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METHODS ARTICLE

Development and Characterization of a Scaffold-Free 3D Spheroid Model of Induced Pluripotent Stem Cell-Derived Human Cardiomyocytes

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Cardiomyocytes (CMs) 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 (iPSCs) of human origin was developed and characterized. Human CMs derived from iPSCs were studied in standard 2D culture and as cardiac microtissues (MTs) formed in hanging drops. Two-dimensional cultures were examined using immunofluorescence microscopy and western blotting, while the cardiac MTs were subjected to immunofluorescence, contractility, and pharmacological investigations. 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 prohypertrophic growth factors with a substantial increase in myofibrils and sarcomeric proteins. In hanging drop cultures, iPSC-derived CMs 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, and extracellular calcium levels. A three-dimensional culture using iPSC-derived human CMs provides an organoid human-based cellular platform that is free of necrosis and recapitulates vital cardiac functionality, thereby providing a 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 of human origin to take into account species-specific gene expression, signaling mechanisms, and electrophysiological properties and for testing antibody therapies.

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New concepts have to be developed to create cardiac model systems to be used for cardiotoxicity testing, ideally in a standard multiwell format, which would also allow medium- to high-throughput screening. Three-dimensional 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 (MTs) 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 MT aggregation in hanging drop techniques as the platform of choice in this study. This study also utilizes induced pluripotent stem cell (iPSC)-derived human cardiomyocytes (CMs), 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 MTs 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 CMs. In this study, we demonstrate that scaffold-free 3D culture of commercially available iPSC-derived CMs is feasible and results in reproducible MTs 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.

Materials and Methods

Culture of iPSC-derived CMs in standard culture

Human iPSC-derived human cardiomyocytes (iCell® Cardiomyocytes) were obtained from Cellular Dynamics International, Inc. (CDI). 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.[13] For standard 2D culture, cryopreserved CMs 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 h, the medium was changed to iCell Maintenance Medium (iCMM; CDI), and then changed every 3 days. One percent penicillin–streptomycin (Gibco, Invitrogen) was added to all culture media. In a specific set of experiments, Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco, Invitrogen) with the addition of 20% fetal calf serum (FCS; PAA Laboratories) and recombinant human insulin-like growth factor-I (IGF-I; Sigma) at 500 ng/mL was used.

Three-dimensional MT culture and viability testing

Cryopreserved CMs 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 CMs 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 MT receiver plate with nonadhesive 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). A spontaneous beating rate over the entire culture period was assessed by manually counting the contraction rate using an inverted microscope for 30 s once every 2 days before medium exchange. For this assessment, the GravityPlus plate was regularly returned to the incubator to keep the temperature constant. The live/dead assay consisting of the fluorescent dyes, calcein-AM and ethidium homodimer-1 (Biotium), was used to determine viability of 2D- and 3D-cultured CMs.

High-resolution calcium imaging

MTs were allowed to attach to laminin-coated glass-bottomed dishes (MatTek) 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, Bio-Rad; Nikon TE300 Eclipse) using 40× oil and 63× water-immersion objectives. The fluorescent probe was excited with a laser line at 488 nm, and 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, and 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 MT contractions and pharmacologic treatments

MTs were transferred from GravityTRAP culture to glass-bottomed dishes (MatTek) for attachment. The medium for these cultures contained 25 mM HEPES (Sigma) to maintain pH. Culture dishes were placed on a heated platform on a Nikon Eclipse TE2000-U inverted microscope and the bath temperature was measured with a wire sensor and temperature controller (Warner Instrument Corp.). MT 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 the medium and replacing the culture medium in the glass-bottomed 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 phosphate-buffered saline (PBS), then fixed with 3% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 10 min, incubated for 30 min with bovine serum

albumin (BSA; Sigma) 1 mg/mL in PBS at room temperature, incubated overnight with primary antibodies at 4°C, washed thrice with PBS, and incubated for 1 h with secondary antibodies coupled to Alexa fluorescent dyes (Invitrogen). 4′,6-diamidino-2-phenylindole (DAPI; Sigma) was added to visualize the nuclei. For whole-mount immunostaining of MTs, spheroids were allowed to adhere onto glass-bottomed dishes (MatTek). After fixation with 3% paraformaldehyde 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 40× and 63× Zeiss oil immersion lenses (Carl Zeiss).

