Cardiac Non-myocyte Cells Show Enhanced Pharmacological Function Suggestive of Contractile Maturity in Stem Cell Derived Cardiomyocyte Microtissues

Stephanie M. Ravenscroft,*,† Amy Pointon,† Awel W. Williams,*, Michael J. Cross,*,1,2 and James E. Sidaway*,†,2

*Department of Molecular and Clinical Pharmacology, MRC Centre for Drug Safety Science, Sherrington Building, the University of Liverpool, Ashton Street, L69 3GE, UK and †Safety and ADME Translational Sciences, AstraZeneca R&D, Cambridge, CB4 0WG, UK

1To whom correspondence should be addressed at Department of Molecular and Clinical Pharmacology, MRC Centre for Drug Safety Science, Sherrington Building, the University of Liverpool, Ashton Street, L69 3GE, UK. E-mail: m.j.cross@liv.ac.uk.

2These authors contributed equally to this study.

ABSTRACT

The immature phenotype of stem cell derived cardiomyocytes is a significant barrier to their use in translational medicine and pre-clinical in vitro drug toxicity and pharmacological analysis. Here we have assessed the contribution of non-myocyte cells on the contractile function of co-cultured human embryonic stem cell derived cardiomyocytes (hESC-CMs) in spheroid microtissue format. Microtissues were formed using a scaffold free 96-well cell suspension method from hESC-CM cultured alone (CM microtissues) or in combination with human primary cardiac microvascular endothelial cells and cardiac fibroblasts (CMEF microtissues). Contractility was characterized with fluorescence and video-based edge detection. CMEF microtissues displayed greater Ca2+ transient amplitudes, enhanced spontaneous contraction rate and remarkably enhanced contractile function in response to both positive and negative inotropic drugs, suggesting a more mature contractile phenotype than CM microtissues. In addition, for several drugs the enhanced contractile response was not apparent when endothelial cell or fibroblasts from a non-cardiac tissue were used as the ancillary cells. Further evidence of maturity for CMEF microtissues was shown with increased expression of genes that encode proteins critical in cardiac Ca2+ handling (S100A1), sarcomere assembly (telethonin/TCAP) and β-adrenergic receptor signalling. Our data shows that compared with single cell-type cardiomyocyte in vitro models, CMEF microtissues are superior at predicting the inotropic effects of drugs, demonstrating the critical contribution of cardiac non-myocyte cells in mediating functional cardiotoxicity.

Key words: cardiac microtissue; cardiotoxicity; hESC-CMs; inotropy; S100A1; contractility.

Drug associated cardiovascular toxicity causes severe morbidity (Schimmel et al., 2004) and is a major reason for attrition during drug discovery and development (Abassi et al., 2012; Albini et al., 2010; Laverty et al., 2011). It occurs at all stages of preclinical and clinical testing and may only be apparent during post-marketing several years after the cessation of treatment (Gwathmey et al., 2009; Mellor, 2011). Drug induced cardiovascular toxicity can result in both functional effects such as arrhythmia and acute alteration of the contractile function (inotropy) of the heart, and morphological (structural) damage to the myocardium (Cross et al., 2015; Laverty et al., 2011). Evaluation of the potential for both types of
Current in vitro approaches to analyse functional and structural cardiotoxicity are focused on utilizing CMs alone (Cross et al., 2015). Here for the first time, we have assessed the ability of adult primary cardiac microvascular endothelial cells and cardiac fibroblasts to induce contractile maturity in hESC-CM in scaffold-free microtissues. In particular, we have assessed the contractile phenotype in the context of pharmacological effects on cardiomyocyte contractility. Spheroid microtissues were formed from hESC-CM alone or in combination with human primary endothelial cells and fibroblasts obtained from either cardiac or non-cardiac (dermal) sources. Compared to all other cell combinations, spheroid microtissues derived from all 3 cardiac cell types showed remarkably enhanced contractile function in response to inotropic drugs, which suggested a greater degree of cardiomyocyte maturity. Evidence of maturity was confirmed at the molecular and structural level. In addition, we show that cardiac specific fibroblasts and endothelial cells enhance and promote maturity through upregulation of the Ca²⁺-binding protein S100A1. We conclude that noncardiomyocyte cells are critical for promoting an authentic cardiac phenotype in complex in vitro models and that such models can be used for more accurate drug safety screening and drug development.

