

1 **Generation and Maturation of Human iPSC-derived** 2 **Cardiac Organoids in Long Term Culture**

3 **Ece Ergir**^{1,2}, **Jorge Oliver-De La Cruz**¹, **Soraia Fernandes**¹, **Marco Cassani**¹, **Francesco Niro**^{1,3},
4 **Daniel Sousa**^{1,3}, **Jan Vrbský**¹, **Vladimír Vinarský**¹, **Ana Rubina Perestrelo**¹, **Doriana Debellis**⁴,
5 **Francesca Cavalieri**^{5,6}, **Stefania Pagliari**¹, **Heinz Redl**^{7,8}, **Peter Ertl**^{2,8}, and **Giancarlo Forte**^{1,9}

6 ¹ Center for Translational Medicine (CTM), St. Anne's University Hospital, International Clinical Research Centre
7 (FNUSA-ICRC), CZ-62500, Brno, Czech Republic

8 ² Faculty of Technical Chemistry, Institute of Applied Synthetic Chemistry & Institute of Chemical Technologies
9 and Analytics, Vienna University of Technology, AT-1040, Vienna, Austria

10 ³ Faculty of Medicine, Department of Biomedical Sciences, Masaryk University, CZ-62500, Brno, Czech Republic.

11 ⁴ Electron Microscopy Facility, Fondazione Istituto Italiano Di Tecnologia, Via Morego 30, IT-16163, Genova, Italy

12 ⁵ Department of Chemical Engineering, The University of Melbourne, Parkville, Victoria 3010, Australia;

13 ⁶ Dipartimento di Scienze e Tecnologie Chimiche, Università degli Studi di Roma Tor Vergata, via della Ricerca
14 Scientifica 1, 00133, Rome, Italy

15 ⁷ Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Center, AT-1200,
16 Vienna, Austria

17 ⁸ Austrian Cluster for Tissue Regeneration, AT-1200, Vienna, Austria

18 ⁹ Department of Biomaterials Science, Institute of Dentistry, University of Turku, FI-20014, Turku, Finland

19
20
21
22
23 **Corresponding author:**

24 Giancarlo Forte, PhD

25 Center for Translational Medicine (CTM)

26 International Clinical Research Center (ICRC)

27 St. Anne's University Hospital

28 Studentska 6, Brno

29 Czech Republic, 62500

30 Tel: +420-543185449

31 giancarlo.forte@fnusa.cz

32

33

34

35 **ABSTRACT**

36

37 Cardiovascular diseases remain the leading cause of death worldwide; hence there is an increasing focus on developing
38 physiologically relevant *in vitro* cardiovascular tissue models suitable for studying personalized medicine and pre-clinical
39 tests. Despite recent advances, models that reproduce both tissue complexity and maturation are still limited.

40 We have established a scaffold-free protocol to generate multicellular, beating and self-organized human cardiac organoids
41 (hCO) *in vitro* from hiPSCs that can be cultured for long term. This is achieved by differentiation of hiPSC in 2D monolayer
42 culture towards cardiovascular lineage, followed by further aggregation on low-attachment culture dishes in 3D. The
43 generated human cardiac organoids (hCOs) containing multiple cell types that physiologically compose the heart, gradually
44 self-organize and beat without external stimuli for more than 50 days. We have shown that 3D hCOs display improved
45 cardiac specification, survival and maturation as compared to standard monolayer cardiac differentiation. We also confirmed
46 the functionality of hCOs by their response to cardioactive drugs in long term culture. Furthermore, we demonstrated that
47 hCOs can be used to study chemotherapy-induced cardiotoxicity.

48 This study could help to develop more physiologically-relevant cardiac tissue models, and represent a powerful platform for
49 future translational research in cardiovascular biology.

50

51 **Keywords:** 3D cardiac organoid, induced pluripotent stem cells, microtissues, tissue engineering, cardiotoxicity

52

53

54

55

56

57 Introduction

58 Cardiovascular diseases (CVD) remain the leading cause of death worldwide¹⁻⁴, and developing new therapies is still a major
59 challenge, since a significant number of drug candidates fail to pass clinical trials, or are withdrawn from the market due to
60 adverse effects⁵⁻⁷. In order to approve safer and more effective therapies, there is an increasing demand to develop faithful
61 models of human heart tissue for pre-clinical research⁷. While recent technologies provide some insight into how human CVDs
62 can be modelled *in vitro*, a comprehensive overview of the complexity of the human heart remains elusive due to the limited
63 cellular heterogeneity, physiological complexity, or maturity of the constructs produced⁸. Furthermore, animal models may not
64 always faithfully reflect the unique features of human biology and disease, and could give rise to ethical concerns⁹⁻¹¹.

65 Induced pluripotent stem cell (iPSC) technology has been revolutionary for the differentiation and derivation of cardiomyocytes
66 for personalized disease modelling and drug testing^{8,12-20}. However, when cultured in 2D as models for development, disease
67 and toxicology^{18,21,22}, cardiomyocytes do not reflect the 3D complexity of the native tissue, where the geometry, the presence
68 of different cell types and their interaction with the extracellular matrix (ECM) play a key role.

69 Early 3D cardiac tissue models called “cardiospheres” were developed by culturing human heart tissue biopsies, or by mixing
70 non-isogenic populations of cardiomyocytes, non-myocytes and biocompatible hydrogels²³⁻²⁵; however, such microtissues
71 generally had limited culture continuity, self-organization and failed to capture the heterogeneity characteristic of organotypic
72 models. Recently, cardiac tissue engineering technologies have enabled the development of more physiologically-relevant tissue
73 models, which entail a higher degree of complexity, organization and dynamics²⁶⁻²⁹, such as engineered heart tissues
74 (EHTs)^{27,30}, isogenic cardiac microtissues^{24,31-36}, and organs-on-a-chip^{33,34,35-42}.

75 Organoids, defined as 3D miniaturized versions of an organ, are emerging as promising tools showing realistic micro-anatomy,
76 and organ specific function^{9,48-50}. In order to be considered as an “organoid”, an *in vitro* model must fulfil specific requirements,
77 including: (1) 3D multicellular composition with organ-specific cell types, (2) self-organization and histological resemblance
78 to the tissue of origin and (3) recapitulation of at least one specialized biological function similar to the organ being modelled<sup>50-
79 52</sup>.

80 Well-established organoids have been already generated for brain, kidneys, intestines, guts, lungs and many other organs^{9,53}.
81 Until recently, organoid models of the heart had not been established yet^{54,55}, as the first such examples only started to emerge
82 in the last couple of years. Notably, early mammalian cardiac organoids showing spontaneous self-organization with distinct
83 atrium- and ventricle- like regions were generated from mouse pluripotent stem cells (PSCs)^{56,57}, or as a part of gastruloids⁵⁸.
84 Shortly afterwards, human PSC-derived cardiac organoid models were described^{55,59-64}, which were developed with different
85 approaches varying from assembling different cardiac cell types⁶¹, followed by other self-organized models more faithful to
86 cardiac-specific development^{55,62-65}.

87 Some features of recently reported human cardiac organoids include being modelled after a single chamber of the heart - namely
88 the left ventricle in the case of “cardioids”⁵⁵, relying on an external ECM scaffold, such as Matrigel^{60,62}, or featuring the co-
89 emergence of gut tissue together with atrial- and ventricular-like regions^{62,64}. While being extremely informative, most of these
90 models lack long-term culture and characterization, which would help them acquire a more mature phenotype, a feature which
91 is usually desirable for more physiologically relevant *in vitro* tissue models⁶⁶.

92 Here, we aimed to establish induced pluripotent stem cell (iPSC)-based, scaffold-free, long-term human cardiac organoids
93 (hCOs), which self-organize to display discrete atrial and ventricular domains, contain multiple cell types of the human heart,
94 and preserve coordinated contractile activity for several months.

95 By combining RNA-sequencing and ultrastructural analysis, we demonstrated that dimensionality and time in culture are crucial
96 mediators of survival, differentiation, collective organization and maturation of hCOs.

97 Finally, we confirmed hCOs qualify as a powerful *in vitro* heart model by proving their response to cardioactive and cardiotoxic
98 drugs in long term culture.

99

100 Results

101 Long term 3D culture drives spontaneous self-organization and increased survival in iPSC- 102 derived human cardiac organoids

103 We aimed to develop a robust differentiation method able to produce cardiac organoids with high reproducibility by using
104 chemically defined culture conditions compatible with drug testing high-throughput analysis.

105 Since 2D monolayer differentiation of induced pluripotent stem cell (iPSC)-derived cardiomyocytes is very well established in
106 literature, and long-term cultures iPSC-derived cardiomyocyte monolayers naturally tend to delaminate into beating clusters,
107 we first performed human iPSC (hereafter hiPSC) differentiation in a 2D system and test whether they could be used to generate
108 3D cardiac aggregates in the absence of any external ECM scaffold.

109 Cardiac differentiation was induced in confluent 2D hiPSC monolayers by sequential modulation of WNT pathway with small
110 molecules, in the absence of insulin⁶⁷ as previously described by Lian et al^{13,14}. First, mesoderm specification was achieved by
111 transient WNT activation through chemical inhibition of GSK3, followed by cardiac mesoderm differentiation through the
112 inhibition of the WNT palmitoleoyltransferase PORCN^{13,14} (Figure 1). When beating cell clusters were observed (day 7), insulin
113 supplement was added to the media, since it was needed for cell survival after early contractile cardiomyocytes emerged during
114 differentiation⁶⁷. On day 15 of differentiation, the monolayer was dissociated into single cells and seeded on round-bottom
115 ultra-low attachment plates in order to induce the spontaneous formation of aggregates (Figure 1). Throughout the study, we
116 used 2D monolayer cultures as controls.

117 After switching the culture from 2D to 3D, we observed the formation of spontaneously beating microtissues. The diameter of
118 the microtissues reached up to 0.9 ± 0.04 mm at day 21 and 1 ± 0.09 mm at day 42 for the long-term culture, in the absence of
119 any external ECM supplementation (Figure 2a, Supplementary video 1 for day 53). The microtissues went on displaying
120 spontaneous contractile activity in long term culture and until at least day 100 (Supplementary video 2 for day 107).

121 When the microtissues were kept in culture for longer time (50 days), discrete cellular clusters and chamber-like structures were
122 observed by histological analysis, which were compatible with a phenomenon of self-organization. This feature could not be
123 observed at an earlier timepoint of day 30 (Supplementary figure 1a).

124 To further monitor this spontaneous cellular organization, we repeated the experiment to generate microtissues from hiPSC
125 expressing GFP-tagged cardiac troponin I (TNNI1) reporter. As expected, we observed the emergence of GFP-expressing
126 contractile cardiomyocytes together with non-tagged cells within the microtissues. With time in culture from day 21 to day 42,
127 the GFP-tagged contractile cardiomyocytes gradually and spontaneously rearranged within the microtissues from a random
128 distribution to one discrete region, and were surrounded by non-GFP tagged cells (Figure 2b).

129 This observation suggested that our 3D differentiation protocol could give rise not only to cardiomyocytes, but also other cell
130 populations, which showed a tendency to self-organize in extended culture times.

131 We then quantified the presence of two of the most represented populations in human heart over time^{68,69} - cardiomyocytes and
132 fibroblasts – in the 3D microtissues by fluorescence-activated cell sorting (FACS) at given time-points (day 30 and day 50 in
133 Figure 2c). The FACS analysis demonstrated that 3D culture is associated with a significant increase in cardiac troponin T2
134 (TNNT2)-positive cardiomyocytes compared to 2D, especially in long term culture (80.9 ± 10.7 % in 3D day 50 vs 15.7 ± 3.7
135 % in 2D day 50, $p < 0.0001$) (Figure 1e). On the contrary, 2D culture was shown to favour the overgrowth of CD90-positive
136 fibroblasts (10.8 ± 6.4 % in 3D day 50 vs 57.5 ± 6.2 % in 2D day 50, $p < 0.0001$) (Figure 2d and Supplementary figure 1b).

137 A common drawback of 3D cultures is the possibility that a necrotic area is induced by the poor oxygen and nutrients diffusion
138 towards the core of the construct⁷⁰. In order to rule out the possibility that this was the case in our 3D cultures, we performed a
139 cell viability assay in which we compared 3D hCOs with 2D cultures at the same timepoint (day 50). We found the overall
140 viability of the 3D hCOs to be significantly higher than standard 2D monolayer culture counterpart (87 ± 13.2 % in 3D day 50
141 vs 58.7 ± 15.1 % in 2D day 50, $p < 0.05$, Figure 2e and f).