Histology and immunolabeling of cryo- and paraffin sections

Cryosections: at least eight MTs per group were collected by gentle spinning in a 1.5-mL tube. The MTs were then fixed with 3% paraformaldehyde in PBS for 15 min, washed with PBS, and stored and embedded in optimal cutting temperature (OCT) compound. Blocks were then cut using a Zeiss cryostat. Frozen sections (10 μm) on slides were air-dried for at least 1 h. After a blocking step with 1% BSA/PBS, primary antibodies in 1% BSA/PBS and 0.3% Tween-20 were applied overnight. For classical histological staining (hematoxylin-fuchsin or Mayer's hemalaun; Sigma) of paraffin sections, MTs were harvested, fixed with 3% paraformaldehyde for 1 h in PBS, collected in agarose plugs, and processed as described previously.[14] 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 coverslip.

Antibodies

To visualize myofibrils in CMs, we used monoclonal antibodies to myomesin (clone B4, obtained from the Developmental Studies Hybridoma Bank, University of Iowa) and polyclonal antibodies to human embryonic heart-specific myomesin.[15] Additionally, polyclonal antibodies to connexin-43 (ab11370; Abcam) and to all-actin (A2066; Sigma) and monoclonal antibodies to the cytoplasmic domain ED-A of fibronectin (sc-59826; Santa Cruz Biotechnology) were used.

Statistical analysis

All values are expressed as mean ± standard deviation. 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 CMs in 2D culture

Cryopreserved iPSC-derived CMs were thawed and cultured according to the manufacturer's protocol using the plating medium for the first 48 h and maintenance medium during the rest of the culture time. After 4–5 days, a dense monolayer of spontaneously contracting CMs had developed. After 10 days in standard 2D culture, cells were fixed and immunostained for sarcomeric and gap junction proteins (Fig. 1A). 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 CMs were cultured all the time in hypertrophy-inducing media consisting of DMEM with the addition of 20% FCS and IGF-I at 500 ng/mL (Fig. 1B). 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 CMs.[16,17] Increased proliferation of nonmuscle 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 to label gap junctions at cell–cell contacts, which were found in the expected pattern (Fig. 1C). The embryonic heart-specific isoform of the sarcomeric M-line protein, myomesin, was found in all cells (Fig. 1D). We performed western blotting (see supplementary materials and methods) for the detection of several proteins that were described in cultures of primary CMs isolated from a rat or mouse (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tec). 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 CMs. The detection of alpha-smooth muscle actin and embryonic heart-specific myomesin isoform suggests that the CMs may be under slightly stressed conditions or indicates a fetal or neonatal pattern of gene expression in these CMs since they are not expressed in unstressed adult human myocardium.[18]

Characterization of MTs made of iPSC-derived CMs

Spheroid MTs assembled in the hanging drop format (GravityPlus) in plating medium for 4 days and were harvested and transferred into nonadhesive GravityTRAP assay plates with a conical well and a flat bottom with the diameter of 1 mm containing maintenance medium (Fig. 2A). 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 Mayer's hemalaun demonstrated the overall MT structure and distribution of nuclei (Fig. 2B, 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 locations inside the MTs were observed (Fig. 2C, asterisk). Immunohistochemistry for cleaved caspase-3 was done using paraffin sections of MTs at different time points (1 day after harvest from the hanging