**MATERIALS AND METHODS**

**Preparation of cardiac microtissues**

hESC-CMs (Cytiva) were obtained from General Electric Healthcare (Hertfordshire, UK). Human induced pluripotent stem cell CMs (hiPS-CMs) were obtained from Cellular Dynamics International (Madison, Wisconsin). Primary human cardiac microvascular endothelial cells (hCMECs), primary human dermal microvascular endothelial cells (hDMECs), primary human coronary artery endothelial cells), primary human cardiac fibroblasts (hCFs), and primary normal human dermal fibroblasts (NhDFs) were obtained from PromoCell (Heidelberg, Germany). All primary cells were subcultured prior to microtissue formation according to supplier’s instructions. All primary cells were detached with pre-warmed Accutase (Sigma, A6964) for 5 min at 37°C, 5% CO₂, centrifuged for 3 min at 1200 g before re-suspension in endothelial basal medium MV2 (PromoCell, C-22221) (supplemented with 5% foetal calf serum (FCS), epidermal growth factor (EGF; 5 ng ml⁻¹), basic fibroblast growth factor (bFGF; 10 ng ml⁻¹), insulin-like growth factor 1 (IGF-1; 20 ng ml⁻¹), vascular endothelial growth factor-A (VEGF-A; 0.5 ng ml⁻¹), Ascorbic Acid (1 μg ml⁻¹) and Hydrocortisone (0.2 μg ml⁻¹). Cell suspensions were counted, diluted and stored at 37°C, 5% CO₂ for up to one hour while hESC-CM cell suspensions were prepared. A vial of Cytiva CMs was thawed and suspended in RPMI 1640 media (Life technologies 61870044) containing b27 supplement (Life technologies 17504) (Pointon et al., 2013). CMEF/DEMF microtissues: cell suspensions were combined together to give a concentration of 500 cells per 100 μl comprising 4 parts hESC-CM, 2 parts hCF/NhDF, and 1 part hCMEC/hDMEC in microtissue media comprising 1 part RPMI (+b27 supplement) to 1 part Endothelial MV2 (+supplements). CME/CMF microtissues: cell suspensions were mixed together to give a concentration of 500 cells per 100 μl comprising 4 parts hESC-CM and 1 part hCF/hCMEC/hDMEC in microtissue media comprising 1 part RPMI (+b27 supplement) to 1 part Endothelial MV2 (+supplements). CME/CMF microtissues: cell suspensions were mixed together to give a concentration of 500 cells per 100 μl comprising 4 parts hESC-CM and 1 part hCF/hCMEC/hDMEC in microtissue media comprising 1 part RPMI (+b27 supplement) to 1 part Endothelial MV2 (+supplements). CME/CMF microtissues: cell suspensions were mixed together to give a concentration of 500 cells per 100 μl comprising 4 parts hESC-CM and 1 part hCF/hCMEC/hDMEC in microtissue media comprising 1 part RPMI (+b27 supplement) to 1 part Endothelial MV2 (+supplements). CME/CMF microtissues: cell suspensions were mixed together to give a concentration of 500 cells per 100μl comprising 4 parts hESC-CM and 1 part hCF/hCMEC/hDMEC in microtissue media comprising 1 part RPMI (+b27 supplement) to 1 part Endothelial MV2 (+supplements).
et al. (2015). After 48 h culture media was refreshed with iCell CM maintenance media. Suspensions were subsequently seeded into round bottom ultra-low adhesion 96-well plates (Corning Costar, CLS7007).

All microtissue plates were incubated at 37°C, 5% CO₂ for 14 days with media refreshed every 3–4 days. Experiments were conducted on microtissues following 14–28 days in culture.

Immunofluorescence

Microtissues were washed in D-phosphate buffered saline (PBS) until no media remained and subsequently fixed in 4% (w/v) para-formaldehyde for 1 h at 4°C, followed by 6 washes in D-PBS and stored at 4°C until processing. Microtissues were permeabilized with 0.5% (v/v) Triton X-100/D-PBS overnight at 4°C. Microtissues were transferred into a falcon tube, centrifuged at 1200g for 2 mins, and block reagent was removed and primary antibody added overnight at 4°C. Microtissues were washed three times with TBST (with 0.1% (v/v) Triton X-100) at RT, each time incubated for 1 h. Secondary antibody was diluted 1:1000 in 1% (w/v) BSA/TBST (with 0.1% (v/v) Triton X-100) and added to the microtissues overnight at 4°C. Microtissues were washed for 1 h with TBST (with 0.1% (v/v) Triton X-100) at RT and stained with Hoechst 33342 (2 μg/ml) in TBST (with 0.1% (v/v) Triton X-100) for 1 h at RT. Microtissues were wash briefly in TBST (with 0.1% (v/v) Triton X-100) before mounting onto microscope slides with ProLong Gold. Microtissues were imaged using a Zeiss AxioObserver inverted fluorescence microscope with Apotome 2, using Plan-Apochromat 20×/0.8 NA objective. Images were acquired using Zeiss Zen software. In order to allow direct comparisons between different microtissues, during image acquisition each microtissue was processed in a blinded manner and equal exposure was applied for each marker.