142 Due to the presence of intrinsic and spontaneous self-organization, cellular heterogeneity, and functional beating in our 3D
143 microtissues over extended culture time, we will hereafter refer to these structures as human cardiac organoids (hCOs).

144

145 **Human iPSC-derived cardiac organoids are composed of multiple heart cell types**

146 One of the key features of *bona fide* organoids is the presence of different cell types typical of the given organ, as well as their
147 ability to self-organize in microstructures similar to the organ being modelled⁵⁰⁻⁵². In recent reports, the formation of cardiac
148 organoids was associated with the developmental co-emergence of elements of endoderm and mesoderm tissues resembling the
149 development of the heart and gut in the embryo^{62,64}.

150 To explore the spatial distribution of different cell subsets in hCOs, cryosections of the constructs at day 50 of culture were
151 immunostained (IF) to detect the presence of cells expressing markers specific of the different populations in the human heart⁶⁹
152 (Figure 3). The same slides were also stained for proteins expressed in the gut.

153 Confocal imaging showed that cells located at the periphery of the hCOs stained positive for marker of epicardial cells Wilms
154 tumor protein 1 (WT1), fibroblast markers TE-7 and ACTA2 (also known as α -smooth muscle actin, or α SMA), which
155 suggested the presence of a fibrotic shell. The core of the construct was composed by cardiac troponin T2 (TNNT2)- and
156 sarcomeric α -actinin (ACTN2)-positive cardiomyocytes.

157 Interestingly, we were able to identify two spatially distinct pools of atrial and ventricular cardiomyocytes within the core of
158 the hCOs, by staining with antibodies directed against ventricular (MYL2) or atrial (MYL7) Myosin Light Chain isoforms.
159 Furthermore, markers for cardiac morphogenesis (GATA4), Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase 2
160 (SERCA2A), and gap junction proteins (Connexin 43, or Cx43) were also observed within the microstructures.

161 Additionally, the analysis showed the presence of discrete areas which stained positive for the endocardial marker NFAT2, and
162 endothelial cell marker CD31. No markers of embryonic or adult gut tissue, or undifferentiated cell markers were detected in
163 day 50 hCOs (Supplementary figure 2).

164 Altogether, these results confirmed that hiPSC-derived long term cultured hCOs contain multiple cell types of the human heart
165 organized in functional domains.

166

167 **Human iPSC-derived cardiac organoids show ultrastructural organization and maturation in long** 168 **term culture**

169 A key feature of the adult heart is the existence of a highly recognizable three-dimensional ultrastructure due to the periodical
170 repetition of the functional units of the contractile apparatus, the sarcomere. The length and the alignment of the sarcomeres,
171 together with the interspacing of myosin-actin myofilaments and the abundance and shape of mitochondria, are considered
172 representative of the maturity of the contractile tissue.

173 We analysed the ultrastructure of the contractile core of the hCOs and assessed how it developed with time in culture by
174 transmission electron microscopy (TEM). TEM analysis clarified that the prototypical contractile apparatus was hardly
175 recognizable in hCOs cultured for 21 days, while highly organized sarcomeres with distinct z-disks and evenly distributed
176 myofilaments could be detected in those cultured for 50 days (Figure 4a, b).

177 The same analysis also demonstrated that longer culture times (day 85) led to the assembly of t-tubules close to the regularly
178 spaced myofibrils, together with the appearance of packed and elongated mitochondria (Figure 4f and Supplementary figure 3).
179 The presence of t-tubules, extensions of the sarcolemma interspersed around the contractile apparatus to maximize the efficiency
180 of calcium exchange, is only found in mature cardiac muscle and was not reported for standard 2D monolayer cultures⁷¹. In our
181 hCOs, we found the sarcomere length to be approximately 1.5 μ m (Figure 4c, d). This value did not change at later time-points
182 (day 85) and is consistent with the values described for young mammalian cardiomyocytes⁷². Meanwhile, Z-band was found to
183 increase slightly in width, but not significantly, with time in culture (700 ± 250 nm on day 85 vs. 450 ± 100 nm on day 50, p
184 <0.0001) as to get closer to the values typical of adult heart⁷² (Figure 4e-g).

185 Furthermore, TEM also demonstrated a higher amount of mitochondria and glycogen accumulation at later time-points (day 50
186 and 85) compared to day 21 (Supplementary figure 3), which suggests a metabolic shift to advanced maturation of
187 cardiomyocytes⁷².

188 All these features indicate hiPSC-derived hCOs undergo structural and metabolic maturation⁶⁶ when cultured for long time in
189 3D.

190

191

3D long term culture induces human iPSC-derived cardiac organoid maturation

192

Contractile cell maturation can be monitored by tracking the evolution of the expression of specific genes encoding for contractile proteins. Hence, we set at investigating the transcriptional landscape of human long-term iPSC-derived 3D hCOs in order to assess the impact of time and dimensionality on the maturation of the contractile cells.

193

194

To this end, we performed bulk RNA-sequencing and differential expression analysis (DE analysis) for hCOs at day 30 and 50 of culture and compared them to 2D monolayer cultures harvested at the same time-points.

195

196

A total of 2975 genes were found to be significantly and differentially regulated in hCOs compared to monolayer cultures at day 30. This number increased to 6437 at day 50 (Figure 5a and Supplementary tables 1 & 2), possibly indicating a bigger divergence between 2D and 3D cultures over time.

197

198

The functional annotation at both time-points revealed that the genes differentially regulated in 3D hCOs had a fingerprint for cardiogenesis, which included the categories of myofibril assembly and sarcomeric organization, muscle contraction and cardiac tissue morphogenesis (Figure 4b). As an example, the top 20 deregulated genes at day 50 between hCOs and monolayer cultures included genes which are well known to be involved in cardiac muscle maturation and function, namely cardiac muscle α -actin (*ACTC1*), sarcomeric α -actinin (*ACTN2*), phospholamban (*PLN*) and myosin heavy chain 7 (*MYH7*) (Figure 5c). On the contrary, 2D cultures showed an increased expression in ECM-related genes, most likely as a result of the predominance of cardiac fibroblast population in monolayer culture (see Figure 2d and Supplementary figure 4).

200

201

Clustering analysis of the genes upregulated in 3D hCOs compared to 2D monolayer cultures at day 50 showed a highly interconnected network of genes involved in heart contraction and sarcomeric organization, which included important structural proteins of the contractile apparatus such as *ACTC1*, *ACTN2*, troponins (*TNNT2*, *TNNC1*, *TNNI1*, *TNNI3*), myosin heavy (*MYH6*, *MYH7*) and light (*MYL2*, *MYL3*) chains, dystrophin (*DMD*), titin (*TTN*), obscurin (*OBSCN*) and myozenin 2 (*MYOZ2*), among many others (Figure 5d).

202

203

In good agreement with the presence of t-tubules in hCOs and their drift towards a more mature intracellular Ca^{2+} -dependent contraction we detected the enhanced expression of the SERCA Ca-ATPase 2 (*AT2A2*) and its inhibitor phospholamban (*PLN*), triadin (*TRDN*), ryanodine receptors (*RYR2* and the Purkinje's cell specific *RYR3*), calsequestrins (*CASQ1*, *CASQ2*), as well as genes related to cardio-renal homeostasis angiotensinogen, atrial natriuretic peptide and corin (*AGT*, *NPPA*, *CORIN*). Interestingly, in accordance with the marked increase in the number of mitochondria observed in the TEM images (see Figure 4a), day 50 hCOs showed an upregulation in the network of genes associated to ATP synthesis coupled electron transport, including several NADH:ubiquinone oxidoreductase and Cytochrome c oxidase subunits.

204

205

206

207

Since long term 3D cultures seemed to be able to promote the maturation of hCOs, we set at comparing the transcriptomic landscape of our *in vitro* constructs with available datasets obtained from adult atrial and ventricular heart tissues^{73,74} (Figure 2d, Supplementary figure 4). We figured using these datasets might help us confirm the results of our confocal analysis hinting at the presence of distinct atrial and ventricular populations in our hCOs (see Figure 3).

208

209

210

211

212

Sample clustering showed that day 50 hCOs were closer to adult heart at transcriptional level, with the most similar levels being associated to the expression of several sarcomere-associated genes, including *TTN*, *ACTN2*, troponins (*TNNT2*, *TNNI3K*, *TTNC1*, *TNNI3*) and Myosin heavy chains (*MYH6*, *MYH7*, *MYH7B*), as well as important components of the calcium handling apparatus (*RYR2*, *ATP2A2*, *PLN*). Consistent with our immunostaining results, day 50 hCOs displayed both atrial (*MYL4*, *MYL7*, *MYBHL*) and ventricular (*MYL2*, *MYL3*, *FHL2*) chamber markers (Figure 5e).

213

214

215

216

217

Overall, our transcriptomics analysis confirmed that, in our 3D hCOs, culture dimensionality is a crucial factor mediating hiPSC-derived cardiac specification and cardiomyocyte maturation at a metabolic, calcium handling and sarcomeric level. The maturation of 3D hCOs is strongly promoted by time in culture.

218

219

Human iPSC-derived cardiac organoids functionally respond to drugs in a dose dependent fashion in long term culture

220

221

An essential feature of *bona fide* organoids is to replicate at least one specialized function of the modelled organ⁵⁰⁻⁵², which in the case of heart tissue can be assessed by the contractile activity of hCOs. As previously indicated, once the hCOs acquired

222

236 spontaneous contractile activity early in culture, the contractility persisted for more than 100 days (Supplementary video 2).
237 In order to test their physiological significance, hCOs were exposed to clinically relevant doses of cardioactive drugs^{75,76} in
238 long-term cultures (> day 50). In detail, isoproterenol and verapamil were used as positive and negative inotropes, respectively.
239 Isoproterenol, a beta-adrenergic agonist, increases the contractile force and beating frequency⁷⁷, while verapamil, a calcium
240 channel blocker, decreases the beating rate⁷⁸. The hCOs were incubated with increasing drug doses (0.01 - 1 μ M) for 15-20
241 minutes and further monitored by live imaging using a confocal microscope.

242 The response to the treatment was evaluated and quantified based on the contraction (Figure 6a) and beating rate (Figure 6b) of
243 the hCOs, analysed through the open-source software tool MUSCLEMOTION^{79,80}. The acquired data shows that increasing
244 concentrations of isoproterenol resulted in enhanced contraction amplitude and beating rate, with the beating rate peak at 1 μ M.
245 As expected, increasing concentrations of verapamil had the opposite effect, with the beating being completely stopped at 1 μ M
246 drug dosage (Supplementary videos 3-8). These observations are in accordance with those reported in previous studies in cardiac
247 microtissues⁸¹ and confirm that hCOs can functionally respond to cardioactive drugs in a dose dependent manner.

248 Finally, we investigated the possibility of modelling drug-induced cardiotoxicity on hCOs, by treating them with doxorubicin
249 (Doxo), a well-known chemotherapeutic agent widely used in the treatment of several types of cancers⁸². The drug is - in fact -
250 also known to have cardiotoxic and pro-fibrotic effects, thus able to cause or exacerbate heart failure *in vivo*⁸³⁻⁸⁸.

251 hCOs were exposed to 0.1 μ g/mL and 1 μ g/mL of Doxo and their morphology and contractile activity was compared with
252 untreated controls (n=6) for the next following 6 days. The culture medium was exchanged every 3 days and hCOs monitored
253 on a daily basis. Figure 6c shows that the morphology of hCOs gradually changed over the course of treatment, and with the
254 organoids displaying more irregular edges when exposed to higher Doxo doses. In addition, clear changes could also be
255 observed in the beating profiles of hCOs treated with Doxo (Figure 6d, e, and Supplementary videos 9-14).

256 We figured the changes in the morphology of hCOs treated with the chemotherapeutics might be due to the induction of cell
257 death by the drug. Therefore, after 6 days of Doxo treatment, we assessed the hCOs viability with a luminescence-based assay.
258 The results in Figure 6e, f revealed that the hCOs treated with 1 μ g/mL Doxo were significantly less viable than both the control
259 group and the group treated with lower drug concentration (0.1 μ g/mL) (Average viability on day 6: 100 \pm 4.1% for ctrl, 86.4
260 \pm 9.3 for 0.1 μ g/mL Doxo, 28.3 \pm 6,6% for 1 μ g/mL Doxo, p<0.0001)
261

262 Discussion

263 In this study we present a straightforward method to generate complex and functional 3D iPSC-derived human cardiac organoids
264 in the absence of an external scaffold.