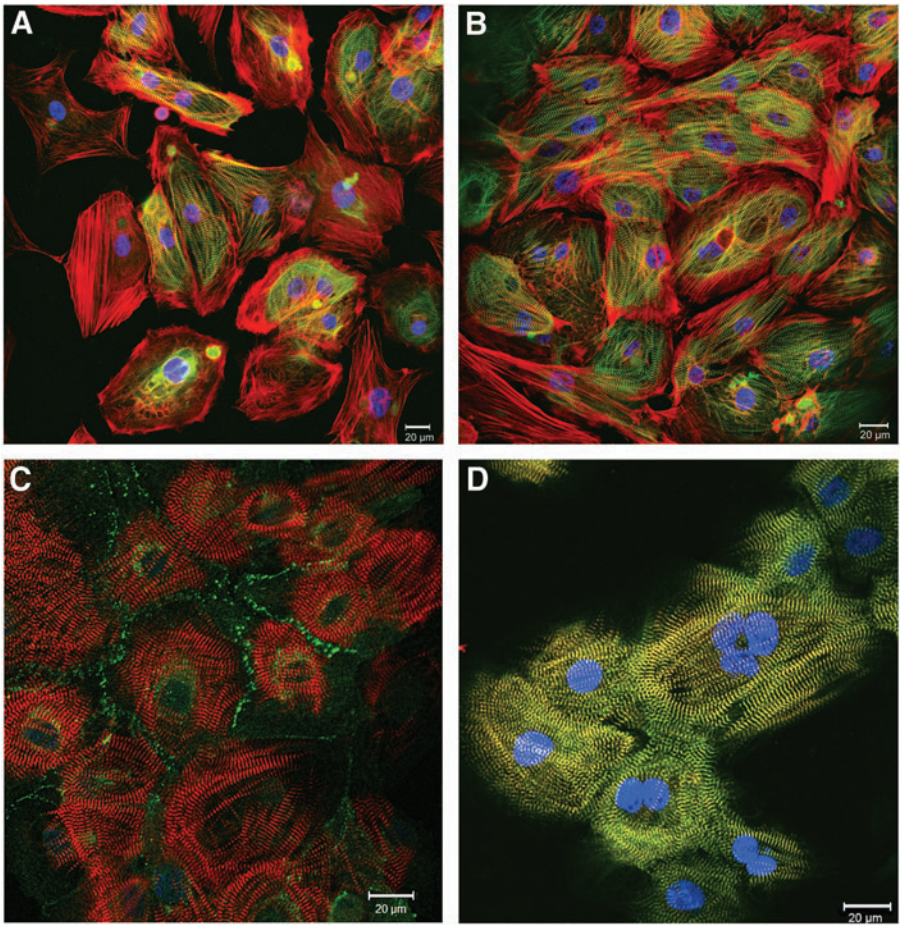

FIG. 1. **(A, B)** Cells were fixed and immunostained for the sarcomeric M-line protein myomesin (*green*), actin (*red*), and DNA (*blue*). **(A)** Cells were cultured in CDI maintenance medium. **(B)** cells were cultured in the medium with 20% fetal calf serum and supplemented with insulin-like growth factor-I (IGF-I) 500 ng/mL. **(C)** Cells were immunostained for the gap junction protein connexin-43 (*green*) and myomesin (*red*). **(D)** Myomesin (*green*) and its embryonic heart isoform (*red*) were found completely colocalizing. Color images available online at www.liebertpub.com/tec

drops, 5 days, and 21 days) in culture. Only few positive cells were found inside MTs in a scattered distribution at all time points (Supplementary Fig. S2). Cryosections of MTs fixed and embedded in OCT were immunostained for myomesin and connexin-43 and stained with DAPI for the labeling of nuclei (Fig. 2D1–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 CMs allowing the attachment to glass-bottomed culture dishes for several days before fixation and with incubation with antibodies to human embryonic heart myomesin, cytoplasmic fibronectin, and DAPI (Fig. 2E1–E3). Myofibrils in CMs 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. 2E1). This intercellular structure corresponds to the typical picture of myofibril organization in a more mature myocardial tissue *in vivo*.[19,20]

Keeping the MT size constant within a series of experiments is a crucial requirement for any investigation using 3D cultures 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. 3A, B). The long-term spontaneous activity has not been characterized so far for iPSC-derived CMs in scaffold-free 3D culture. Generally, all MTs made of iPSC-derived CMs showed spontaneous contractions directly after transfer into the GravityTRAP. 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 sizes over the course of 30 days in GravityTRAP culture every 2days, followed by medium exchange (Fig. 3C, above). It was observed that smaller MTs consisting of 2500 cells showed a consistently higher spontaneous contraction rate compared with 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. 3C, 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. 3D). A positive control for cardiotoxicity was provided by treating MTs with the broad-range tyrosine kinase inhibitor sunitinib malate at 0.1 µM overnight. 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. 3D). Those single necrotic cells might also explain the pockets of debris occasionally found by histology analysis (Fig. 3C, asterisk).

