Development and characterization of a scaffold-free 3D spheroid model of iPSC-derived human cardiomyocytes

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Abstract

Purpose: Cardiomyocytes are terminally differentiated cells in the adult heart and ischemia and cardiotoxic compounds can lead to cell death and irreversible decline of cardiac function. As testing platforms, isolated organs and primary cells from rodents have been the standard in research and toxicology, but there is a need for better models that more faithfully recapitulate native human biology. Hence, a new *in vitro* model comprising the advantages of 3D cell culture and the availability of induced pluripotent stem cells (iPSC) from human origin was developed and characterized.

Methods: Human cardiomyocytes (CMs) derived from induced pluripotent stem cells (iPSCs) were studied in standard 2D culture and as cardiac microtissues (MTs) formed in hanging drops. 2D cultures were examined using immunofluorescence microscopy and Western blotting while the cardiac MTs were subjected to immunofluorescence, contractility, and pharmacological investigations.

Results: iPSC-derived CMs in 2D culture showed well-formed myofibrils, cell-cell contacts positive for connexin-43, and other typical cardiac proteins. The cells reacted to pro-hypertrophic growth factors with a substantial increase in myofibrils and sarcomeric proteins. In hanging drop cultures, iPSC-derived cardiomyocytes formed spheroidal MTs within 4 days showing a homogeneous tissue structure with well-developed myofibrils extending throughout the whole spheroid without a necrotic core. MTs showed spontaneous contractions for more than 4 weeks that were recorded by optical motion tracking, sensitive to temperature, and responsive to electrical pacing. Contractile pharmacology was tested with several agents known to modulate cardiac rate and viability. Calcium-transients underlay the contractile activity and were also responsive to electrical stimulation, caffeine-induced Ca^{2+}-release, extracellular calcium levels.

Conclusions: 3D culture using iPSC-derived human cardiomyocytes provides an organoid human-based cellular platform that is free of necrosis and recapitulates vital cardiac functionality, thereby providing new and promising relevant model for the evaluation and development of new therapies and detection of cardiotoxicity.

Introduction

Traditional cell culture in flat, rigid polystyrene dishes or similar culture formats, where the cells grow in a monolayer, has served its purpose well for many years and will continue to do so for certain applications in future. Nevertheless, it became evident during the past decade that this system is limited when it comes to reliably predicting efficacy and safety of new drugs. One third of all drug withdrawal from 1990 to 2006 has occurred for direct cardiotoxicity (1) indicating that commonly used model systems lack the predictive power for early and accurate detection of this type of toxicity. The majority of drug attrition occurs in late stages of development when the costs incurred are already very high (2). Therefore, there is a clear need for *in vitro* models that faithfully replicate the tissue environment. For the heart this means consideration of dynamic factors such as motion and stretch, electrical communication and paracrine stimuli from neighboring cells within the tissue. Furthermore, the cells should be from human origin in order to take into account species-specific gene expression, signaling mechanisms, electrophysiological properties and for testing antibody therapies.

New concepts have to be developed to create cardiac models systems to be used for cardiotoxicity testing, ideally in a standard multi-well format, which would also allow medium to high throughput screening. 3D-culture is a new alternative to the classical approaches and can help bridge the gap between 2D-culture and tissue (3,4). At present, 3D culture systems exist in many variants for different tissue types and applications. Nevertheless, 3D-culture is not yet widely used in cardiovascular research and toxicology. Gravity-enforced assembly of microtissues was found to be applicable to a variety of cell types for use in toxicology and drug development(5-7), Therefore, we have adapted self-assembling, scaffold-free, spheroidal microtissue aggregation in hanging drop techniques as the platform of choice in this study. This study also utilizes iPSC-derived human cardiomyocytes, which can be differentiated at industrial scale and quality from both healthy and patient populations, thus providing a substrate for both efficacy and safety studies. These cells have been used in a number of projects so far and their electrophysiological, genetic and toxicological properties have been characterized(8-12). Hanging drop microtissues of iPSC-CMs can be produced in a consistent manner, do not require the addition of extracellular matrix proteins, and overcome the sourcing, handling, and phenotypic hurdles associated with primary human cardiomyocytes. Here we demonstrate that scaffold-free 3D culture of commercially available iPSC-derived cardiomyocytes is feasible and results in reproducible microtissues that reliably respond to external electrical, pharmacological and physical stimuli and show long-term viability. Morphological and functional characterization emphasizes that this model may become a valuable tool for substance safety testing in the future.