Gene expression analysis

Adult left ventricle, foetal heart, and smooth muscle total RNA were purchased from U.S. Biologic (Memphis, USA). Adult left ventricle heart total RNA was obtained from one 21-year-old normal male donor with no reported concomitant disease. hESC-CM total RNA was obtained from hESC-CMs cultured as described in Pointon et al. (2013). RNA was harvested following 72 h in culture as per the recommended manufacturer instructions. All primary cells were detached with prewarmed Accutase (Sigma, A6964) for 5 min at 37°C, 5% CO₂, centrifuged for 3 min at 1200 × g before lysis in RLT buffer (Qiagen) and stored at −80°C until processing. Microtissues were pooled and transferred into a falcon tube, centrifuged at 1200 × g for 2 mins and re-suspended in prewarmed PBS. Microtissues were re-centrifuged at 1200 × g for 2 min and lysed in RLT buffer (Qiagen). Total RNA was extracted using an RNeasy Mini Kit (Qiagen). A NanoDrop spectrophotometer (NanoDrop Technologies) was used to quantify RNA samples.

RNA samples were diluted to 20 ng and reverse transcribed using the SuperScript III first-strand synthesis supermix (Life technologies). cDNA samples were preamplified using the TaqMan PreAmp Master mix kit with TaqMan Probes (Supplementary Table S1), 10 cycles and final stock diluted 1:250. qRT-PCR was performed using 2.5 μl of preamplified cDNA and TaqMan Gene Expression master mix. qRT-PCR reactions were performed in a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California), 2.5 μl of preamplified cDNA was used with TaqMan Gene Expression probes (Supplementary Table S1) and master mix to monitor amplification under standard cycling conditions. GAPDH was used as an endogenous control. Relative quantification of gene expression was performed using the ΔΔCt relative quantitation method. The mean Ct value was calculated for each gene and normalized to the endogenous control, fold change relative to hESC-CM monolayer was determined and data expressed as a percentage of adult ventricle heart expression levels.

Solutions and compounds

Compounds were obtained from Sigma-Aldrich (Dorset, UK) or the AstraZeneca compound collection. Compounds were initially formulated in DMSO as a 1000× stock solution and subsequently serially diluted using DMSO. The DMSO stock solutions were diluted 1000× into the cardiac microtissue media to give the final test concentrations. The final vehicle concentration was 0.1% (v/v) DMSO. Where compounds were insoluble in DMSO, PBS, (Sigma) was used. Caffeine was formulated in microtissue media at 10 mM.

Video-based edge detection of contractions

Microtissues were transferred onto 0.1% (w/v) gelatin-coated 13 mm plastic coverslips (Thermanox) within a 12-well plate and incubated at 37°C, 5% CO₂ for at least 1 h before mounting onto the IonOptix contractility system. Contractile properties of microtissues were assessed using a video-based edge detection method (IonOptix, Dublin, Ireland) (Harmer et al., 2012). A gelatin-attached microtissue was placed in a perfusion chamber (FHC Inc., Bowdoinham, Maine) mounted at the stage of an inverted Nikon TE200 microscope (Nikon UK, Surrey, UK). The microscope stage (Prior Scientific Instruments, Cambridge, UK) was motorized and controlled remotely. Microtissues were continuously perfused from a gravity fed system at 2 ml/min with microtissue media heated to 37 ± 1°C using a line heater (Cell MicroControls, Norfolk, Virginia). The microtissues were field stimulated with suprathreshold voltage at a pacing frequency of 1 or 1.5 Hz, with a bipolar pulse of 6 ms duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a MyoPacer EP stimulator (IonOptix). Microtissues were imaged at 240 Hz using an IonOptix Myocam-S CCD camera. Digitized images were displayed within the IonWizard acquisition software (IonOptix). The edge of the microtissue of interest was aligned parallel to the video raster line, by means of a semi-automated dovecote prism optical system that could be controlled remotely (Cairn Research, Faversham, UK). Optical intensity data were collected representing dark and light bands at the edge of the microtissue of interest. The IonWizard software analyzes the periodicity in the optical density by means of a fast Fourier transform algorithm. The output of this analysis is a direct real-time measurement of microtissue contraction.