265 Our study has demonstrated that long-term cultured 3D hCOs have structural and functional characteristics resembling native
266 human heart such as: (1) spontaneous self-organization; (2) presence of multiple cell types of the heart belonging to epicardium,
267 myocardium and endocardium layers; (3) ultrastructural organization and maturation of cardiac muscle sarcomeres and
268 mitochondria; (4) distinct atrial and ventricular regions; (5) improved survival, cardiac specificity and maturation at
269 transcriptional level; (6) long-term functional beating in the absence of external stimuli; (7) functional response to cardioactive
270 and cardiotoxic drugs in a dose- and time-dependent fashion.

271 When compared to standard 2D cardiomyocytes culture, 3D hCOs showed an enhanced survival rate. More importantly, their
272 cellular composition appeared to be more representative of the physiological conditions in the heart, although their overall
273 phenotype appeared to be close to foetal heart^{81,89}.

274 While the effects of long term culture in promoting the maturation of PSC-derived cardiomyocytes is well established^{90,91}, 2D
275 culture is usually challenging in the long run, as the cardiomyocytes tend to delaminate from the traditional tissue culture
276 plates⁹⁰, leaving non-myocyte cell populations such as fibroblasts to overgrow.

277 One of the greatest advantages of our model is that there is no need for an external scaffold as the hCOs self-organize and
278 survive for a long time in static culture conditions. Other models have been developed in the absence of an external ECM,
279 however they usually require the differentiation of other stromal cells (such as epicardial or fibroblastic cells) separately,
280 followed by their assembly with cardiac myocytes^{34,55}.

281 On the contrary, by following the original 2D cardiac differentiation protocol by Lian et al^{13,14} and simply switching the
282 dimensionality from 2D to 3D, we have generated long-term 3D hCOs in which epicardial cells, fibroblasts, and endocardial
283 cells emerge spontaneously together with cardiomyocytes, without the need to set up separate lineage differentiation protocols
284 and further assembly. In addition, the spontaneous beating of our hCOs was observed for more than 100 days as, to this date,
285 only reported by Silva et al. as well. The latter paper, though, reported that the developmental co-emergence of gut also occurred
286 together with the heart tissue⁶⁴.

287 In our model, a distinct gut tissue was not observed after 50 days of culture through immunofluorescence analysis. Histological
288 analysis of the hCOs has shown distinct populations of WT1-positive epicardial cells, TE-7-positive fibroblast-like cells,
289 NFAT2-positive endocardial cells along with a distinct core of cardiomyocytes. CD31-positive endothelial cells could also be
290 detected, however at almost negligible levels. This finding might be explained by the fact that in native human heart endothelial
291 cells have different developmental origins⁹², and we did not supplement our culture media with endothelial-lineage favouring
292 factors, like VEGF, during our study.

293 Another remarkable feature of the hCOs is the representation of distinct atrial and ventricular cardiomyocyte populations, which
294 were clearly identified by confocal imaging of MYL2 and MYL7. Although with significant differences, a similar phenomenon
295 of atrial/ventricular cardiomyocyte subtype distinction was also shown by Israeli and colleagues, which appeared to be blunted
296 with long term culture⁶³. Instead, the herein reported hCOs revealed higher cardiac-specific cellular heterogeneity and
297 maturation in terms of histological, ultrastructural, and transcriptional-levels in long term 3D cultures compared to shorter
298 culture times and standard 2D monolayer culture.

299 The superior maturity and complexity of our long-term 3D hCOs could be also demonstrated at the ultrastructural level by our
300 TEM analysis over long culture times. At day 50 and 85, our hCOs displayed clearly aligned myofibers resulting in well-
301 organized sarcomeres, as compared to earlier time points. These morphological adaptations, which are hallmarks of mature
302 cardiac tissues⁷², were further supported by transcriptomics analysis, where hCOs showed an increased expression of genes
303 related to cardiac contraction and sarcomeric structures.

304 The electrophysiology and calcium handling properties of immature cardiomyocytes diverge in crucial ways from their mature
305 counterpart. While immature cardiomyocytes can rely on the calcium released at the cell periphery to initiate sarcomere
306 contraction, mature cardiomyocytes develop plasma membrane invaginations (t-tubules) which are wrapped around the
307 myofibrils as to make the process of calcium release and re-uptake faster and more efficient^{93,94}. The first polarization triggers

308 the release of calcium stored in the sarcoplasmic reticulum by RYR2, which is back to the sarcoplasmic reticulum by SERCA2
309 (sarco/endoplasmic reticulum Ca^{2+} -ATPase). The analysis of hCOs confirmed the presence of t-tubules at the longer time-points
310 (> day 50), the upregulation of some of the genes involved in their biogenesis (*JPH2*, *ACTN2*, *NEXN*) and sarcomeric calcium
311 management (*RYR2*, *PLN*, *SERCA*), hence demonstrating long-term 3D culture determines functional maturation of hCOs.

312 Finally, cardiomyocytes also experience several metabolic adaptations during *in vivo* maturation in order to generate the
313 required amount of ATP⁹⁵. Our ultrastructural results are compatible with this metabolic switch, showing that hCOs acquired
314 elongated mitochondria distributed around the contractile apparatus with time in culture. This idea is further reinforced by the
315 upregulation in long term 3D cultured hCOs of genes related to chain electron transport-linked phosphorylation.

316 Functional analysis of the 3D hCOs proved that they were responsive to clinically relevant doses of cardioactive and cardiotoxic
317 drugs. This is the first step required to consider the generation of patient-specific models of disease for drug testing.

318 As for modelling chemotherapy-induced cardiotoxicity, while doxorubicin is a well-established chemotherapeutic known to
319 cause heart failure as a side effect⁸³⁻⁸⁸, the *in vitro* modelling in cardiac microtissues is still not well-established due to the
320 limitations so far encountered in reproducing the physiological cellular heterogeneity, and cardiomyocyte maturity⁶¹.
321 Furthermore, it is crucial to consider the influence of cell-cell interactions of cardiomyocyte vs non-myocytes - especially
322 fibroblasts - and cell-ECM interactions⁹⁶. Our hCOs platform offers the advantages of long-term viability, cellular heterogeneity
323 and advanced maturity *in vitro*, rendering it a physiologically relevant alternative for future studies. For instance, further clinical
324 parameters such as the cardio-fibrotic potential of doxorubicin treatment^{86,87,97}, the secretion of cardiac troponins and natriuretic
325 peptides as clinically relevant markers of chemotherapy-induced cardiotoxicity⁹⁸ or simply the potential for personalized
326 medicine could be exploited by using this iPSC-derived hCOs⁹⁹.

327 Nevertheless, despite the remarkable structural and functional maturation in the long-term culture model as compared to
328 traditional monolayers, our hCOs still displays features of the foetal rather than the adult heart. For example, myocytes of our
329 hCOs have ~1.5 μm length of sarcomeres (as in foetal mammalian hearts), whereas in adult hearts, this length is usually 2 μm ⁷².
330 We believe the adoption of additional stimuli (i.e.: dynamic culture, mechanical conditioning and electrical stimulation) might
331 in the future help further improve the model here described¹⁰⁰.

332 In a longer perspective, *in vitro* cardiotoxicity studies should not underestimate the importance of multi-organ interactions.
333 While drugs in their native chemistries may not always result in direct cardiac damage¹⁰¹, their metabolic by-products might
334 cause cardiotoxicity *in vivo*^{101,102}.

335 In conclusion, we have established a simple procedure to prepare human induced pluripotent stem cell-derived cardiac organoids
336 recapitulating some key features of the human heart, and displaying improved maturation and functionality in long term culture,
337 thus enhancing the potential of *in vitro* platforms to be used in translational research applications.

338
339

340 **Methods**

341 **Stem cell culture and maintenance**

342 The human iPSC cell line DF 19–9–7 T (iPS, karyotype: 46, XY) was purchased from WiCell (Madison, WI, USA), and the
343 Troponin I1 reporter iPSC line (TNNI1-iPS) was purchased from Coriell Institute (Cat.no. AICS-0037- 172, Camden, New
344 Jersey, USA). All iPSC cells were cultured and maintained in feeder-free conditions as previously described^{103,104} on Growth
345 Factor Reduced Matrigel®-coated plates, (1:100 in DMEM/F12, Corning) in complete Essential 8™ Medium (E8, Thermo
346 Fisher Scientific) containing penicillin/streptomycin (P/S) (0.5%, VWR), incubated at 37°C, 5% CO₂.

347 **hiPSC-derived monolayer cardiac differentiation and maintenance**

348 Cardiac differentiation from hiPSCs was performed following the Wnt signalling modulation protocol by Lian et.al.^{13,14}, with
349 slight modifications as previously described¹⁰³. Briefly, prior to cardiovascular differentiation, hiPSCs were dissociated into
350 single cells (TrypLE Select, Thermo Fisher Scientific) and re-seeded onto 12-well Matrigel-coated plates with 2.0×10^5
351 cells/cm², with complete Essential 8 medium including Y27632 Rock Inhibitor (RI) (1:4000 dilution from 10 μM stock, 2.5 μM
352 final, Selleck chemicals, Houston, TX, USA). The next day, the medium was replaced with complete Essential 8 medium
353 without RI, and the medium exchange was performed daily until the cells reached 100% confluency. On day 0, to start
354 mesoderm differentiation, the medium was changed with RPMI 1640 with L-Glutamine (Biosera) media supplemented with
355 P/S, B-27™ supplement minus insulin (1×, Thermo Fisher Scientific) and CHIR99021 (8 μM, Sigma-Aldrich). On day 2, the
356 medium was exchanged with RPMI 1640+B-27 minus insulin (RPMI+B27-Ins), supplemented with IWP-2 (5 μM, Selleck
357 chemicals). On day 4, the medium was replaced with RPMI+B27-Ins, and medium exchange was performed every 2 days until
358 the cells started beating (usually on day 7, but may depend on the iPSC line). Once beating clusters started to emerge, the
359 medium was replaced with RPMI 1640+B-27™ supplement (RPMI+B27+Ins) (1×, Thermo Fisher Scientific), and medium
360 exchange was performed every 2-3 days until day 15 of differentiation. From day 15 on, the cells were used either for generation
361 of 3D hCOs, or were continued to be cultured as 2D monolayers as controls, with medium exchange every 3-4 days until the
362 end of experiments.

363 **hiPSC-derived cardiac organoid generation and maintenance**

364 On day 15 of monolayer cardiac differentiation, cells were dissociated by putting 0.5 mL StemPro™ Accutase™ (Gibco) per
365 well, and incubating in room temperature or in 37°C incubator for 10-20 minutes with occasional mechanical pipetting, until
366 the cells were visually dissociated. After sufficient dissociation, Accutase™ was stopped by putting 3mL RPMI 1640 with 20%
367 Knockout Serum Replacement (Gibco) per well, and the cells were pelleted by centrifugation at 150xg for 3 minutes. The
368 resulting pellet was resuspended with RPMI/B27+Ins + P/S + RI (1:2000 dilution from 10 μM stock, 5 μM final), counted by
369 LUNA™ cell counter (Logos) , and seeded 150,000 cells/well in 96-well round bottom ultra-low attachment plates (Corning
370 Costar 7007) at a volume of 150 μL/well. The plates were then centrifuged at 200xg for 5 minutes and incubated at 37°C, 5%
371 CO₂. As 2D control, either 200,000 cells were seeded in Matrigel-coated 96-well tissue culture plates at a volume of 200
372 μL/well, or 1.5×10^6 cells were seeded on Matrigel-coated 24-well tissue culture plates in 1 mL volume per well, or left
373 undetached in 12-well plates. After 48 hours, medium was replaced with RPMI/B27+Ins without RI from hCOs and 2D-controls
374 that were re-seeded. From this point on, medium was changed partially every 2-3 days with RPMI/B27+ Ins +P/S until day 30,
375 then every 3-4 days at least until day 50, or longer.

376 **Cell viability with Calcein AM/EthD-1 staining**

377 3D hCO and 2D culture control viability was assessed with LIVE/DEAD™ Viability/Cytotoxicity Kit, for mammalian cells
378 (Thermo Fisher Scientific), according to manufacturer's instructions, and confocal imaging was performed with Zeiss LSM 780
379 confocal microscope, the viability was later quantified by image processing with ImageJ (v1.53o).