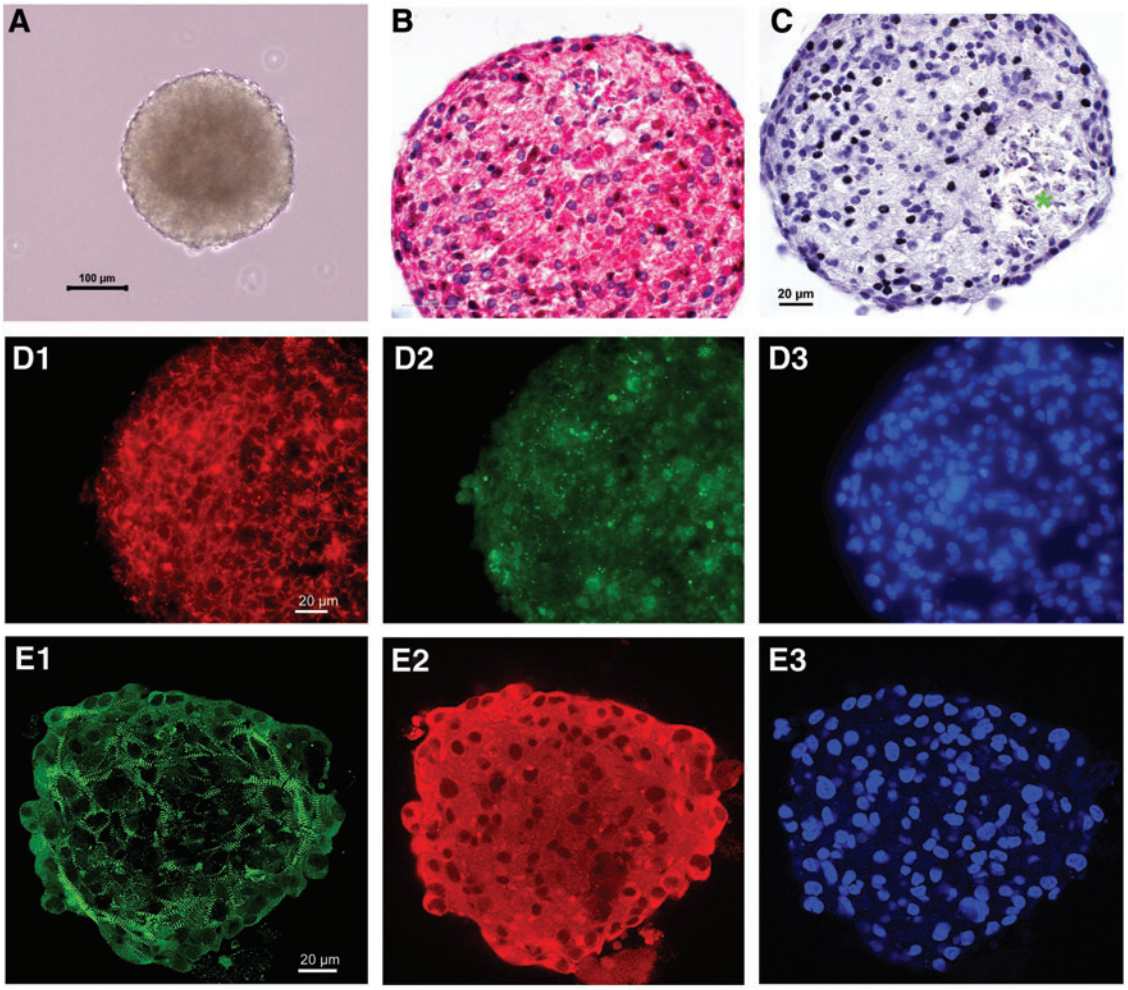

FIG. 2. Demonstration of morphology and localization of typical proteins in cardiac microtissues (MTs) using different techniques. MTs, after 3 weeks in culture, were fixed and processed for histology and immunohistochemistry. **(A)** Phase-contrast image of an unstained MT. **(B, C)** Paraffin sections stained using hematoxylin-fuchsin in pink **(B)** or Mayer's hemalaun in blue **(C)**. *Green asterisk* in **(C)** indicates cellular debris. **(D1–D3)** Cryosections were stained for myomesin in red **(D1)**, connexin-43 in green **(D2)**, and for DNA in blue **(D3)**. **(E1–E3)** MT was permeabilized and immunostained as a whole mount. Confocal images of single optical sections are shown for embryonic heart myomesin in green **(E1)**, cytoplasmic fibronectin in red **(E2)**, and DNA in blue **(E3)**. Color images available online at www.liebertpub.com/tec

Necrotic cells were usually found directly after thawing of the cryopreserved CMs (Fig. 3D). On average, we counted six times more calcein-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 MT. This assumption was corroborated by immunohistochemical 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 (Supplementary Fig. S2).

Calcium handling in MT

Calcium handling is a fundamental element of excitation–contraction coupling (EC coupling) in the single CM, and disorders affecting the electromechanical 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 CMs for cytosolic calcium signals and their response to external stimuli at high spatial and temporal resolution using fluo-4 as the 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. 4A). 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. 4B), was occasionally proceeded by spontaneous and local Ca^{2+} release events in the form of Ca^{2+} sparks or wavelets (Fig. 4B). MTs were then subjected to electrical field stimulation. MTs responded to the external pacing and adapted to different stimulation frequencies, as shown in Figure 4C, during the switch from 0.2 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