Each compound response was measured from separate randomly selected microtissues on triplicate independent occasions, only one microtissue was assessed at a time. After microtissues were added to the microtissue stage, perfusion with microtissue media (with 0.1% (v/v) DMSO as the vehicle control) was immediately started. At this time field stimulation was also started, and microtissues were left to acclimatize for approximately 2 min at approximately 37°C, after which at least 30s of basal contractions were recorded for each microtissue. Test compounds were applied in a cumulative manner with each concentration applied for 250s or until steady-state was reached.
achieved. Every 3–4 days, 10 μM verapamil was applied as a positive control, additionally beat rate was assessed at the start and end of the experimental time frame (14–28 days) to ensure consistency. Data from any microtissue batches that either did not elicit a response to 10 μM verapamil and/or varied in beat rate >10% over the time period were rejected.

Initial analysis was performed using the IonWizard software. For each test condition, data for 10–15 contractions were averaged, using the stimulation time as common reference point, to give a single representative monotonic contractility transient. This transient was analysed using the ‘monotonic transient analysis’ function of the IonWizard software. From this analysis, peak height, which indicates the percentage of peak contraction relative to the resting length was used to quantify microtissue dynamics. Concentration-effect curves were plotted for each compound and expressed relative to vehicle (0.1% (v/v) DMSO).

Data analysis
Concentration-effect curves were plotted for each compound and expressed relative to vehicle ± SD. The molar concentration of test compound producing 50% inhibition (IC50) or 50% response (EC50), was calculated using GraphPad Prism (La Jolla, California) to fit data to a 4-parameter nonlinear regression curve. Each IC50/EC50 was generated in triplicate for each compound and geometric means were subsequently calculated. F-tests to compare the concentration response curves respectively were performed using GraphPad Prism (La Jolla, California). T-tests and ANOVA to compare the conditions were performed using GraphPad Prism (La Jolla, California).

RESULTS
Development of Cardiac Microtissues
hESC-CMs were cultured either alone (CM microtissues) or with hCMECs and hCFs (CMEF microtissues) in 96-well round-bottomed ultra-low adhesion plates. Following two weeks in culture, microtissues with uniform size and shape, measuring 200 μm in diameter, with equal light exposure were formed (Figs. 1A and 2A). Immunofluorescence imaging of specific cardiac cell types: cardiomyocyte (alpha actinin, ACTN2), endothelial (CD31), and fibroblast markers (collagen 1, COL1A1) was utilized to determine the morphology and cellular architecture of the microtissues. The presence of hCMECs (CD31) (Figure 1B) and hCFs (collagen 1) (Figure 1C) were confirmed in the CMEF microtissue and located in close proximity to the hESC-CMs, displaying even cell type distribution throughout. Staining of α-actinin highlights hESC-CMs within the CMEF and CM microtissues. Differences in cardiac striations between the CM and CMEF microtissues were revealed following imaging of α-actinin with the latter displaying striations with enhanced organization and elongation (Figs. 1D and 2D).