380 **Immunofluorescence (IF)**

381 Organoid fixation, cryosectioning, and immunostaining was modified from Perestrelo et al⁹⁶. Briefly, 3D hCOs were collected
382 with low binding pipette tips, and fixed in 4% PFA (Santa Cruz Biotechnology) supplemented with 0.03% eosin (Sigma
383 Aldrich) for 2 hours at room temperature, followed by washing with 1xPBS (Lonza), and embedding in 30% sucrose solution
384 at 4C until they sink at the bottom (~2 days). The organoids were then embedded in OCT solution (Leica), frozen in cassettes
385 embedded in isopentane (VWR) cooled with dry ice, and were stored in -80°C until further processing. The frozen organoids
386 were cryosectioned by cryotome (Leica CM1950) onto Menzel Gläser, SuperFrost® Plus slides (Thermo Fisher Scientific) at
387 10µM thickness.

388 For IF analysis, the organoid sections were washed with PBS for 2x5 min at RT, followed by permeabilization with 0.2% Triton
389 X-100 (Sigma Aldrich) for 5 min. Blocking was done by 2.5% bovine serum albumin (BSA) (Biowest) in PBS for our at room
390 temperature. Primary antibodies were incubated overnight at 4°C (Supplementary table 3). The next day, the samples were
391 washed with PBS, followed by appropriate Alexa-conjugated secondary antibodies (Supplementary table 3). Counterstaining
392 was done by DAPI (Roche), and organoid slides were mounted with Mowiol®4-88 (Sigma Aldrich). Confocal imaging was
393 performed with Zeiss LSM 780 confocal microscope.

394 Further histological procedures can be found in Supplementary methods.

395 **Organoid dissociation**

396 For FACS and RNA isolation, organoids were collected in Eppendorf tubes with low binding pipettes on day 30 and 50, and
397 dissociated with MACS MultiTissue Dissociation Kit 3 (Miltenyi Biotec) according to manufacturer's instructions. As controls,
398 2D monolayer cultures were directly dissociated on the plates with the same kit.

399 **Microscopy and image analysis**

400 Confocal imaging was performed with Zeiss LSM 780 confocal microscope. Light microscopy imaging was performed with
401 Leica DM IL LED inverted microscope, and fluorescence images were collected with Leica DMI4000 B inverted fluorescence
402 microscope. Image analysis was performed with ImageJ (v1.53o).

403 **RNA isolation, bulk RNA-sequencing and DE analysis**

404 For RNA isolation, 3D hCOs and 2D controls were dissociated by using MACS MultiTissue Dissociation Kit 3 on day 30 and
405 day 50. After cell dissociation, RNA was extracted with High Pure RNA Isolation Kit (Roche) according to manufacturer's
406 instructions, and quantified with Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). Quality control for RNA
407 integrity number (RIN) was also determined with Agilent 2100 Bioanalyzer.

408 Bulk RNA-sequencing and DE analysis procedure is further described in Supplementary methods.

409 Gene ontology (GO) enrichment analysis for biological processes was performed with EnrichR web-tool¹⁰⁵⁻¹⁰⁷. Clustering
410 analysis of upregulated genes was done with Cytoscape¹⁰⁸.

411 RNA-seq data from this study were also compared to adult human heart RNA-seq data from BioProjects PRJNA667310⁷³ and
412 PRJNA628736⁷⁴ obtained from the NCBI BioProject database (<https://www.ncbi.nlm.nih.gov/bioproject/>) (Accession numbers:
413 SRR12771050, SRR12771052, SRR11620685, SRR11620686) by using Biojupies web-tool¹⁰⁹.

414 **Fluorescent activated cell sorting (FACS)**

415 Cells dissociated with Accutase on Day 15 of differentiation, and with MACS Multi-tissue dissociation kit 3 (Miltenyi Biotec
416 Ca No. 130-110-204) at day 30 and 50, along with corresponding 2D controls were analysed by Beckman Coulter MoFlow
417 Astrios Cell Sorter (Beckman Coulter Life Sciences) as described previously^{96,104}, with CD90-APC, TNNT2-FITC antibodies,
418 and their corresponding unstained controls(Supplementary table 3). Cell populations were quantified by using FlowJo software
419 V10 (Tree Star).

120 **Transmission Electron Microscopy (TEM) sample preparation and analysis**

121 hCOs were transferred to new well-plates with low binding pipettes, and washed 2 x with PBS. Fixation was done by 1.5%
122 PFA+ 1.5% glutaraldehyde (Sigma Aldrich) diluted in RPMI 1640 culture media for 1 hour, then washed with 0.1 M cacodylate
123 buffer (pH 7.4) (Sigma Aldrich) for 3 times 5 minutes each, and incubated overnight at 4°C in cacodylate with glutaraldehyde.
124 The next day, samples were rewashed 3 times with cacodylate buffer, and incubated at 4°C in cacodylate buffer until sending
125 the samples, protected from light. The organoids were post-fixed for 1.5 hours with 1% osmium tetroxide in 0.1 M cacodylate
126 buffer and washed for 3 times with 0.1 M cacodylate buffer, 10 minutes each, then stained with 1% uranyl acetate in milli-Q
127 water overnight at 4°C, followed by washing in milli-Q water. The samples were then dehydrated in an ascending EtOH series
128 using solutions of 70%, 90%, 96% and 3 times 100% for 10 minutes each, incubated in propylene oxide (PO) 3 times for 20
129 minutes before incubation in a mixture of PO and Epon resin overnight, and incubated in pure Epon for 2 hours and embedded
130 by polymerizing Epon at 68°C for 48 hours. Ultra-thin sections of 70 nm were cut using a Leica Ultracut EM UC 6 Cryo-
131 ultramicrotome. TEM images were collected with a JEOL JEM 1011 electron microscope and recorded with a 2 Mp charge
132 coupled device camera (Gatan Orius).

133 **Drug treatments**

134 Isoproterenol hydrochloride (Sigma Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich), and Verapamil
135 hydrochloride (Sigma Aldrich) in methanol (VWR) to prepare 1 M stock solutions. Each drug solution was 10- fold serially
136 diluted in RPMI/B27+Ins media to make final concentrations between 0.0001-1 μ M. As untreated controls, complete
137 RPMI/B27+Ins media supplemented with DMSO or methanol were used. For data collection, the hCOs with drug-supplemented
138 media were incubated for 10 minutes at 37°C, 5% CO₂ each time the drug concentration changed, followed by live imaging with
139 video acquisition per condition with Zeiss LSM 780 confocal microscope. Chemotherapy-induced cardiotoxicity was evaluated
140 in hCOs treated with RPMI/B27+Ins supplemented with doxorubicin (Doxo) (Sigma Aldrich), at 0.1 μ g/mL and 1 μ g/mL
141 concentrations, or plain RPMI/B27+Ins as control for 6 days. Growth medium was exchanged every 3 days, and organoid
142 morphology and beating were monitored daily by live video recording with Zeiss LSM 780 confocal microscope for 6 days. On
143 day 6 of drug treatment, the organoids were collected for evaluating viability.

144 **Live video acquisition and contraction analysis**

145 To evaluate the contractile function of organoids, live image sequences were acquired with Zeiss LSM 780 Confocal microscope
146 using transmitted light mode (37°C, 5% CO₂), as equivalent to 60 fps for 15 seconds; where the image sequences were later
147 turned into .avi files with ImageJ. The beating rates were counted and calculated manually from the video analysis. and
148 representative contraction graphs were derived with the open-source video analysis software MUSCLEMOTION^{79,80} per
149 providers' instructions.

150 **Luminescence-based cell viability assay**

151 hCO viability after 6 days of doxo treatment was assessed by CellTiter-Glo® Luminescent Cell Viability Assay Kit (Promega)
152 according to manufacturer's instructions. After the hCOs were dissociated by the assay treatment, the samples were transferred
153 to a clear-bottom 96-well white assay plate (Corning Costar 3610) and cell viability was quantified with Centro LB 960
154 Microplate Luminometer (Berthold Technologies).

155 **Statistics**

156 Data are expressed as mean \pm SD. Statistical analysis was performed using Microsoft Excel 2010 and GraphPad Prism v6.01
157 using one-way ANOVA (with Sidak's multiple comparisons tests or Tukey's multiple comparisons test) and two-way ANOVA
158 tests (with Sidak's multiple comparisons test or Dunnett's multiple comparisons test), where $p < 0.05$ was considered to be
159 significantly different as denoted with asterisks [(*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$, (****) $p \leq 0.0001$]. Sample sizes
160 of independent experiments were described in the figure legends where applicable.

161 **Data availability**

162 The RNA-sequencing data obtained and presented in this study were generated at CF Genomics of CEITEC, and the dataset
163 will be freely available after getting an accession number. The data that support the findings of this study are available upon
164 reasonable request.

165

166 **References**

- 167 1. Abbafati, C. *et al.* Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic
168 analysis for the Global Burden of Disease Study 2019. *Lancet (London, England)* **396**, 1204 (2020).
- 169 2. Namara, K. M., Alzubaidi, H. & Jackson, J. K. Cardiovascular disease as a leading cause of death: how are pharmacists
170 getting involved? *Integr. Pharm. Res. Pract.* **8**, 1 (2019).
- 171 3. Roth, G. A. *et al.* Global Burden of Cardiovascular Diseases and Risk Factors, 1990–2019: Update From the GBD 2019
172 Study. *J. Am. Coll. Cardiol.* **76**, 2982–3021 (2020).
- 173 4. Heidenreich, P. A. *et al.* Forecasting the impact of heart failure in the united states a policy statement from the american
174 heart association. *Circ. Hear. Fail.* **6**, 606–619 (2013).
- 175 5. Seruga, B., Ocana, A., Amir, E. & Tannock, I. F. Failures in Phase III: Causes and Consequences. *Clin Cancer Res* **21**,
176 4552–4560 (2015).
- 177 6. McNaughton, R., Huet, G. & Shakir, S. An investigation into drug products withdrawn from the EU market between
178 2002 and 2011 for safety reasons and the evidence used to support the decision-making. *BMJ Open* **4**, (2014).
- 179 7. Khakoo, A. Y., Yurgin, N. R., Eisenberg, P. R. & Fonarow, G. C. Overcoming Barriers to Development of Novel
180 Therapies for Cardiovascular Disease: Insights From the Oncology Drug Development Experience. *JACC Basic to*
181 *Transl. Sci.* **4**, 269–274 (2019).
- 182 8. Cho, S., Lee, C., Skylar-Scott, M. A., Heilshorn, S. C. & Wu, J. C. Reconstructing the heart using iPSCs: Engineering
183 strategies and applications. *J. Mol. Cell. Cardiol.* **157**, 56–65 (2021).
- 184 9. Kim, J., Koo, B. K. & Knoblich, J. A. Human organoids: model systems for human biology and medicine. *Nature*
185 *Reviews Molecular Cell Biology* **21**, 571–584 (2020).
- 186 10. Marshall, J. J. & Mason, J. O. Mouse vs man: Organoid models of brain development & disease. *Brain Res.* **1724**,
187 (2019).
- 188 11. Gorzalczany, S. B. & Rodriguez Basso, A. G. Strategies to apply 3Rs in preclinical testing. *Pharmacol. Res. Perspect.*
189 **9**, (2021).
- 190 12. Takahashi, K. *et al.* Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell* **131**,
191 861–872 (2007).
- 192 13. Lian, X. *et al.* Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of
193 canonical Wnt signaling. *Proc. Natl. Acad. Sci. U. S. A.* **109**, E1848–E1857 (2012).
- 194 14. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin
195 signaling under fully defined conditions. *Nat. Protoc.* **8**, 162–175 (2013).
- 196 15. Sadahiro, T., Yamanaka, S. & Ieda, M. Direct Cardiac Reprogramming: Progress and Challenges in Basic Biology and
197 Clinical Applications. *Circ. Res.* **116**, 1378–1391 (2015).
- 198 16. Burridge, P. W. *et al.* Chemically defined generation of human cardiomyocytes. *Nat. Methods* **11**, 855–860 (2014).
- 199 17. Burridge, P. W. *et al.* Modeling cardiovascular diseases with patient-specific human pluripotent stem cell-derived
200 cardiomyocytes. *Methods Mol. Biol.* **1353**, 119–130 (2014).
- 201 18. Burridge, P. W. *et al.* Human induced pluripotent stem cell-derived cardiomyocytes recapitulate the predilection of
202 breast cancer patients to doxorubicin-induced cardiotoxicity. *Nat. Med.* **22**, 547–556 (2016).
- 203 19. Sharma, A. *et al.* Derivation of highly purified cardiomyocytes from human induced pluripotent stem cells using small
204 molecule-modulated differentiation and subsequent glucose starvation. *J. Vis. Exp.* e52628 (2015). doi:10.3791/52628
- 205 20. Sharma, A. *et al.* Use of human induced pluripotent stem cell-derived cardiomyocytes to assess drug cardiotoxicity.
206 *Nat. Protoc.* **13**, 3018–3041 (2018).
- 207 21. Lan, F. *et al.* Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in
208 patient-specific induced pluripotent stem cells. *Cell Stem Cell* **12**, 101–113 (2013).
- 209 22. Sharma, A. *et al.* Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes as an In Vitro Model for
210 Coxsackievirus B3-Induced Myocarditis and Antiviral Drug Screening Platform. *Circ. Res.* **115**, 556–566 (2014).
- 211 23. Li, T.-S. *et al.* Cardiospheres recapitulate a niche-like microenvironment rich in stemness and cell-matrix interactions,
212 rationalizing their enhanced functional potency for myocardial repair. *Stem Cells* **28**, 2088–98 (2010).
- 213 24. Nguyen, D. C. *et al.* Microscale generation of cardiospheres promotes robust enrichment of cardiomyocytes derived
214 from human pluripotent stem cells. *Stem cell reports* **3**, 260–8 (2014).
- 215 25. Richards, D. J. *et al.* Inspiration from heart development: Biomimetic development of functional human cardiac
216 organoids. *Biomaterials* **142**, 112–123 (2017).
- 217 26. Zimmermann, W.-H. Tissue Engineering of a Differentiated Cardiac Muscle Construct. *Circ. Res.* **90**, 223–230 (2001).
- 218 27. Weinberger, F., Mannhardt, I. & Eschenhagen, T. Engineering Cardiac Muscle Tissue: A Maturing Field of Research.
219 *Circ. Res.* **120**, 1487–1500 (2017).
- 220 28. Dhahri, W., Romagnuolo, R. & Laflamme, M. A. Training heart tissue to mature. *Nat. Biomed. Eng.* **2**, 351–352
221 (2018).

