCACCCTCTCGGAGATCAAG RV: TCCCTTCGCTTCTGAGTTAGG		

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### Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

757 **Table 3:** Top30 differentially upregulated and down-regulated genes in DMD-CO versus DMD-Iso-  
758 CO.

	ID	Gene Name	baseMean	log2FoldChange	lfcSE	stat	pvalue	padj
Differentially Upregulated Genes	13088	MGP	3880.521907	5.120819425	0.187135	27.36434	7.29E-165	5.44E-161
	13862	MYL1	2675.966929	7.262145462	0.269213	26.9755	2.87E-160	1.60E-156
	4878	COL1A2	14733.15743	3.549526614	0.166558	21.31101	8.97E-101	2.87E-97
	9557	HAPLN1	1152.713973	6.021041431	0.284317	21.17721	1.55E-99	4.33E-96
	14864	OGN	1806.551067	5.301108486	0.251858	21.04799	2.39E-98	5.93E-95
	10461	IGF1	2063.104984	3.348551855	0.171565	19.51771	7.76E-85	1.58E-81
	3270	C1R	836.7627027	3.594911367	0.186023	19.32509	3.30E-83	6.16E-80
	6414	DCN	6535.506649	3.199249115	0.168451	18.99221	1.98E-80	3.16E-77
	26211	SSPN	1223.773057	3.454929985	0.184017	18.77501	1.21E-78	1.69E-75
	10472	IGFBP3	2242.532837	3.187765804	0.174276	18.29151	9.67E-75	1.14E-71
	12548	LUM	1859.659818	3.902455102	0.213459	18.28201	1.15E-74	1.29E-71
	3273	C1S	505.5575661	4.532842321	0.25232	17.96466	3.69E-72	3.44E-69
	16517	PRRX1	2296.492692	3.165414194	0.176446	17.93981	5.77E-72	5.16E-69
	13878	MYL9	2014.301562	3.047364639	0.171162	17.80398	6.58E-71	5.66E-68
	4832	CNTN4	438.9813428	7.862840625	0.451874	17.40051	8.18E-68	6.53E-65
	6893	DPP6	575.4551543	8.475259781	0.487454	17.3868	1.04E-67	8.01E-65
	12815	MATN4	464.8395192	5.461265657	0.315708	17.29848	4.83E-67	3.60E-64
	1178	ACTC1	2989.236594	4.07554558	0.236959	17.19934	2.69E-66	1.88E-63
	4877	COL1A1	17931.90071	3.760557058	0.221112	17.00747	7.23E-65	4.90E-62
	15297	PCDH5	332.9803767	4.835046959	0.285038	16.96279	1.55E-64	9.90E-62
	24364	S100A6	1991.282073	3.116793963	0.185219	16.82763	1.53E-63	9.51E-61
	11066	KCTD12	2475.653645	2.587393647	0.153899	16.81233	1.98E-63	1.20E-60
	13886	MYLPF	895.0795595	4.39539839	0.275249	15.96882	2.11E-57	1.18E-54
	27539	TNNI1	1506.276513	3.591374007	0.225007	15.96117	2.38E-57	1.30E-54
	11676	LEPR	1025.641125	3.609301571	0.229042	15.75825	6.03E-56	3.14E-53
	6784	DNAJC15	250.7545201	4.796254742	0.305622	15.69341	1.68E-55	8.53E-53
	3362	K2orf40	1709.870968	2.953046306	0.189313	15.59879	7.42E-55	3.53E-52
	11319	KLHL41	358.1653311	4.180634798	0.268513	15.56955	1.17E-54	5.46E-52
	7375	ELN	3805.846419	2.592306878	0.16688	15.534	2.04E-54	9.32E-52
	14268	NEXN	1403.167628	3.120924866	0.201871	15.45996	6.46E-54	2.84E-51
Differentially Down-regulated Genes	8257	FEZF2	2147.508792	-7.297943854	0.236509	-30.857	4.52E-209	1.01E-204
	6732	DMRTA2	1410.513095	-6.781574008	0.239105	-28.3624	5.89E-177	6.59E-173
	11722	LHX2	3925.790741	-4.554419135	0.20393	-22.3332	1.76E-110	7.87E-107
	15965	PMEL	1206.380481	-4.518113856	0.211711	-21.341	4.73E-101	1.76E-97
	18101	RORB	4525.902649	-4.172628967	0.20232	-20.6239	1.67E-94	3.74E-91
	7899	FAM19A5	746.1460517	-8.183408649	0.426594	-19.1831	5.12E-82	8.81E-79
	6352	DAPL1	1233.729736	-5.235519232	0.276173	-18.9574	3.84E-80	5.72E-77
	7421	EMX2	2184.20914	-3.94754281	0.214817	-18.3763	2.03E-75	2.68E-72
	7422	EMX2OS	843.8705185	-3.862243505	0.210282	-18.367	2.41E-75	3.00E-72
	26756	TBR1	1184.062948	-4.109792654	0.226273	-18.163	1.01E-73	1.08E-70
	9992	HMX1	602.267519	-5.86682695	0.323561	-18.132	1.78E-73	1.81E-70
	28131	TYR	391.1820556	-4.982659101	0.275952	-18.0563	7.04E-73	6.85E-70
	2364	ARX	634.2942288	-5.00821928	0.286738	-17.4662	2.59E-68	2.15E-65
	1658	ALDH1A3	643.2412907	-3.696045432	0.213864	-17.2822	6.40E-67	4.62E-64
	25017	SIX3	1428.324473	-3.282550679	0.193132	-16.9964	8.73E-65	5.74E-62
	2492	ATOH7	469.676258	-7.115070692	0.426177	-16.6951	1.42E-62	8.37E-60
	17083	RAX	577.8533812	-4.099396951	0.256498	-15.9822	1.70E-57	9.75E-55
	9702	HES5	1171.343235	-2.936085758	0.184229	-15.9371	3.50E-57	1.86E-54
	7420	EMX1	323.5042159	-6.926075308	0.44215	-15.6645	2.64E-55	1.31E-52
	22627	RP11-89K21.1	382.2280006	-4.214816465	0.269141	-15.6603	2.83E-55	1.37E-52
	14893	ONECUT3	285.5907893	-5.959062474	0.384535	-15.4968	3.65E-54	1.63E-51
	12209	LINC01551	307.298716	-6.914384951	0.44911	-15.3957	1.75E-53	7.52E-51
	28033	TTYH1	2791.568121	-2.459452396	0.162906	-15.0974	1.68E-51	6.61E-49
	14611	NR2E1	319.4212395	-4.893422773	0.326486	-14.9882	8.77E-51	3.17E-48
	4464	CHAC1	995.4565501	-2.920428254	0.195376	-14.9477	1.61E-50	5.72E-48
	25400	SLC7A5	861.7666728	-2.941521316	0.200486	-14.6719	9.75E-49	3.16E-46
	16129	POU4F2	369.4507945	-3.970980557	0.271888	-14.6052	2.60E-48	8.20E-46
	13703	MTHFD2	1961.02617	-2.063767517	0.141852	-14.5487	5.95E-48	1.82E-45
	24961	SHMT2	3444.426553	-1.989370244	0.137842	-14.4322	3.25E-47	9.81E-45
	4621	CKB	15898.89106	-1.934043819	0.138295	-13.9849	1.93E-44	5.32E-42

## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

759 **Table 4:** List of identified TOP20 KEGG pathways based on the upregulated DEGs in DMD-CO  
 760 (Cut-off log<sub>2</sub>FC > 1.5, Cut-off p-value < 0.05)

Pathway	Total	Expected	Hits	P.Value	FDR
ECM-receptor interaction	82	8.64	37	8.86E-16	2.82E-13
Protein digestion and absorption	90	9.48	36	2.06E-13	3.27E-11
Complement and coagulation cascades	79	8.32	33	4.89E-13	4.27E-11
Focal adhesion	199	21	57	5.37E-13	4.27E-11
PI3K-Akt signaling pathway	354	37.3	75	1.29E-09	8.18E-08
Hypertrophic cardiomyopathy (HCM)	85	8.95	27	7.44E-08	3.94E-06
Dilated cardiomyopathy	91	9.59	27	3.56E-07	1.62E-05
Amoebiasis	96	10.1	27	1.15E-06	4.59E-05
Calcium signaling pathway	188	19.8	42	1.48E-06	5.23E-05
Renin secretion	69	7.27	20	1.72E-05	0.000546
Proteoglycans in cancer	201	21.2	41	2.11E-05	0.000611
Pathways in cancer	530	55.8	85	3.49E-05	0.000926
Regulation of actin cytoskeleton	214	22.5	42	4.39E-05	0.00107
PPAR signaling pathway	74	7.8	20	5.25E-05	0.00119
cGMP-PKG signaling pathway	166	17.5	34	9.70E-05	0.00206
Cytokine-cytokine receptor interaction	294	31	52	0.000106	0.0021
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	72	7.58	19	0.000114	0.00214
Retinol metabolism	67	7.06	18	0.000134	0.00237
Maturity onset diabetes of the young	26	2.74	10	0.000175	0.00293
Vascular smooth muscle contraction	132	13.9	28	0.000212	0.00336

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## Patient-Derived Cardiac Organoids to Model DMD Cardiomyopathy and Disease Progression

773 **Table 5:** List of genes identified for the selected KEGG pathways from Table 3, based on the  
774 upregulated DEGs in DMD-CO.