Material and Methods

Culture of induced pluripotent stem cell (iPSC)-derived cardiomyocytes in standard culture

Human iPSC-derived human cardiomyocytes (iCell® Cardiomyocytes) were obtained from Cellular Dynamics International Inc. (CDI, Madison, WI, USA). Nascent CMs are allowed to mature until day 32 at which point they are frozen for shipping. The details of the procedure were published previously (21). For standard 2D culture, cryopreserved cardiomyocytes were rapidly thawed, then diluted in iCell Plating Medium (iCPM, CDI) and seeded into culture dishes (Nunc) coated with 0.1% gelatin in water. After 48 hours, medium was changed to iCell Maintenance Medium (iCMM, CDI) and then changed every three days. 1% penicillin–streptomycin (Gibco, Invitrogen) was added to all culture media. In a specific set of experiments, DMEM with Glutamax (Gibco, Invitrogen) with the addition of 20% fetal calf serum (PAA Laboratories) and recombinant human insulin-like growth factor-I (IGF-I) (Sigma) at 500 ng/ml was used.

3D microtissue culture and viability testing

Cryopreserved cardiomyocytes were diluted in iCPM and directly seeded in hanging droplets in a volume of 40 µl medium per droplet using the GravityPlus™ system (InSphero). The starting number of viable cardiomyocytes was at least 2500, or as indicated in the results section, and adjusted according to the reported viability parameter of the production lot. After 4 days in the hanging drop without medium change, the spheroids were transferred to a 96-well microtissue receiver plate with non-adhesive surface (GravityTRAP™, InSphero) in a volume of 70 µl iCMM per well. In some experiments, the alpha 1 adrenergic agonist phenylephrine was added in the final concentration of 100 µM and combined with 30 µM ascorbic acid (Sigma). Spontaneous beating rate over the entire culture period was assessed by manually counting contraction rate using an inverted microscope for 30 seconds once every 2 days before medium exchange. For this assessment, the GravityPlus™ plate was regularly returned to the incubator in order to keep the temperature constant. The Live/Dead assay consisting of the fluorescent dyes calcein-AM and ethidium homidimer-1 (Biotium) was used to determine viability of 2D- and 3D-cultured cardiomyocytes.

High-resolution calcium imaging

MTs were allowed to attach to laminin-coated glass bottom dishes (MatTek, Ashland MA, USA) overnight and then loaded with fluo-4-AM purchased from Life Technologies dissolved in DMSO (30 min at room temperature, de-esterification for 10 min). Images were obtained using a laser-scanning confocal microscope (MicroRadiance, BioRad; Nikon TE300 Eclipse) using 40x oil and 63x water-immersion objectives. The fluorescent probe was excited with a laser line at 488 nm, emission was collected above 510 nm. Line-scan images were recorded at a rate of 50 lines/s. Changes in fluorescence are shown as $\Delta F/F_0$. External bath solution contained (in mM): 140 NaCl, 5.4 KCl, 1.1 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, 10 Glucose

(pH 7.4). For rapid solution exchange for the modulation of extracellular calcium (from 1.8 to 0.5 mM Ca^{2+}) and caffeine-treatment (10 mM), a custom-built buffer switching system was used. All experiments were performed at room temperature.

Assessment of microtissue contractions and pharmacologic treatments

MTs were transferred from GravityTrap^{TM} culture to glass-bottom dishes (MatTek) for attachment. Medium for these cultures contained 25 mM HEPES (Sigma) in order to maintain pH. Culture dishes were placed on a heated platform on a Nikon Eclipse TE2000-U inverted microscope and bath temperature was measured with a wire sensor and temperature controller (Warner Instrument Corp.). Microtissue motion was recorded at a video frequency of 240 Hz (MyoCam, IonOptix,) using the SoftEdge detection method and processed with IonWizard analysis software (IonOptix). Different pharmacological agents were tested on spontaneously contracting MTs at 37°C by solving the respective chemical in medium and replacing the culture medium in the glass bottom-dish before video analysis.