CMEF Microtissues Display Improved Contractile Properties
To determine the contraction and relaxation kinetics of the microtissues, we used the IonOptix cell geometry measurement system and IonWizard software. This allowed measurement of movement of microtissues in real time at 37 °C, with a continuous flow of cell culture media, while spontaneously beating and following field stimulation at 0.5, 1, 2, and 3 Hz (i.e. equivalent to 30, 60, 120, and 180 beats/min). Under these conditions both microtissues displayed typical spontaneous synchronized contractility transients, with a spontaneous beat rate of 54 ± 6 and 24 ± 6 beats/min in CMEF and CM microtissues, respectively (mean ± SD, n = 5) (Figs. 3A and B); P value < .01. This spontaneous beat rate for each tissue remained constant over the period of study (14–28 days) confirming the viability of tissues (Figs. 3A and B). Furthermore, both microtissues were able to maintain synchronized, typical contraction transients following field stimulation (7 V for 6 ms) at 0.5, 1, 2, and 3 Hz (Figs. 3E and F), resulting in a mean beat rate of 29 ± 1.1, 59 ± 1.5, 90 ± 1.5, and 123 ± 8.5 beats/min, respectively in CMEF microtissues and 21 ± 3, 58 ± 4.1, 91 ± 3.2, and 119 ± 5.5 beats/min, respectively in CM microtissues (n = 3 ± SD). As changes in [Ca2+]i are intrinsically linked to changes in cardiomyocyte contraction, we investigated the relationship between these parameters in both microtissues. In field-stimulated (0.5, 1, 2, and 3 Hz) CM and CMEF microtissues, the rise in [Ca2+]i occurred before the initiation of contraction (Figs. 3G and H). Both microtissues showed a frequency–response relationship: as the frequency increased, the amplitude of the [Ca2+]i and contraction transients decreased (n = 3) (Figs. 3I and J). However, the CMEF microtissue...
produced Ca\textsuperscript{2+} transients of that were of higher amplitude at 1, 2, and 3 Hz. In order to assess the functionality of Ca\textsuperscript{2+} handling, microtissues were acutely exposed to caffeine (10 mM) through a short timescale exposure in the perfused media and contraction transients monitored during spontaneously beating conditions. CMEF microtissues produced an immediate transient increase in contraction number and amplitude, followed by cessation of contraction (Figure 3K). Whereas in CM microtissues; contractions gradually decreased in both number and amplitude prior to cessation of contraction (Figure 3L).

Due to these observed differences in Ca\textsuperscript{2+} handling, the pharmacological response following 1.5 Hz field stimulation to
A known in vivo negative (verapamil) and positive (dobutamine) inotrope was assessed to determine the suitability of these microtissues for the identification and investigation of changes in cardiac contractility. Contractility transients were recorded following vehicle control (0.1% (v/v) DMSO) and each compound on three independent occasions (n = 3). To take into account differences in basal contraction kinetics between microtissues, all data was normalized to the vehicle response (0.1% DMSO (v/v)) in each microtissue. Consequently any differences observed are a direct result of compound exposure. In both CMEF and
FIG. 3. Functional characterization of CMEF and CM microtissues. Contractile properties (edge) and Ca\(^{2+}\) kinetics were assessed using a video-based edge detection method (IonOptix, Dublin, Ireland). Microtissues were formed and incubated for 14 days prior to measurement of the spontaneous beat rate in both CMEF (A) and CM microtissues (B) at the start (day 14) and end (day 28) of the experimental period. Plates were incubated at 37°C, 5% CO\(_2\) for 14 days with media refreshed every 3–4 days. Experiments were conducted on microtissues following 14–28 days in culture. Representative trace showing contraction (edge) kinetics in spontaneous
CM microtissues, 10 μM verapamil reduced the contractility transients (Figs. 3M and N), whereas following 10 μM dobutamine the CMEF microtissue produced a positive inotropic response with an increase in contractile amplitude, but no effect was observed in the CM microtissue (Figs. 3O and P). This data demonstrates the potential utility of microtissues for the detection and investigation of cardiac contractility in vitro.

The potential of cardiac microtissues to detect a pharmacologically diverse panel of 12 inotropic compounds with in vivo changes in cardiac contractility (5 negative and 7 positive inotropes) and 5 noninotropic compounds without in vivo changes in cardiac contractility were assessed following field stimulation at 1.5 Hz. Cumulative concentration response curves and corresponding IC_{50} values were obtained for each compound in both CMEF and CM microtissues. Overall, all positive inotropes tested were detected with an increase in amplitude of contraction in CMEF microtissues. In comparison, in CM microtissues 4 out of 7 positive inotropes were detected; however, the direction of response was the reverse to that observed in vivo for all except epinephrine (Table 1). For example, the Na⁺/K⁺-ATPase membrane pump inhibitor, digoxin, increased the amplitude of contractility transients in CMEF microtissues, producing an EC_{50} value of 3.54 μM. In contrast, in CM microtissues a decrease in the amplitude of contractility transients was observed, resulting in an IC_{50} value of 0.4 μM. Additionally, following exposure to 5 negative inotropes, all were correctly detected in CMEF, but only 3 (diltiazem, doxorubicin, verapamil) were detected in the CM microtissue (Table 1). For example, the β1-adrenergic receptor antagonist, atenolol, resulted in IC_{50} values of 0.94 μM and above 100 μM in CMEF and CM microtissues respectively. This data indicates the superior utility of CMEF microtissues compared to CM microtissues with respect to in vitro to in vivo pharmacological correlation and relevance.