Hypertrophic cardiomyopathy or Dilated cardiomyopathy		Arrhythmogenic right ventricular cardiomyopathy (ARVC)		PPAR signaling pathway		PI3K-Akt signaling pathway				ECM-receptor interaction	
Gene	Log <sub>2</sub> FC	Gene	Log <sub>2</sub> FC	Gene	Log <sub>2</sub> FC	Gene	Log <sub>2</sub> FC	Gene	Log <sub>2</sub> FC	Gene	Log <sub>2</sub> FC
CACNA1S	7.5297	CACNA1S	7.5297	FABP4	8.1837	IBSP	9.2927	NOS3	2.1560	IBSP	9.2927
ITGB3	5.2163	ITGB3	5.2163	MMP1	5.0562	G6PC	5.8632	FGF10	2.1540	ITGB3	5.2163
ACTC1	4.0755	ACTN2	3.4819	OLR1	3.9245	GNG10	5.6788	FGF1	2.1206	COL1A1	3.7606
MYL2	4.0557	ITGA11	3.2052	ACSL5	3.6310	EFNA2	5.2888	ANGPT2	2.0804	COL1A2	3.5495
TNNC1	3.7984	ITGA8	2.5419	CYP4A22	3.6060	ITGB3	5.2163	PIK3CG	2.0143	COL6A5	3.4788
MYBPC3	3.6837	ITGA4	2.4919	FABP1	3.5066	EREG	4.1616	ITGA9	1.8600	COL4A4	3.3955
IGF1	3.3486	ITGA10	2.3803	APOA1	3.1635	FGF7	3.7761	TEK	1.8581	VTN	3.2546
ITGA11	3.2052	RYR2	2.3080	APOC3	3.0915	COL1A1	3.7606	IL4R	1.8530	ITGA11	3.2052
TNNT2	3.1617	ITGA1	2.1960	APOA5	3.0506	COL1A2	3.5495	NTF3	1.8446	COL6A3	2.8668
TTN	3.0126	ITGA9	1.8600	PPARG	2.7145	COL6A5	3.4788	OSMR	1.7646	LAMA4	2.8110
ITGA8	2.5419	LAMA2	1.7542	SLC27A6	2.3279	HGF	3.4238	PCK1	1.7554	THBS2	2.7463
ITGA4	2.4919	PKP2	1.7517	CYP4A11	2.1472	COL4A4	3.3955	LAMA2	1.7542	THBS1	2.7432
ITGA10	2.3803	SGCD	1.7042	FABP3	2.0050	IGF1	3.3486	COL6A6	1.7356	COL2A1	2.6382
AGT	2.3747	CACNA1C	1.6742	PCK1	1.7554	VTN	3.2546	THBS4	1.7279	ITGA8	2.5419
RYR2	2.3080	ITGA3	1.4188	LPL	1.7078	ITGA11	3.2052	COL4A1	1.7006	ITGA4	2.4919
TPM2	2.2657	CACNB2	1.4144	ACOX2	1.5419	FLT1	3.1137	AREG	1.6861	COL6A2	2.4325
ITGA1	2.1960	LMNA	1.4088	PLTP	1.1501	TLR2	3.0622	LAMA3	1.6558	LAMB1	2.4208
TGFB3	2.1664	ITGA5	1.0834	ACADL	1.1032	IGF2	2.8811	ERBB4	1.6241	COL9A1	2.4000
TPM1	1.9245	CTNNA3	1.0382	CD36	1.0910	COL6A3	2.8668	FGFR4	1.5331	COL4A3	2.3868
ITGA9	1.8600			EHHADH	1.0699	CHRM2	2.8540	FGF5	1.5194	ITGA10	2.3803
LAMA2	1.7542					LAMA4	2.8110	PDGFD	1.4253	FN1	2.3529
SGCD	1.7042					THBS2	2.7463	FGFR2	1.4223	COL9A3	2.3099
CACNA1C	1.6742					THBS1	2.7432	ITGA3	1.4188	ITGA1	2.1960
ITGA3	1.4188					PDGFRA	2.6431	PDGFRB	1.3871	ITGA9	1.8600
CACNB2	1.4144					COL2A1	2.6382	JAK3	1.3675	LAMA2	1.7542
LMNA	1.4088					IL6R	2.5559	PDGFB	1.3395	COL6A6	1.7356
ITGA5	1.0834					ITGA8	2.5419	CREB3L1	1.3243	THBS4	1.7279
						ITGA4	2.4919	TNC	1.3076	COL4A1	1.7006
						COL6A2	2.4325	PIK3AP1	1.2975	LAMA3	1.6558
						LAMB1	2.4208	PDGFA	1.2740	ITGA3	1.4188
						COL9A1	2.4000	VEGFC	1.2521	TNC	1.3076
						COL4A3	2.3868	GNB4	1.2110	CD44	1.2547
						ITGA10	2.3803	KITLG	1.1125	HSPG2	1.2435
						FN1	2.3529	COL4A2	1.1124	SV2B	1.2219
						COL9A3	2.3099	ITGA5	1.0834	COL4A2	1.1124
						FGF23	2.2844	CDKN1A	1.0739	CD36	1.0910
						ITGA1	2.1960	LPAR3	1.0707	ITGA5	1.0834
								CCND2	1.0585		