Immunocytochemistry of 2D-cultured cells and whole-mount staining of MTs

Cells cultured on gelatin-coated polystyrene culture dishes (Nunc) were washed with PBS then fixed with 3% para-formaldehyde in PBS for 15 min, permeabilized with 0.2% Triton-X100 (Sigma) in PBS for 10 min, incubated for 30 min with bovine serum albumin (Sigma) 1 mg/mL in PBS at room temperature, incubated over night with primary antibodies at 4 °C, washed 3 times with PBS and incubated 1 h with secondary antibodies coupled to Alexa fluorescent dyes (Invitrogen). DAPI (Sigma) was added to visualize the nuclei. For whole-mount immunostaining of MTs, spheroids were allowed to adhere onto glass-bottom dishes (MatTek). After fixation with 3% para-formaldehyde for 1 h in the cold, permeabilization and antibody incubation steps in 1%BSA/PBS/10% Tween-20 (AppliChem) were prolonged to 1 day each before examination by confocal microscopy. Preparations were examined on a Zeiss LSM 5 Exciter confocal microscope using 40x and 63x Zeiss oil immersion lenses (Carl Zeiss).

Histology and immunolabeling of cryo- and paraffin sections

Cryosections: at least 8 MTs per group were collected by gentle spinning in a 1.5-ml tube. The MTs were then fixed with 3% para-formaldehyde in PBS for 15 min, washed with PBS, stored in and embedded in OCT compound. Blocks were then cut using a Zeiss cryostat. Frozen sections (10 µm) on slides were air dried for at least 1 hour. After a blocking step with 1%BSA/PBS, primary antibodies in 1%BSA/PBS and 0.3% Tween20 were applied overnight. For classical histological staining (hematoxylin-fuchsin or mayers-haemalaun (Sigma)) of paraffin sections, MTs were harvested, fixed with 3% para-formaldehyde for 1 hour in PBS, collected in agarose plugs and processed as described previously(13). For immunohistochemical labeling of paraffin sections, MTs were harvested and fixed as described. Primary antibodies were detected with the Ventana iVIEW DAB detection kit, resulting in a brown reaction product. Sections were counterstained with hematoxylin and covered with a glass cover slip.

Antibodies

To visualize myofibrils in cardiomyocytes, we used monoclonal antibodies to myomesin (clone B4, obtained from the Developmental Studies Hybridoma Bank, Univ. of Iowa) and polyclonal antibodies to human embryonic heart-specific myomesin(14). Additionally, polyclonal antibodies to connexin-43 (ab11370, Abcam), to all-actin (A2066, Sigma) and monoclonal antibodies to the cytoplasmic domain ED-A of fibronectin (sc-59826, St. Cruz Biotech) were used.

Statistical analysis

All values are expressed as mean ± S.D. Statistical analysis of differences observed between the groups was performed by Student's unpaired t-tests. Statistical significance was accepted at the level of $p < 0.05$.

Results

Characterization of iPSC-derived cardiomyocytes in 2D-culture

Cryopreserved iPSC-derived cardiomyocytes were thawed and cultured according to the manufacturer's protocol using plating medium for the first 48 hours and maintenance medium during the rest of the culture time. After 4-5 days, a dense monolayer of spontaneously contracting cardiomyocytes had developed. After 10 days in standard 2D-culture, cells were fixed and immunostained for sarcomeric and gap junction proteins (Fig. 1 A). The majority of cells showed well-formed myofibrils positive for the sarcomeric protein myomesin and actin-stress fibers. In another line of experiments, iPSC-derived cardiomyocytes were cultured all the time in hypertrophy-inducing media consisting of DMEM with the addition of 20%FCS and insulin-like growth factor 1 (IGF-I) at 500 ng/ml (Fig. 1 B). Under these conditions, the sarcomeric cytoskeleton appeared very prominent in the immunostained cells suggesting a hypertrophic response to this growth factor with enhanced expression of muscle proteins as has been described in cultures of primary neonatal and adult rat cardiomyocytes (15,16). Increased proliferation of non-muscle cells was also observed in this rich medium (not shown). Cells cultured in maintenance medium were fixed and immunostained for myomesin and for connexin-43 in order to label gap junctions at cell-cell contacts, which were found in the expected pattern (Fig. 1 C). The embryonic heart-specific isoform of the sarcomeric M-line protein myomesin was found in all cells (Fig. 1 D). We performed Western blotting for the detection of several proteins that were described in cultures of primary cardiomyocytes isolated from rat or mouse (supplemental figure 1). Among them, cytoskeleton/sarcomeric proteins (beta myosin heavy chain, sarcomeric alpha actinin, alpha-smooth muscle actin, desmin, embryonic-heart myomesin, MLC2v, MLC2a), receptor tyrosine kinases (ErbB2, ErbB4, VEGFR2), proteins involved in Ca^{2+} handling (ryanodine receptor2, sodium-calcium exchanger, sarcoplasmic reticulum ATPase2a), and a heat shock protein (HSP70) were detected in the standard-cultured iPSC-derived cardiomyocytes. The detection of alpha-smooth muscle actin and embryonic heart specific myomesin isoform suggests that the cardiomyocytes may be under slightly stressed conditions, or indicates a fetal or neonatal pattern of gene expression in these cardiomyocytes, since they are not expressed in unstressed adult human myocardium (17).