**Contractile Pharmacology Is Dependent upon a Cardiac tri-Culture System**

In order to further explore these differences in responses between CMEF and CM microtissues, three additional microtissues were evaluated. The first additional microtissue contained hESC-CMs and hCMECs (CME microtissue), the second contained hESC-CMs and hCF (CMF microtissue) and the third microtissue contained both endothelial and fibroblasts from a dermal vascular bed with hESC-CMs (DMEF microtissue; Supplementary Figure S1). Two negative (atenolol, lapatinib) and two positive (digoxin, dobutamine) inotropic pharmacologically diverse compounds were assessed to identify if one particular cell type within the CMEF microtissue was responsible for the differences observed. Following exposure to the β1-adrenergic receptor antagonist, atenolol, a negative inotropic response was observed in both the CMEF and CMF microtissues, resulting in IC_{50} values of 0.94 μM; no effect was observed in CM, CME, or DMEF microtissues (Figure 4A). However the EGFR-1/EGFR-2 (Her2) inhibitor, lapatinib (Albanell and Gascon, 2005), produced

| TABLE 1. Contractile Responses of CMEF and CM Microtissues to Known Pharmacological Agents |
|---------------------------------------------------------------|
| **Compound** | **Drug Target** | **CMEF Microtissue IC_{50}/EC_{50} (μM)** | **CM Microtissue IC_{50}/EC_{50} (μM)** | **In Vivo Effect** |
|----------------|----------------|------------------------------------------|------------------------------------------|-------------------|
| Amoxicillin | β-lactam antibiotic | NE | - | NE |
| Paracetamol | Cyclooxygenase inhibitor | NE | - | NE |
| Sildenafil | PDE-5 inhibitor | NE | - | NE |
| Enalapril | Angiotensin-converting enzyme inhibitor | NE | - | NE |
| Tolbutamide | ATP-sensitive potassium channel inhibitor | NE | - | NE |
| Atenolol | β1-adrenergic receptor antagonist | 0.94 | NE | -ve inotrope |
| Verapamil | L-type Ca²⁺ channel blocker | 0.11 | 1 | -ve inotrope |
| Diltiazem | L-type Ca²⁺ channel blocker | 0.38 | 2.47 | -ve inotrope |
| Doxorubicin | Intercalates DNA | 1.16 | 1.16 | -ve inotrope |
| Lapaatinib | EGFR-1/EGFR-2 (Her2) inhibitor | 0.86 | NE | -ve inotrope |
| Epinephrine | Nonselective adrenergic receptor agonist | 0.07 | 0.0012 | +ve inotrope |
| Glibenclamide | ATP-sensitive potassium channel inhibitor | 0.8 | NE | +ve inotrope |
| Levosimendan | Calcium sensitizer (binds cardiac troponin C) | 0.13 | NE | +ve inotrope |
| Digoxin | Inhibits Na⁺/K⁺-ATPase membrane pump | 3.54 | 0.4 | +ve inotrope |
| Dobutamine | β1-adrenergic receptor agonist | 0.29 | 0.03 | +ve inotrope |
| Isoproterenol | β1-adrenergic receptor agonist | 0.0004 | 0.00009 | +ve inotrope |
| Milrinone | PDE-3 inhibitor | 83.68 | NE | +ve inotrope |

Contractile response of CMEF and CM microtissues to a panel of 12 pharmacologically diverse inotropic compounds. Drug targets and known in vivo effects are shown. IC_{50}/EC_{50} shown (n = 3). Direction of inotropic response highlighted with arrows (↑ increase in inotropy, ↓ decrease in inotropy). Correct prediction compared to in vivo effect highlighted in green, incorrect prediction compared with in vivo effect highlighted in red.