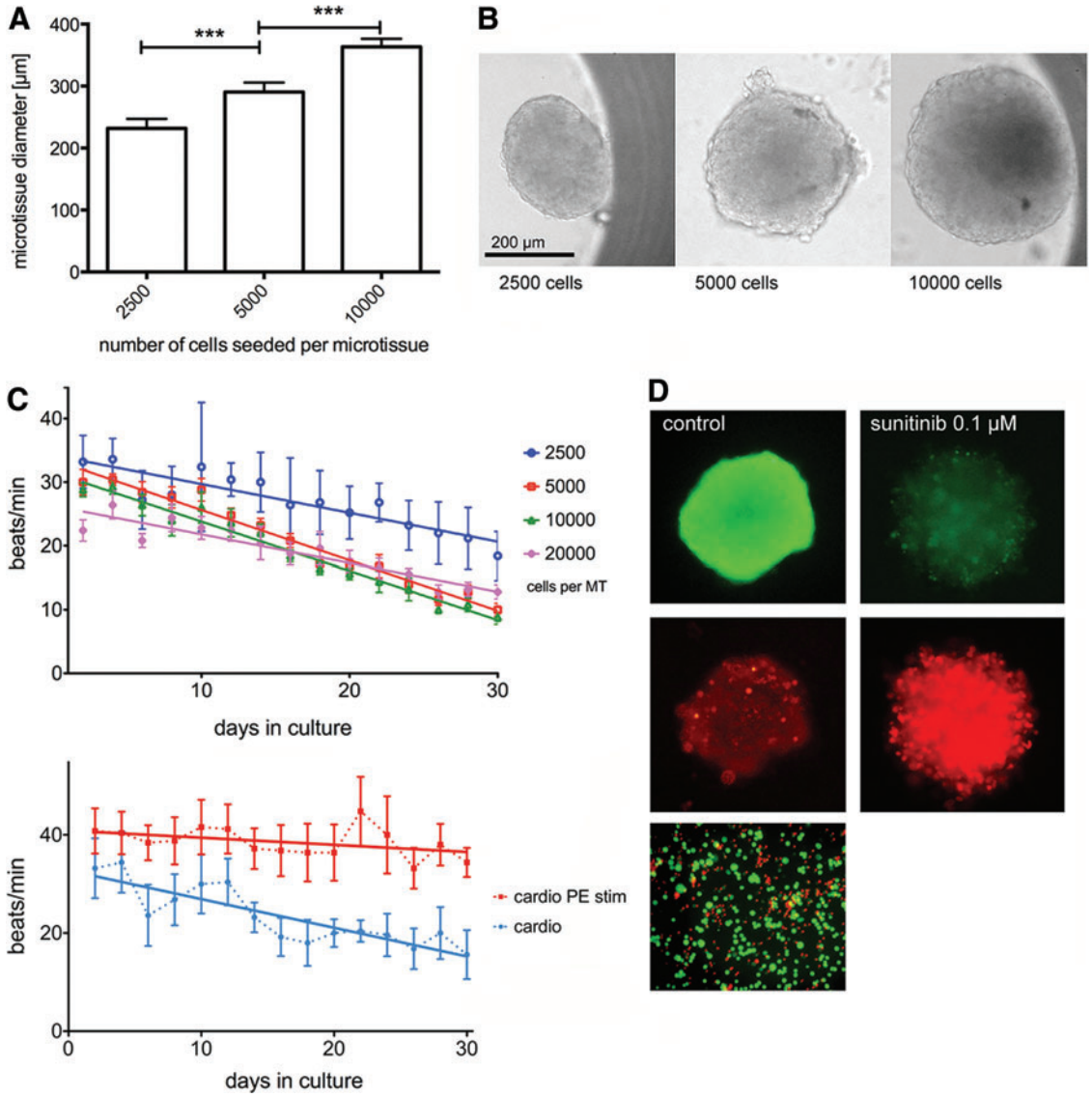

FIG. 3. **(A, B)** Microtissues made of three different starting numbers of cardiomyocytes were photographed after 10 days in GravityTRAP™ culture and their diameters were measured ($n = 8$ MT per group, ***$p < 0.001$). **(C)** Rate of spontaneous contractions over 1-month culture time was plotted for different sizes of MTs (*above*) and for the size of 2500 cells with and without 100 µM phenylephrine stimulation (*below*). **(D)** Live/Dead staining. *Green* fluorescent calcein-AM demonstrates viable cells throughout the spheroid. *Red* fluorescent ethidium homodimer staining labels the nuclei in necrotic cells. Viable and necrotic cells are shown combined before seeding (*left column*, *below*). A positive control for cell death was provided by sunitinib treatment overnight (*right column*). Color images available online at www.liebertpub.com/tec

events and contractions, while addition of 10 mM caffeine, an agonist of the sarcoplasmic reticulum (SR) Ca^{2+} release channel ryanodine receptor, resulted in massive Ca^{2+} release from the sarcoplasmic reticulum (Fig. 4D, E). Rapid decay of the Ca^{2+} transient and recommencement of the spontaneous beating activity demonstrate functional Ca^{2+} removal mechanisms and efficient Ca^{2+} reuptake 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 MTs for several hours on an inverted microscope and by 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 laboratory to record contractility of single isolated CMs from the adult rat heart.[21] The system follows the motion of any object showing high contrast (i.e., the unidirectional displacement of the edge of an MT) and displays the result as a line graph. MTs adherent on glass-bottomed culture dishes were allowed to adapt to a 37°C temperature, and several recordings of 60 s duration were performed. Figure 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