Characterization of microtissues made of iPSC-derived cardiomyocytes

Spheroid microtissues assembled in the hanging drop format (GravityPLUS^{TM}) in plating medium for 4 days and were harvested and transferred into non-adhesive GravityTRAP^{TM} assay plates with a conical well and a flat bottom with the diameter of 1 mm containing maintenance medium (Fig. 2 A). MTs were fixed after 3 weeks with medium changes every 3 days and subjected to several methods of histology and immunocytochemistry such as paraffin embedding and histological staining of sections, immunolabeling of cryosections, whole-mount immunostaining and confocal microscopy of intact MTs (Fig. 2). Staining of paraffin sections from large MTs made of 10'000 cells with either hematoxylin-fuchsin or Mayers haemalaun demonstrated

the overall MT structure and distribution of nuclei (Fig. 2 B, C). General tissue staining of MTs showed an equal distribution of cells without a necrotic core, layering of different cell types or vacuolization. Occasionally, “pockets” of cellular debris in seemingly random location inside the MTs were observed (Fig. 2, C, asterisk). Immunohistochemistry for cleaved caspase-3 was done using paraffin sections of MTs at different time points (1 day after harvest from the hanging drops, 5 days and 21 days) in culture. Only few positive cells were found inside MTs in a scattered distribution at all time points (supplemental fig. 2). Cryosections of MTs fixed and embedded in OCT were immunostained for myomesin, connexin-43 and stained with DAPI for the labeling of nuclei (Fig. 2 D1-D3). Cells showing sarcomeric striation were found over the entire area of the sections. Punctuated connexin-43 patterns indicating gap junctions were also detected on sections. The whole-mount preparation of MTs in combination with confocal microscopy allowed investigation of the distribution of proteins in the entire MT without physical sectioning. Good results could be obtained using our protocol with comparably small MTs of 2500 cardiomyocytes allowing the attachment to glass bottom culture dishes for several days before fixation and with incubation with antibodies to human embryonic heart myomesin, cytoplasmic fibronectin, and DAPI (Fig. 2 E1-E3). Myofibrils in cardiomyocytes were found to be partially aligned with the curvature of the outer layer of the spheroid, and in this region myofibrils often continue linearly from one cell to the next (Fig. 2 E1). This intercellular structure corresponds to the typical picture of myofibril organization in a more mature myocardial tissue *in vivo* (18,19).

Keeping the MT size constant within a series of experiments is a crucial requirement for any investigation using 3D cultures in order to obtain reproducible results. We have evaluated the relationship between the initial number of seeded cells and the resulting MT diameter as measured from photographs taken at day 10 in culture (Fig. 3 A, B). The long-term spontaneous activity has not been characterized so far for iPSC-derived cardiomyocytes in scaffold-free 3D-culture. Generally, all MTs made of iPSC-derived cardiomyocytes showed spontaneous contractions directly after transfer into the GravityTRAP^{TM}. MTs seeded with less than 2500 cells did not form any spheroid bodies but only loosely adherent clusters. We then assessed the rate of spontaneous contractions of MTs of different size over the course of 30 days in GravityTRAP^{TM} culture every two days followed by medium exchange (Fig. 3 C above). It was observed that smaller MTs consisting of 2500 cells showed a consistently higher spontaneous contraction rate compared to larger MTs. In another experiment, the effect of phenylephrine, an alpha 1 adrenergic receptor agonist, on the spontaneous contraction rate of small MTs (2500 cells) (Fig. 3 C below) was evaluated. Addition of this compound increased and stabilized the spontaneous beating rate over culture time. We then evaluated cellular viability in the 3D culture after 5 days in culture and used the live/dead-assay (Fig. 3 D). A positive control for cardiotoxicity was provided by treating MTs with the broad-range tyrosine kinase inhibitor sunitinib malate at 0.1 µM over night. All untreated MTs showed an overall strong green fluorescent signal corresponding to live cells, but some necrotic cells were found in a scattered distribution in the red channel (Fig. 3 D). Those single necrotic cells might also explain the pockets of debris occasionally found by histology analysis (Fig. 3 C, asterisk). Necrotic cells were usually found directly after thawing of the cryopreserved cardiomyocytes (Fig. 3 D). On average we counted 6 times more calceine-AM positive cells than necrotic cells (not shown). We assume that necrotic cells originate from the initial pool of cryopreserved cells, which become incorporated into the microtissue. This assumption was corroborated by immunhistochemical staining for cleaved caspase-3 at three different time points in culture that showed a