Contracting CMEF and CM microtissues (C), the number of contractions over a period of time were quantified (D) (n > 3, mean ± SD). Representative recordings of CMEF (O) and CM microtissue (P) contractions following field stimulation at 0.5, 1, 2, and 3 Hz. Representative traces following measurement of Ca²⁺ (red trace) and contraction transients (edge) (blue trace) in (Q) CMEF and (R) CM microtissues following field stimulation at 0.5, 1, 2, 3 Hz with 7 V for 6 ms. Summary of the frequency-response relationship for contraction (edge) (O) and [Ca²⁺], (Q) at 0.5, 1, 2, and 3 Hz, n = 3, (±) SD in both CMEF (open bars) and CM microtissues (solid bars). A representative experiment showing the effect of 10 mM caffeine on contraction transients elicited at 1.5 Hz stimulation in CMEF (O) and CM (P) microtissues 20s of vehicle control (0.1% DMSO) contraction transients (left side) and > 10 s of 10 μM caffeine contraction transients (right side) are shown. Representative contracility transients elicited at 1.5 Hz stimulation in CMEF (Q) and CM (P) microtissues following 10 μM dobutamine. 20 s of basal contraction transients (left side) and 20 s of 10 μM dobutamine contraction transients (right side) are shown. *P-value < .01. (Full color version available online.)
a negative inotropic response in both CMEF and CME microtissues resulting in IC50 values of 0.86 and 1 μM respectively; no effect was observed in CM, CMF, and DMEF microtissues (Figure 4B).

Following exposure to the known in vivo positive inotropes, dobutamine, and digoxin, a positive inotropic response was only observed in the CMEF microtissue resulting in EC50 values of 0.3 and 3.54 μM, respectively, the converse response was observed in CM, CME, and CMF microtissues, resulting in IC50 values of 0.02, 0.01, and 0.001 μM respectively following dobutamine and 0.7, 0.9, and 0.3 μM respectively following digoxin (Figs. 4C and D). DMEF microtissues displayed no response to either positive inotrope. These results highlight the requirement of both endothelial and fibroblast cells of cardiac origin to be present for the enhanced pharmacological response to be observed. To confirm that the enhanced pharmacological response of the multicellular microtissue was not restricted to microtissues composed of hESC-derived CMs we also utilized iPSc-derived CMs. Two microtissues containing hiPS-CMs were prepared; hiPS-CMs in combination with hCMECs and hCF (hiPS CMEF) and hiPS-CMs alone (hiPS CM). Following exposure to the known in vivo positive inotrope, dobutamine, a positive inotropic response was again only observed in the hiPS CMEF microtissue, resulting in an EC50 value of 0.39 μM in hiPS CMEF; the converse response was observed in the hiPS CM microtissue, resulting in an IC50 value of 0.1 μM (Figure 4E). These results indicate the suitability of both hESC-CM and hIPC-CM microtissues to detect changes in cardiac contractility.

**FIG. 4.** The contractile response of cardiac microtissue models to known inotropic compounds. Concentration-effect curves of CMEF, CM, DMEF, CME, and CMF microtissues to (A) atenolol, (B) lapatinib, (C) dobutamine, and (D) digoxin. E, concentration-effect curves of microtissues containing hiPS-CMs alone or in combination with hCMECs and hCF response to dobutamine. Data expressed relative to vehicle (0.1% (v/v) DMSO) (n = 3 ± SD). *P-value < .05.
In order to provide further mechanistic insight into possible explanations for these enhanced physiologically relevant responses in CMEF microtissues, gene expression analysis of a panel of genes involved in microtubule and sarcomere assembly (S100A1; S100 calcium-binding protein A1, TCAP; titin-cap), cardiovascular function (ADRB1; β1-adrenergic receptor, PDE3A; phosphodiesterase 3, KCND3; potassium voltage-gated channel Kv4.3), nitric oxide production (NOS3; nitric oxide synthase 3) and cardiomyocyte maturity (MYH6; myosin heavy chain 6, MYH7; myosin heavy chain 7) were evaluated. When compared with the expression of these genes in monolayer hESC-CMs and CM microtissues, CMEF microtissues showed increased expression of S100A1, TCAP, PDE3A, NOS3 and KCND3 (P value < .01) with fold changes of 0.007, 0.12, 0.07, 0.23, and 0.1 in CMEF microtissues and 0.0007, 0.03, 0.03, 0.08, and 0.05 in CM microtissues compared to adult ventricular tissue, respectively (Figs. 5A–D and F). These expression levels are comparable to those observed in foetal cardiac tissue. Whereas, ADRB1 expression was similar to adult ventricle tissue in both CMEF and CM microtissues with fold changes of 1.9 and 1.3 (P value < .05), respectively, this is compared with a fold change of 0.02 in monolayer hESC-CMs (Figure 5E). MYH7, a predominantly adult myosin chain, was expressed at equal levels in CMEF and CM microtissues with a fold change of 0.15 relative to adult ventricle tissue, an expression level comparable to foetal heart and hESC-CM monolayer. MYH6, predominantly a foetal myosin chain, was highly expressed in CMEF and CM microtissues, 13- and 5-fold, respectively (P value < .01), compared with adult ventricle (Figs. 5G and H).