- 522 29. Hirt, M. N., Hansen, A. & Eschenhagen, T. Cardiac Tissue Engineering. *Circ. Res.* **114**, (2014).
- 523 30. Schaaf, S. *et al.* Human Engineered Heart Tissue as a Versatile Tool in Basic Research and Preclinical Toxicology.
- 524 *PLoS One* **6**, e26397 (2011).
- 525 31. Mathur, A. *et al.* Human iPSC-based cardiac microphysiological system for drug screening applications. *Sci. Rep.* **5**, 1–
- 526 7 (2015).
- 527 32. Ma, Z. *et al.* Self-organizing human cardiac microchambers mediated by geometric confinement. *Nat. Commun.* **6**,
- 528 7413 (2015).
- 529 33. Giacomelli, E. *et al.* Three-dimensional cardiac microtissues composed of cardiomyocytes and endothelial cells co-
- 530 differentiated from human pluripotent stem cells. *Dev.* **144**, 1008–1017 (2017).
- 531 34. Giacomelli, E. *et al.* Human-iPSC-Derived Cardiac Stromal Cells Enhance Maturation in 3D Cardiac Microtissues and
- 532 Reveal Non-cardiomyocyte Contributions to Heart Disease. *Cell Stem Cell* **26**, 862–879.e11 (2020).
- 533 35. Giacomelli, E., Sala, L., Oostwaard, D. W. van & Bellin, M. Cardiac microtissues from human pluripotent stem cells
- 534 recapitulate the phenotype of long-QT syndrome. *Biochem. Biophys. Res. Commun.* **572**, 118–124 (2021).
- 535 36. Tsan, Y. C. *et al.* Physiologic biomechanics enhance reproducible contractile development in a stem cell derived
- 536 cardiac muscle platform. *Nat. Commun.* **12**, 1–16 (2021).
- 537 37. Grosberg, A., Alford, P. W., McCain, M. L. & Parker, K. K. Ensembles of engineered cardiac tissues for physiological
- 538 and pharmacological study: Heart on a chip. *Lab Chip* **11**, 4165–4173 (2011).
- 539 38. Shim, J., Grosberg, A., Nawroth, J. C., Parker, K. K. & Bertoldi, K. Modeling of cardiac muscle thin films: pre-stretch,
- 540 passive and active behavior. *J. Biomech.* **45**, 832–41 (2012).
- 541 39. Zhang, Y. S. *et al.* From cardiac tissue engineering to heart-on-a-chip: Beating challenges. *Biomed. Mater.* **10**, (2015).
- 542 40. Wang, G. *et al.* Modeling the mitochondrial cardiomyopathy of Barth syndrome with induced pluripotent stem cell and
- 543 heart-on-chip technologies. *Nat. Med.* **20**, 616–623 (2014).
- 544 41. Marsano, A. *et al.* Beating heart on a chip: a novel microfluidic platform to generate functional 3D cardiac
- 545 microtissues. *Lab Chip* **16**, 599–610 (2016).
- 546 42. Ugolini, G. S. *et al.* On-chip assessment of human primary cardiac fibroblasts proliferative responses to uniaxial cyclic
- 547 mechanical strain. *Biotechnol. Bioeng.* (2016). doi:10.1002/bit.25847
- 548 43. Ugolini, G. S., Visone, R., Redaelli, A., Moretti, M. & Rasponi, M. Generating Multicompartmental 3D Biological
- 549 Constructs Interfaced through Sequential Injections in Microfluidic Devices. *Adv. Healthc. Mater.* **6**, (2017).
- 550 44. Occhetta, P. *et al.* A three-dimensional *in vitro* dynamic micro-tissue model of cardiac scar formation. *Integr. Biol.* **10**,
- 551 174–183 (2018).
- 552 45. Schneider, O., Zeifang, L., Fuchs, S., Sailer, C. & Loskill, P. User-Friendly and Parallelized Generation of Human
- 553 Induced Pluripotent Stem Cell-Derived Microtissues in a Centrifugal Heart-on-a-Chip. *Tissue Eng. - Part A* **25**, 786–
- 554 798 (2019).
- 555 46. Khademhosseini, A. *et al.* Microfluidic patterning for fabrication of contractile cardiac organoids. *Biomed.*
- 556 *Microdevices* **9**, 149–57 (2007).
- 557 47. Skardal, A. *et al.* Multi-tissue interactions in an integrated three-tissue organ-on-a-chip platform. *Sci. Rep.* **7**, 8837
- 558 (2017).
- 559 48. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid
- 560 technologies. *Science (80-.)*. **345**, 1247125–1247125 (2014).
- 561 49. Clevers, H. Modeling Development and Disease with Organoids. *Cell* **165**, 1586–1597 (2016).
- 562 50. Lancaster, M. A. & Huch, M. Disease modelling in human organoids. *DMM Dis. Model. Mech.* **12**, dmm039347
- 563 (2019).
- 564 51. Sasai, Y. Next-generation regenerative medicine: organogenesis from stem cells in 3D culture. *Cell Stem Cell* **12**, 520–
- 565 530 (2013).
- 566 52. Sasai, Y. Cytosystems dynamics in self-organization of tissue architecture. *Nature* **493**, 318–326 (2013).
- 567 53. Schutgens, F. & Clevers, H. Human Organoids: Tools for Understanding Biology and Treating Diseases. *Annu. Rev.*
- 568 *Pathol. Mech. Dis.* **15**, 211–234 (2020).
- 569 54. Corrò, C., Novellasdemunt, L. & Li, V. S. W. A brief history of organoids. *Am. J. Physiol. - Cell Physiol.* **319**, C151–
- 570 C165 (2020).
- 571 55. Hofbauer, P. *et al.* Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* **184**, 3299–3317.e22 (2021).
- 572 56. Andersen, P. *et al.* Precardiac organoids form two heart fields via Bmp/Wnt signaling. *Nat. Commun.* **9**, 1–13 (2018).
- 573 57. Lee, J. *et al.* In vitro generation of functional murine heart organoids via FGF4 and extracellular matrix. *Nat. Commun.*
- 574 **11**, (2020).
- 575 58. Rossi, G. *et al.* Capturing cardiogenesis in gastruloids. *Cell Stem Cell* **28**, 230–240.e6 (2021).
- 576 59. Voges, H. K. *et al.* Development of a human cardiac organoid injury model reveals innate regenerative potential.
- 577 *Development* **144**, 1118–1127 (2017).
- 578 60. Mills, R. J. *et al.* Functional screening in human cardiac organoids reveals a metabolic mechanism for cardiomyocyte
- 579 cell cycle arrest. *Proc. Natl Acad. Sci. USA* **114**, E8372–E8381 (2017).

- 580 61. Richards, D. J. *et al.* Human cardiac organoids for the modelling of myocardial infarction and drug cardiotoxicity. *Nat.*
581 *Biomed. Eng.* **4**, 446–462 (2020).
- 582 62. Drakhlis, L. *et al.* Human heart-forming organoids recapitulate early heart and foregut development. *Nat. Biotechnol.*
583 **39**, 737–746 (2021).
- 584 63. Lewis-Israeli, Y. R. *et al.* Self-assembling human heart organoids for the modeling of cardiac development and
585 congenital heart disease. *Nat. Commun.* **12**, (2021).
- 586 64. Silva, A. C. *et al.* Co-emergence of cardiac and gut tissues promotes cardiomyocyte maturation within human iPSC-
587 derived organoids. *Cell Stem Cell* **28**, 2137–2152.e6 (2021).
- 588 65. Drakhlis, L., Devadas, S. B. & Zweigerdt, R. Generation of heart-forming organoids from human pluripotent stem cells.
589 *Nat. Protoc.* **16**, 5652–5672 (2021).
- 590 66. Yang, X., Pabon, L. & Murry, C. E. Engineering adolescence: maturation of human pluripotent stem cell-derived
591 cardiomyocytes. *Circ. Res.* **114**, 511–23 (2014).
- 592 67. Lian, X., Zhang, J., Zhu, K., Kamp, T. J. & Palecek, S. P. Insulin inhibits cardiac mesoderm, not mesendoderm,
593 formation during cardiac differentiation of human pluripotent stem cells and modulation of canonical Wnt signaling can
594 rescue this inhibition. *Stem Cells* **31**, 447 (2013).
- 595 68. Pinto, A. R. *et al.* Revisiting Cardiac Cellular Composition. *Circ. Res.* **118**, 400 (2016).
- 596 69. Litviňuková, M. *et al.* Cells of the adult human heart. *Nature* **588**, 466–472 (2020).
- 597 70. Zuppinger, C. 3D Cardiac Cell Culture: A Critical Review of Current Technologies and Applications. *Front.*
598 *Cardiovasc. Med.* **6**, 87 (2019).
- 599 71. Ahmed, R. E., Anzai, T., Chanthra, N. & Uosaki, H. A Brief Review of Current Maturation Methods for Human
600 Induced Pluripotent Stem Cells-Derived Cardiomyocytes. *Front. Cell Dev. Biol.* **8**, 178 (2020).
- 601 72. Yang, X., Pabon, L. & Murry, C. E. Engineering adolescence: maturation of human pluripotent stem cell-derived
602 cardiomyocytes. *Circ. Res.* **114**, 511–523 (2014).
- 603 73. A novel approach for the diagnosis of dilated cardiomyopathy (DCM), ID 667310 - BioProject - NCBI. Available at:
604 <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA667310>. (Accessed: 9th December 2021)
- 605 74. RNA-seq of heart tissues from healthy individuals and DMD patients, ID 628736 - BioProject - NCBI. Available at:
606 https://www.ncbi.nlm.nih.gov/bioproject?LinkName=sra_bioproject&from_uid=10669020. (Accessed: 9th December
607 2021)
- 608 75. Guo, L. *et al.* Estimating the Risk of Drug-Induced Proarrhythmia Using Human Induced Pluripotent Stem Cell-
609 Derived Cardiomyocytes. *Toxicol. Sci.* **123**, 281–289 (2011).
- 610 76. Mannhardt, I. *et al.* Human Engineered Heart Tissue: Analysis of Contractile Force. *Stem Cell Reports* **7**, 29–42 (2016).
- 611 77. Vandecasteele, G. *et al.* Muscarinic and beta-adrenergic regulation of heart rate, force of contraction and calcium
612 current is preserved in mice lacking endothelial nitric oxide synthase. *Nat. Med.* **5**, 331–334 (1999).
- 613 78. Schwinger, R. H. G., Böhm, M. & Erdmann, E. Negative inotropic properties of isradipine, nifedipine, diltiazem, and
614 verapamil in diseased human myocardial tissue. *J. Cardiovasc. Pharmacol.* **15**, 892–899 (1990).
- 615 79. Sala, L. *et al.* Musclemotion: A versatile open software tool to quantify cardiomyocyte and cardiac muscle contraction
616 in vitro and in vivo. *Circ. Res.* **122**, e5–e16 (2018).
- 617 80. van Meer, B. J. *et al.* Quantification of Muscle Contraction In Vitro and In Vivo Using MUSCLEMOTION Software:
618 From Stem Cell-Derived Cardiomyocytes to Zebrafish and Human Hearts. *Curr. Protoc. Hum. Genet.* **99**, 1–21 (2018).
- 619 81. Thavandiran, N. *et al.* Functional arrays of human pluripotent stem cell-derived cardiac microtissues. *Sci. Reports* **2020**
620 *101* **10**, 1–13 (2020).
- 621 82. Thorn, C. F. *et al.* Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet. Genomics* **21**, 440
622 (2011).
- 623 83. Octavia, Y. *et al.* Doxorubicin-induced cardiomyopathy: From molecular mechanisms to therapeutic strategies. *J. Mol.*
624 *Cell. Cardiol.* **52**, 1213–1225 (2012).
- 625 84. Takemura, G. & Fujiwara, H. Doxorubicin-Induced Cardiomyopathy. From the Cardiotoxic Mechanisms to
626 Management. *Prog. Cardiovasc. Dis.* **49**, 330–352 (2007).
- 627 85. Silva, D., Santos, D., Coeli, R. & Goldenberg, S. Doxorubicin-Induced Cardiotoxicity: From Mechanisms to
628 Development of Efficient Therapy. *Cardiotoxicity* (2018). doi:10.5772/INTECHOPEN.79588
- 629 86. Tanaka, R. *et al.* Reactive fibrosis precedes doxorubicin-induced heart failure through sterile inflammation. *ESC Hear.*
630 *Fail.* **7**, 588–603 (2020).
- 631 87. Levick, S. P. *et al.* Doxorubicin-Induced Myocardial Fibrosis Involves the Neurokinin-1 Receptor and Direct Effects on
632 Cardiac Fibroblasts. *Heart. Lung Circ.* **28**, 1598–1605 (2019).
- 633 88. Page, R. L. *et al.* Drugs That May Cause or Exacerbate Heart Failure. *Circulation* **134**, e32–e69 (2016).
- 634 89. Gilsbach, R. *et al.* Dynamic DNA methylation orchestrates cardiomyocyte development, maturation and disease. *Nat.*
635 *Commun.* **5**, (2014).
- 636 90. Pasqualini, F. S., Nesmith, A. P., Horton, R. E., Sheehy, S. P. & Parker, K. K. Mechanotransduction and Metabolism in
637 Cardiomyocyte Microdomains. *Biomed Res. Int.* **2016**, (2016).