scattered distribution of few positive cells inside MTs and no increase over culture time (supplemental fig. 2).

Calcium handling in MT

Calcium handling is a fundamental element of excitation-contraction coupling (EC-coupling) in the single cardiomyocyte and disorders affecting the electro-mechanical coupling are causal for the development of heart failure and life-threatening arrhythmias. In toxicology, a model system of heart muscle needs to be able to properly react to known modulators of cardiac function. We therefore tested MTs made of iPSC-derived cardiomyocytes for cytosolic calcium signals and their response to external stimuli at high spatial and temporal resolution using fluo-4 as calcium-sensitive fluorescent probe (Fig. 4). The calcium indicator only partially penetrated into MTs as shown by optical sections comprising the surface and, in two different MTs, the center regions of the MTs (Fig. 4 A). The line-scans were obtained from single cells in the periphery of MTs. The spontaneous contractile activity of MTs, which can be recognized by a temporally highly synchronized global Ca^{2+} transient (Fig. 4 B), was occasionally proceeded by spontaneous and local Ca^{2+} release events in form of Ca^{2+} sparks or wavelets (Fig. 4 B). MTs were then subjected to electrical field stimulation. MTs responded to the external pacing and adapted to different stimulation frequencies, as shown in figure 4 C during the switch from 0.2 Hz to 0.5 Hz. Using a rapid superfusion system, MTs were exposed to low Ca^{2+} concentrations, leading to interruption of the spontaneous Ca^{2+} release events and contractions, while addition of 10 mM caffeine, an agonist of the SR Ca^{2+} release channel ryanodine receptor, resulted in massive Ca^{2+} release from the sarcoplasmic reticulum (Fig. 4 D, E). Rapid decay of the Ca^{2+} transient and re-commencement of the spontaneous beating activity demonstrate functional Ca^{2+} removal mechanisms and efficient Ca^{2+} re-uptake into the SR, which is needed for the Ca^{2+} release for the next contraction.

Measurement of spontaneous contractile activity by optical motion tracking

A video motion tracking system (IonOptix) was used to measure the contractile activity of microtissues for several hours on an inverted microscope and using a heated stage platform equipped with a wire sensor for temperature measurements in the culture medium. A similar system has been used in our lab to record contractility of single isolated cardiomyocytes from the adult rat heart(20). The system follows the motion of any object showing high contrast (i.e. the unidirectional displacement of the edge of a microtissue) and displays the result as a line graph. Microtissues adherent on glass-bottom culture dishes were allowed to adapt to 37°C temperature and several recordings of 60 second duration were performed. Fig. 5 shows the spontaneous MT beating rate after adaptation to 37°C and examples of motion of the same MT after drug addition or temperature change at different time points. The general beta-adrenergic receptor agonist isoproterenol was used at 1 µM and led to an increase of spontaneous beating rate from 40 to 70 beats per minute (bpm) (Fig. 5 A). Blebbistatin is a cell-permeable inhibitor for non-muscle myosin II ATPase and was used at a final concentration of 10 µM. This compound led to a decrease of the amplitude of MT motion and finally, the motion ceased 5 minutes after addition (Fig. 5 B). Doxorubicin is a cancer chemotherapeutic drug with well-known cardiotoxic effects, which are related to the formation of reactive oxygen species, changes in

Ca^{2+} handling and mitochondrial function. The addition of 100 µM doxorubicin led to a change in the regularity of contractions as shown in a recording after 72 minutes (Fig. 5 D). Besides the drug response, temperature-dependence of spontaneous MT activity was assessed in the range of 20°C up to 45°C (Fig. 5 E). MT contractions did not entirely stop at lower temperatures and showed a steep increase in frequency in the physiological range. Hyperthermic tachycardia was reversible upon return to normothermic conditions.