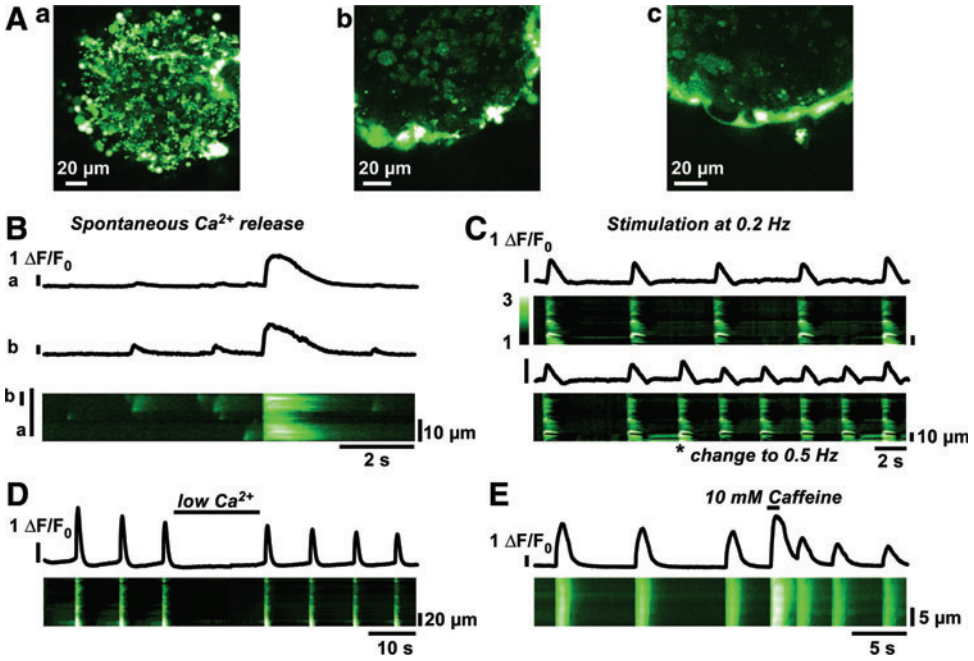

FIG. 4. Microtissues on glass coverslips were loaded with fluo-4-AM (shown in green) and analyzed using confocal line-scan imaging. The dye penetrated the first outer layers of the spheroid **(A)**. Different optical sections show the surface of the MT **(Aa)** and two different cross-sections **(Ab, Ac)**. Details of calcium release events were recorded for spontaneous releases **(B)**, during electrical field stimulation at 0.2 and 0.5 Hz **(C)**, under temporary reduction of external calcium (from 1.8 to 0.5 mM) **(D)**, and during addition of the RyR agonist caffeine **(E)**. Line-scan images and corresponding line profiles are shown. Color images available online at www.liebertpub.com/tec

agonist, isoproterenol, was used at 1 µM and led to an increase of spontaneous beating rate from 40 to 70 beats per minute (Fig. 5A). Blebbistatin is a cell-permeable inhibitor for nonmuscle 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 min after addition (Fig. 5B). 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 min (Fig. 5D). Besides the drug response, temperature dependence of spontaneous MT activity was assessed in the range of 20°C up to 45°C (Fig. 5E). 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 CM 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 with CMs from adult animals or human tissue samples. CMs derived from hiPSCs offer a constant supply of human cardiac cells without ethical concerns. These cells exhibit ionic currents and channel gating properties quantitatively similar to human CMs,[8,13] express relevant cardiac marker proteins, and lack the expression of typical stem cell markers.

Three-dimensional 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 the high-throughput screening process.[13,22] MTs in the 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, nonadhesive 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 the 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 MT has to be checked for differentiation, functionality, and viability. Scaffold-based cardiac constructs promote the linear alignment of CMs 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 CMs 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 nonproliferating iPSC-derived CMs for the generation of MTs and characterized their viability and specific cardiac features. The cells aggregated and formed 3D MTs with a defined size within 4 days in the hanging drop. Spontaneous contractile activity persisted for at least