- 538 91. Grancharova, T. *et al.* A comprehensive analysis of gene expression changes in a high replicate and open-source dataset
539 of differentiating hiPSC-derived cardiomyocytes. *Sci. Reports 2021 111* **11**, 1–21 (2021).
- 540 92. Meilhac, S. M. & Buckingham, M. E. The deployment of cell lineages that form the mammalian heart. *Nat. Rev.*
541 *Cardiol.* **15**, 705–724 (2018).
- 542 93. Zhang, X. H. *et al.* Regionally diverse mitochondrial calcium signaling regulates spontaneous pacing in developing
543 cardiomyocytes. *Cell Calcium* **57**, 321 (2015).
- 544 94. Karbassi, E. *et al.* Cardiomyocyte maturation: advances in knowledge and implications for regenerative medicine. *Nat.*
545 *Rev. Cardiol.* **17**, 341 (2020).
- 546 95. Schaper, J., Meiser, E. & Stammler. Ultrastructural morphometric analysis of myocardium from dogs, rats, hamsters,
547 mice, and from human hearts. *Circ. Res.* **56**, 377–391 (1985).
- 548 96. Perestrelo, A. R. *et al.* Multiscale Analysis of Extracellular Matrix Remodeling in the Failing Heart. *Circ. Res.* **128**, 24–
549 38 (2021).
- 550 97. Rodrigues, P. G. *et al.* Early myocardial changes induced by doxorubicin in the nonfailing dilated ventricle. *Am. J.*
551 *Physiol. - Hear. Circ. Physiol.* **316**, H459–H475 (2019).
- 552 98. Dolci, A., Dominici, R., Cardinale, D., Sandri, M. T. & Panteghini, M. Biochemical markers for prediction of
553 chemotherapy-induced cardiotoxicity systematic review of the literature and recommendations for use. *Am. J. Clin.*
554 *Pathol.* **130**, 688–695 (2008).
- 555 99. Ho, B. X., Pek, N. M. Q. & Soh, B. S. Disease modeling using 3D organoids derived from human induced pluripotent
556 stem cells. *Int. J. Mol. Sci.* **19**, (2018).
- 557 100. Marchianò, S., Bertero, A. & Murry, C. E. Learn from Your Elders: Developmental Biology Lessons to Guide
558 Maturation of Stem Cell-Derived Cardiomyocytes. *Pediatr. Cardiol.* **40**, 1367–1387 (2019).
- 559 101. Xu, T., Wu, L., Xia, M., Simeonov, A. & Huang, R. Systematic Identification of Molecular Targets and Pathways
560 Related to Human Organ Level Toxicity. *Chem. Res. Toxicol.* **34**, 412–421 (2021).
- 561 102. Hoang, P. *et al.* Engineering spatial-organized cardiac organoids for developmental toxicity testing. *Stem Cell Reports*
562 **16**, 1228–1244 (2021).
- 563 103. Pagliari, S. *et al.* YAP–TEAD1 control of cytoskeleton dynamics and intracellular tension guides human pluripotent
564 stem cell mesoderm specification. *Cell Death Differ.* **2020 284** **28**, 1193–1207 (2020).
- 565 104. Vrbský, J. *et al.* Evidence for discrete modes of YAP1 signaling via mRNA splice isoforms in development and
566 diseases. *Genomics* **113**, 1349–1365 (2021).
- 567 105. Chen, E. Y. *et al.* Enrichr: Interactive and collaborative HTML5 gene list enrichment analysis tool. *BMC*
568 *Bioinformatics* **14**, (2013).
- 569 106. Kuleshov, M. V. *et al.* Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. *Nucleic Acids*
570 *Res.* **44**, W90–W97 (2016).
- 571 107. Xie, Z. *et al.* Gene Set Knowledge Discovery with Enrichr. *Curr. Protoc.* **1**, e90 (2021).
- 572 108. Shannon, P. *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks.
573 *Genome Res.* **13**, 2498–2504 (2003).
- 574 109. Torre, D., Lachmann, A. & Ma’ayan, A. BioJupies: Automated Generation of Interactive Notebooks for RNA-Seq Data
575 Analysis in the Cloud. *Cell Syst.* **7**, 556–561.e3 (2018).
- 576
577
578

579 **Acknowledgements**

580 We thank Václav Hejret, Jana Vašíčková, and Jana Bartoňová for scientific advice and technical assistance. We acknowledge
581 the CF Genomics of CEITEC supported by the NCMG research infrastructure (LM2018132 funded by MEYS CR)
582 Bioinformatics for their support with obtaining scientific data presented in this paper. We are grateful to Ivana Andrejčinová.,
583 Marco de Zuani and Jan Frič for the NFAT2 and ASCL1 antibodies. We also thank Romana Vlčková, Hana Duřová, and
584 Helena Ďuríková for their support on continuation of the study.

585 Giancarlo Forte, Stefania Pagliari and Vladimír Vinarský were supported by the European Regional Development Fund -
586 Project ENOCH (No. CZ.02.1.01/0.0/0.0/16_019/0000868). Jorge Oliver De La Cruz and Soraia Fernandes were supported
587 by the European Social Fund and European Regional Development Fund-Project MAGNET
588 (CZ.02.1.01/0.0/0.0/15_003/0000492). Marco Cassani, an iCARE-2 fellow, has received funding from Fondazione per la
589 Ricerca sul Cancro (AIRC) and the European Union's Horizon 2020 research and innovation programme under the Marie
590 Skłodowska-Curie Grant Agreement No. 800924. Francesco Niro and Daniel Sousa have received funding from the European
591 Union's Horizon 2020 research and innovation programme under grant agreement No 860715.

592

593 **Author contributions statement**

594 E.E. conceptualized the study, designed and performed the experiments, analysed the data and drafted the manuscript.
595 J.O.D.L.C., S.F., M.C., V.V., A.R.P. and S.P. provided essential information and techniques, and assisted in designing,
596 planning, carrying out and interpreting experiments. F.N. and D.S. performed experiments and analysed the data. J.V. and
597 J.O.D.L.C. assisted with bioinformatics analysis. M.C. and D.D. performed the TEM analysis. F.C. and H.R. assisted in
598 funding acquisition, and manuscript revision. P.E. provided study supervision and manuscript revision. G.F. provided study
599 supervision, funding acquisition, paper conceptualization and manuscript finalization. All authors contributed in
700 interpretation, and presentation of data as well as manuscript editing. All authors reviewed and approved the final version.