Discussion

Neonatal rodent cardiomyocyte preparations are presently the most widely used cell type for the investigation of different cardiovascular research topics *in vitro*, since their isolation from tissue and maintenance is relatively easy compared to cardiomyocytes from adult animals or human tissue samples. Cardiomyocytes derived from hiPSC offer a constant supply of human cardiac cells without ethical concerns. These cells exhibit ionic currents and channel gating properties quantitatively similar to human cardiomyocytes (8,21) and express relevant cardiac marker proteins, and lack the expression of typical stem cell markers.

3D culture models have rarely been used in cardiovascular research so far and many questions about their functional properties and biological relevance remain unresolved, which currently slows progress in the field. However, new advances in 3D-culture have recently been stimulated by the needs of tissue engineering, stem cell research and the demand for better models in drug development. Adapted analysis methods and automation-compatible technologies make it easier now to implement these cultures in high throughput screening process (13,22). Microtissues in form of cellular spheroids take advantage of the inherent capability of many cell types to aggregate and can be obtained by the hanging-drop technique, non-adhesive culture surfaces or by using rotating culture vessels. The first method has the advantage that MTs obtain the same size and shape when seeded with the same starting number of cells. Self-aggregating spheroids do not require a scaffold consisting of artificial materials or extracellular matrix proteins in form of matrigel. This is beneficial for several applications, since no animal-derived products with poor batch to batch reproducibility and the risk of disease transmission or potential interactions of screening compounds with the scaffold material have to be considered (23). On the other hand, not every cell type can be used to form spheroids by self-aggregation and the resulting microtissue has to be checked for differentiation, functionality and viability. Scaffold-based cardiac constructs promote the linear alignment of cardiomyocytes and, by direct attachment to sensors, have been used to measure contractile force and other physiologic parameters (24). The hanging drop method for cell culture, as we have used here, is actually an older concept and even the use of cardiomyocytes to produce “mini hearts” dates back to the early seventies (25). A couple of pioneering studies of the last decade have provided technological innovations and a better understanding of this type of 3D culture and its potential for applications in tissue engineering and drug development (5,26,27).

We have used non-proliferating, iPSC-derived cardiomyocytes for the generation of MTs and characterized their viability and specific cardiac features. The cells aggregated and formed 3D microtissues with defined size within 4 days in the hanging drop. Spontaneous contractile activity persisted for at least 1 month in GravityTrap^{TM} culture with regular medium changes, and the addition of the alpha-adrenergic receptor agonist phenylephrine enhanced and stabilized this activity. The internal structure of the MTs showed a homogenous tissue without central necrosis. Most cells assumed a spherical shape and were filled with myofibrils. Such a tissue structure is reminiscent of the fetal mammalian heart. Regional differentiation of strands of connected cells was observed in whole-mount preparations, and it remains to be investigated in detail, if these groups of cells represent foci of contractile activity in the MTs. Pharmacological inhibition of myosin ATPase or stimulation of the beta-

adrenergic system modulated cardiac functionality of the MTs in an expected manner.

High-resolution calcium imaging using fluo-4 and confocal microscopy revealed spontaneous local Ca^{2+} release events and global Ca^{2+} transients, which could be modulated by electrical field stimulation, and a functional SR Ca^{2+} store as revealed by caffeine depletion. These results show that microtissues generate cardiac-like calcium transients, which in turn translate into contractions. The MTs rapidly responded to pharmacological and electrical stimuli, which suggests that the cells are adequately differentiated into the cardiogenic lineage. Hence, disturbances in these properties by cardiotoxic drugs would be effective in this system. Moreover, MT contractions could be enforced by electrical field pacing to reveal abnormalities in EC-coupling after exposure to different compounds. The fact that spontaneous contractile activity is strongly temperature dependent might be a challenge for a drug testing process that is not at least semi-automatic. Medium exchange and the addition of drugs outside of an incubator or warming chamber require temperature control and adaptation. Others have noted this issue with the same cell type in 2D culture (28).

In conclusion, reproducible, scaffold-free 3D culture of iPSC-derived cardiomyocytes is feasible. Microtissues show long-term, stable functionality and respond to electrical, pharmacological and physical stimuli. This promising model warrants further study on the outcome of 3D culture conditions on cardiac toxicology and pathophysiology.

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Author Disclosure Statement

W.M., I. A. and J.K. are employees of InSphero AG. B.A. is an employee of Cellular Dynamics International. Other authors report no competing financial interests.

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