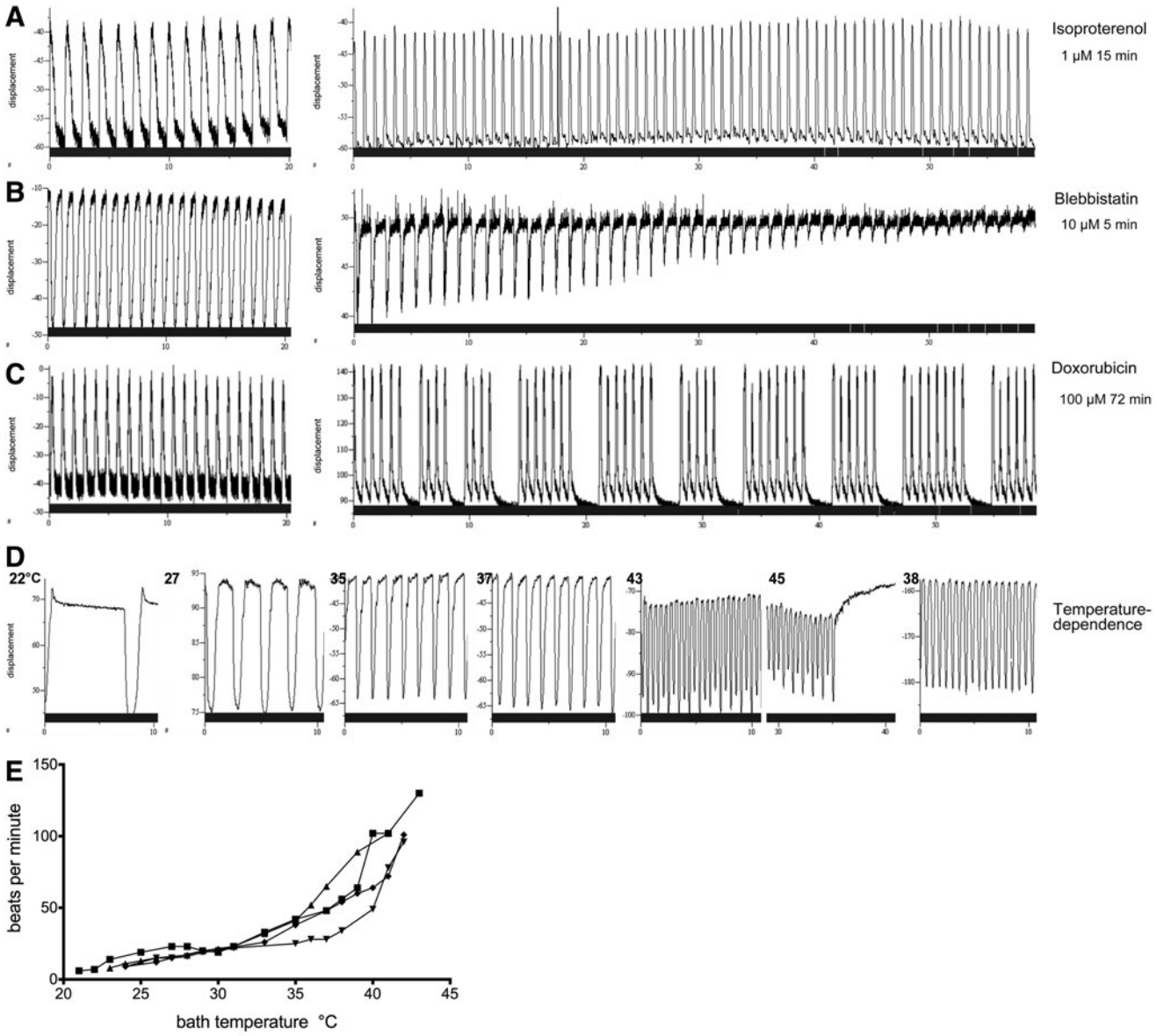

FIG. 5. Spontaneous contractions of MTs were measured by real-time video motion tracking. **(A–C)** Cultures were allowed to adapt to 37°C, and then an initial sequence of 60 s before and several sequences after the addition of a drug were recorded as indicated. Representative traces are shown. **(D, E)** MTs were kept in the cold (22°C), then the bath temperature was steadily increased within 1 h while recordings were taken. **(E)** Plots of four individual experiments for the temperature dependence of spontaneous contraction rate.

1 month in GravityTRAP 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 MTs generate cardiac-like calcium transients, which in turn translate into contractions. The MTs rapidly responded to pharmacological and electrical stimuli, which suggest 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 semiautomatic. The 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 CMs is feasible. MTs 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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Disclosure Statement

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

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