701

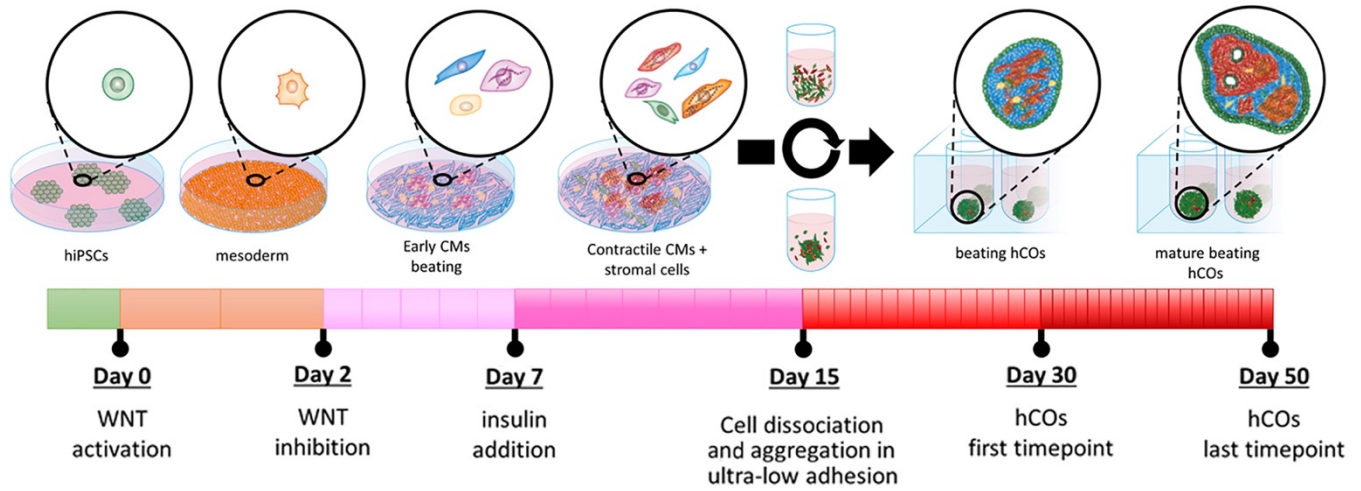
702 **Competing interests**

703 None

704

705

706 **Figures:**



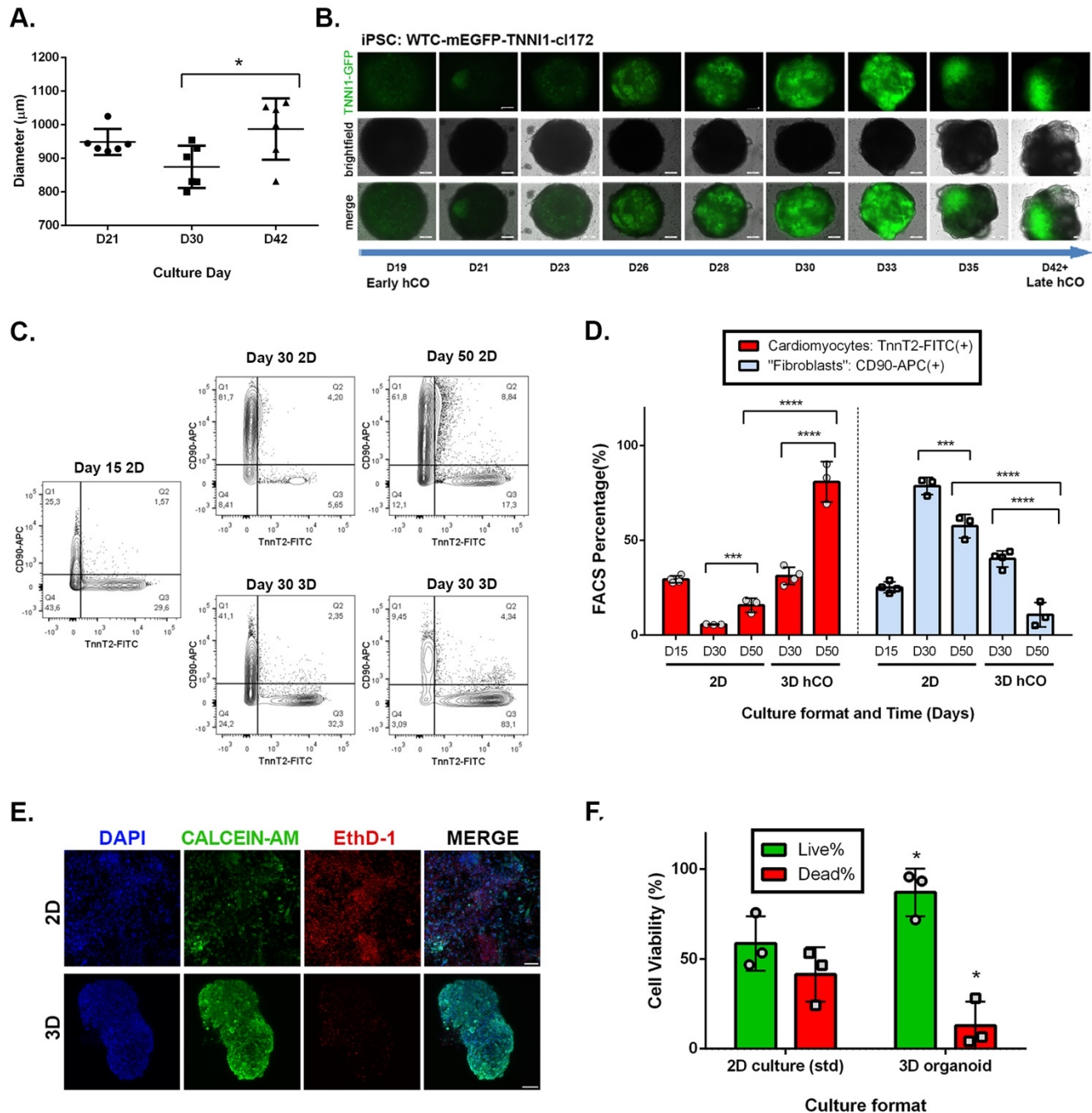
707

708

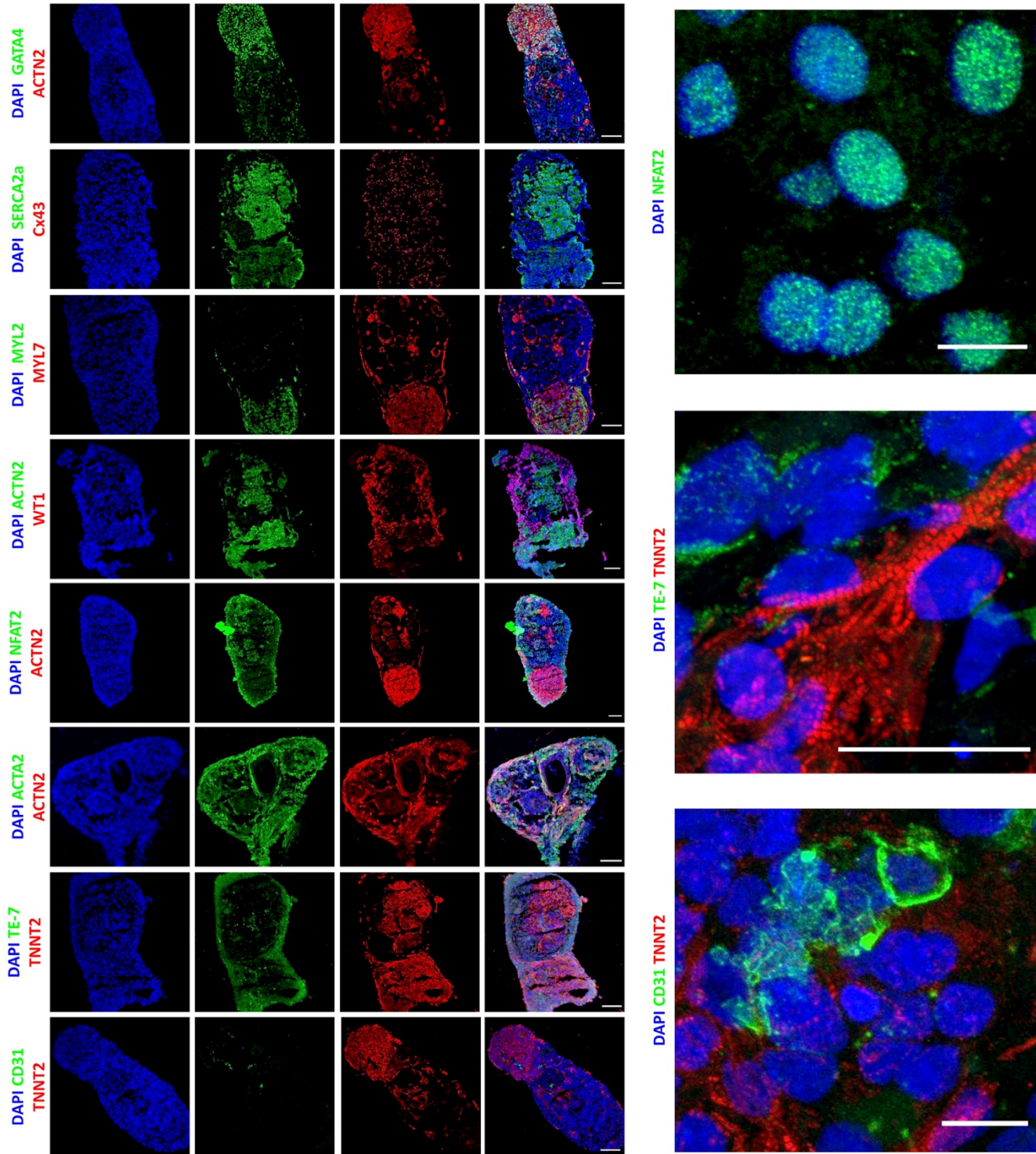
709

710

Figure 1. Schematic workflow for the generation and long-term culture of hiPSC derived cardiac organoids. Light green cells represent hiPSCs, red cells represent cardiomyocytes, dark green cells α SMA+ cells, other colours represent other non-myocytes.



711
 712 **Figure 2. Human Cardiac Organoids Spontaneously Self-Organize and Improve Survival in Long Term Culture. a.**
 713 Average diameter of 3D hCOs over time (n=6, Mean \pm SD). **b.** Monitoring spontaneous self-organization of hiPSC-derived
 714 cardiomyocytes within 3D organoids expressing GFP-tagged Cardiac Troponin I reporter in hCOs over culture time (Scale bar =
 715 200 μm). **c.** Representative FACS images for of 2D vs 3D on different days of culture, with cardiomyocytes (TNNT2-FITC), and
 716 «fibroblasts» (CD90-APC). **e.** Quantification of FACS data with different cell populations with respect to culture dimensionality
 717 and time (n=3, Mean \pm SD). **e.** Cell viability of 3D hCOs compared to standard 2D cardiac differentiation culture with Calcein/AM
 718 staining on day 50, green=live, red= dead. (Scale bar = 200 μm). **f.** Quantification of cell viability on day 50 (n= 3 organoids for
 719 3D, or 3 wells per condition for 2D) (n=3, Mean \pm SD)



720

721

722

723

724

725

726

Figure 3. Long term culture of hCOs recapitulates advanced morphology and cellular heterogeneity *in vitro*. Immunofluorescence analysis showing the co-specification of multiple cell type markers in 3D hCO sections on day 50 including cardiomyocytes (ACTN2, TNNT2), atrial (MYL7) and ventricular (MYL2) cardiomyocyte subtypes, epicardial cells (WT1), endocardial cells (NFAT2), smooth muscle/fibroblastic cells (ACTA2), “fibroblasts” (TE-7), endothelial cells (CD31), counterstained with DAPI (blue). Additionally, cardiac morphogenesis (GATA4), calcium ATPase (SERCA2A), and gap junction proteins (Cx43) can be observed. Full organoid scale bar = 100µm, Close-up scale bar = 10 µm.

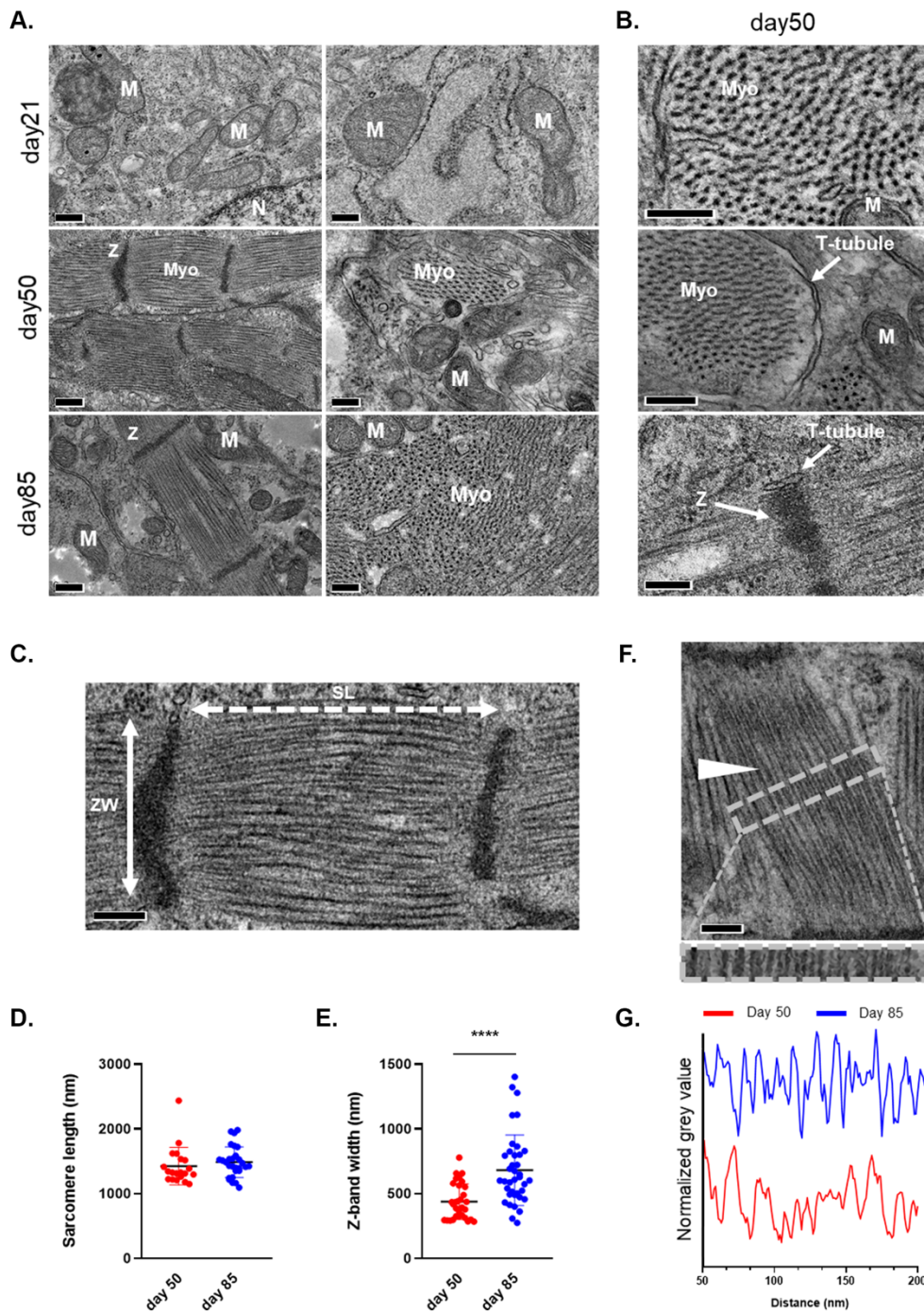


Figure 4. Ultrastructural analysis of hCOs indicates enhanced maturation in 3D environment over time. a. TEM images of hCOs showing increasing ultrastructural organization over time, from day 21 to day 85: Cardiomyocyte myofibers (Myo), Mitochondria (M) Z-band (Z), T-tubules. Scale bars = 200 nm. **b.** Close-up images on day 50. Scale bars are 200 nm. **c.** Representative micrograph showing sarcomere length (SL; white dotted two-headed arrow) and z-band width (ZW; cross sectional length; white solid two-headed arrow) with the corresponding sarcomere length. **d.** day 50, 1400 ± 300 ; day 85, 1500 ± 200 ; $N > 20$, Mean \pm SD)- and z-band width (**e**; day 50, 450 ± 100 nm; day 85 700 ± 250 nm; $N > 30$, Mean \pm SD). Scale bar = 200 nm. **f.** Micrograph showing myofiber organization (white arrow) with their relative organization as defined by the plot profile intensity on day 50 vs day 85 of the area indicated by the grey dashed box of "figure 4f". Scale bar = 200 nm.

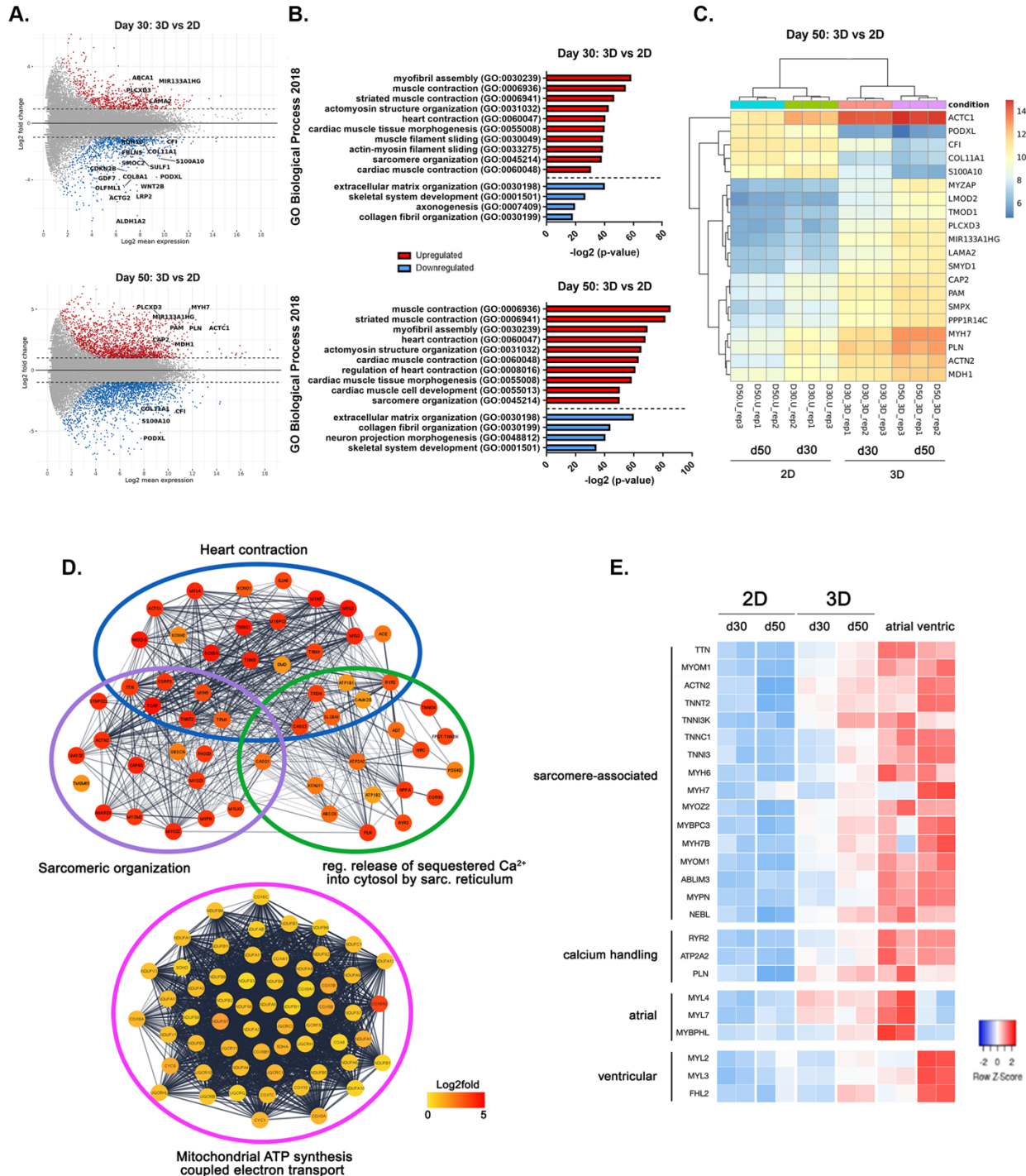


Figure 5. 3D long term hCO cultures induce improved cardiac specification and cardiomyocyte maturation at the transcriptional level. **a.** MA plot of the differentially regulated genes between 2D monolayer and 3D hCOs at day 30 (up) and day 50 (down). **b.** Graph representing the -log₂value adjusted p-value of significantly upregulated (red) or downregulated (blue) GO Biological process categories when comparing 3D hCOs and 2D monolayers at day 30 (up) and day 50 (down). **c.** Heatmap representing the log₂fold change for the top 20 differentially regulated genes between 2D monolayer and 3D hCOs at day 50. **d.** STRING network of the upregulated genes at day 50 hCOs as compared to monolayer cultures for the indicated GO biological processes categories by Cytoscape (Kappa score = 0.3). Log₂fold change is color-coded represented for the nodes. **e.** Heatmap encoding for log₂fold changes in selected cardiac genes for the 3D hCOs and the 2D monolayers at both time-points in comparison with available datasets for adult human atrial and ventricular tissues.

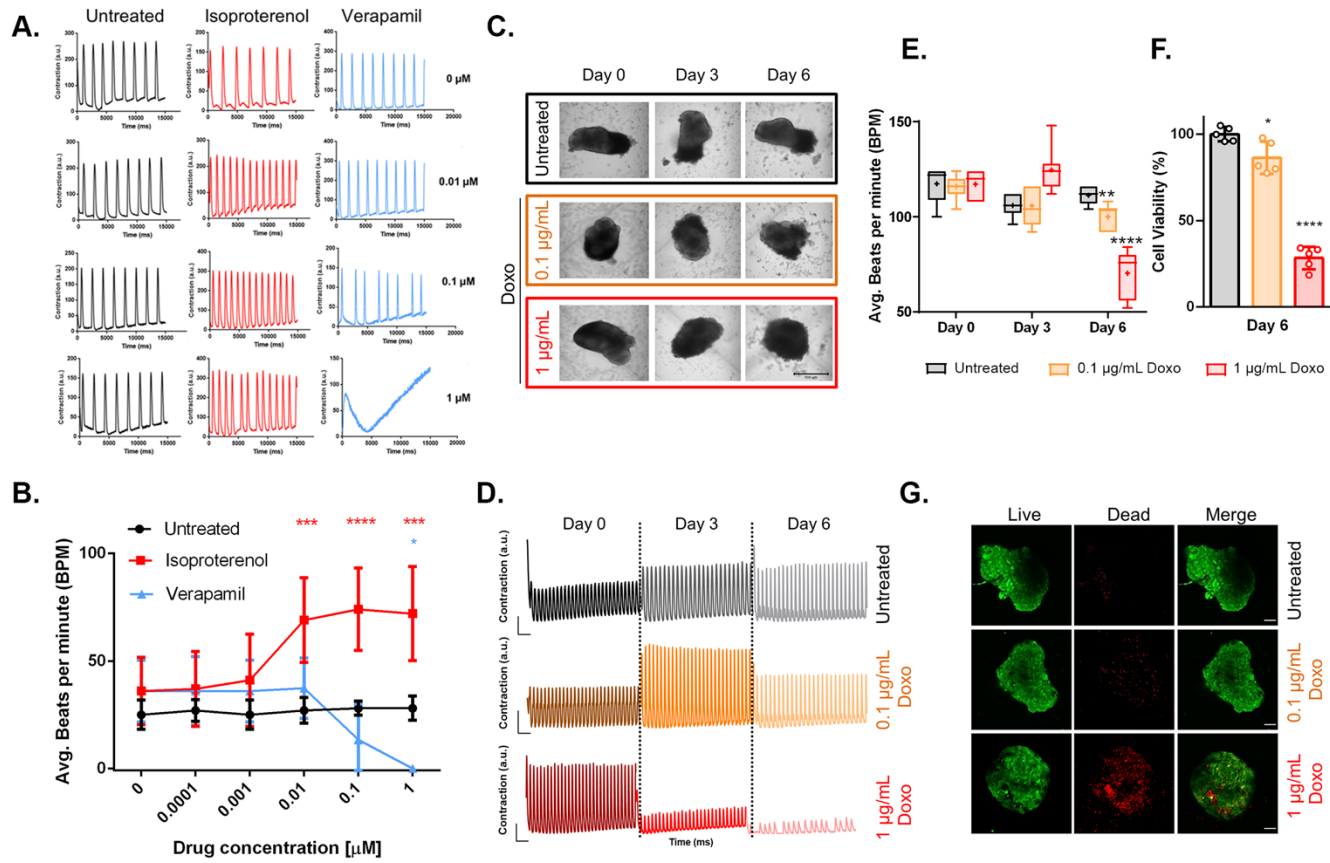


Figure 6. 3D hCOs show functional response to cardioactive and cardiotoxic drugs in a dose and time dependent manner.
a. Representative contraction amplitude plots of long-term (>day 50) hCOs in response to untreated vs increasing doses of Isoproterenol and Verapamil. **b.** Average beating rates of hCOs in response to untreated vs increasing doses of Isoproterenol and Verapamil. (n=3, Mean \pm SD) **c.** Morphology of long-term (>day 50) hCOs in response to untreated vs increasing doses of Doxorubicin (Doxo) over 6 days. (Scale bar = 500 μm). **d.** Representative contraction amplitude plots of hCOs in response to untreated vs increasing doses of Doxo over 6 days (t=15s). **e.** Average beating rates of hCOs in response untreated vs increasing doses of Doxo over 6 days. (n= 6, Mean \pm SD) **f.** Luminescence-based quantification of cell viability of 3D hCOs on day 6 of Doxo treatment normalized to untreated samples (untreated vs 0.1 $\mu\text{g/mL}$ vs 1 $\mu\text{g/mL}$) (n= 6, Mean \pm SD). **g.** Representative cell viability of hCOs on day 6 of Doxo treatment (untreated vs 0.1 $\mu\text{g/mL}$ vs 1 $\mu\text{g/mL}$) with Calcein/AM staining, green =live, red= dead. (Scale bar = 100 μm)

List of supplementary materials

Supplementary video 1– Representative beating hCO

Supplementary video 2 – Day 100+ organoid video

Supplementary Figures:

SI1– a. H&E staining and IF on D30 vs D50 hCO, b. 2d monolayer vs 3d hCO on day 50

SI2 – IF hCOs for undifferentiated cells & gut tissue

SI3 - Supplementary figure 3 – Additional TEM images

SI4 – Supplementary transcriptomics analyses for differentially regulated genes between day 30 and day 50 of culture for 3D hCOs and 2D monolayer culture.

Supplementary Methods – Histology (H&E), RNAseq and DE analysis.

Supplementary videos (3-8) – Beating hCOs before & after max dose of cardioactive drugs

Supplementary videos (9-14) – Beating hCOs before & after doxorubicin on Day 0 and Day 6

Supplementary table 1 – log2changes and p-values for the different comparisons

Supplementary table 2 – GO BP clustering analysis for the differentially regulated genes at the different comparisons

Supplementary table 3 – List of antibodies used in the study (IF + FACS)