**S100A1 Regulates Contractile Maturity**

S100A1, a cardiac Ca\(^{2+}\) regulator (Maco et al., 2001), showed enhanced upregulation specifically within CMEF microtissues (Figure 5A). Considering the different caffeine-ICR responses of the CMEF microtissues we hypothesized that S100A1 may have a role in the contractile maturity of the CMEF microtissues. To confirm this, short interfering RNA (siRNA)-mediated knockdown of S100A1 was performed in CMEF microtissues and its effect on caffeine response studied. Partial knockdown of S100A1 mRNA expression by 42% ± 2.58 (mean ± SD) (Figure 6A), resulted in a complete loss of the transient increase in CMEF contraction amplitude following caffeine exposure (Figs. 6B and C).

**DISCUSSION**

The human heart is composed of CMs, fibroblasts and endothelial cells, which facilitate normal cardiac development and physiological cardiac function (Brutsaert, 2003). Here we have shown that adult cardiac nonmyocyte cells can promote...
contractile maturity of embryonic stem cell derived CMs in vitro by co-culturing as a 3D microtissue to better recapitulate the in vivo cellular physiology and pharmacological response.

The advent of stem-cell derived human CMs offers the potential for more physiologically relevant preclinical safety testing for drug-induced cardiotoxicity and disease understanding. However, it has been widely reported that hESC-CMs structurally resemble embryonic CMs and lack the complex communications required for efficient excitation-contraction coupling (Kehat et al., 2003). In the adult CM, isoproterenol, a β1-adrenergic receptor agonist, displays positive inotropy (increase in contraction amplitude), and positive chronotropy (increase in contraction rate). hESC-CMs elicit positive chronotropy but no positive inotropy suggesting hESC-CMs have some degree of β1-adrenergic receptor expression, but again highlighting the immaturity of this cell model (Robertson et al., 2013). Here we have demonstrated the relatively low level β1-adrenergic receptor expression found in hESC-CMs can be increased using three dimensional cell culturing (Figure 5E).

The enhanced in vitro to in vivo pharmacological correlation observed in the CMEF microtissues compared with the CM microtissues was evident across a range of different negative inotropic drugs targeting a diverse set of targets (Table 1), eg, the β1-adrenergic receptor (atenolol) and EGFR-1/EGFR-2 receptor (lapatinib) and positive inotropic drugs targeting the β1-adrenergic receptor and Na/K+ ATPase (digoxin). The ability of atenolol to inhibit contraction in the absence of exogenous catecholamines can be potentially explained by either the inverse agonist properties of β-adrenoceptor antagonists (Varma et al., 1999) or endogenous secretion of catecholamines by the microtissue (Sorriento et al., 2012). The positive pharmacological correlation evident in the CMEF was restricted to cardiac endothelial cells and fibroblasts, as substitution with dermal equivalents of microvascular endothelial cells and fibroblasts in the microtissue did not reconstitute the positive correlation. This difference between the effect of CMEF microtissues compared with CM microtissues was not restricted to the use of hESC-derived CMs as a similar effect on contractility with dobutamine was observed using iPSc-derived CMs (Figure 4E).

This positive pharmacological effect was not due to differences in cellular architecture within the microtissues as the dermal nonmyocyte cells and cardiac nonmyocyte cells formed multicellular microtissues with a similar structure. However, differences in gene expression between the different multicellular microtissues suggest that the cardiac derived nonmyocytes are capable of inducing a transcriptional program within the CMs that results in pharmacological maturity. Analysis of gene expression in the different cardiac microtissues revealed that the Ca2+ binding protein S100A1 was upregulated in the CMEF microtissue relative to the CM microtissue (Figure 5A). The importance of this gene upregulation was confirmed using a partial siRNA-mediated knockdown CMEF model which highlighted a loss of inotropic response to a short timescale exposure of the Ca2+ mobilizing agent caffeine. Studies have

![FIG. 6. S100A1 regulates contraction in response to caffeine. CMEF microtissues were formed and incubated for 14 days prior to transfection with 10 nM nonsilencing siRNA (–ve control) and 10 nM S100A1 siRNA. A, RNA was extracted from microtissues and expression of GAPDH and S100A1 mRNA analysed by qRT-PCR (n = 3, ± SD). Contractile response recorded using the IonOptix cell geometry system under vehicle control (0.1% v/v DMSO) and 10 mM caffeine in (B) nonsilencing siRNA control CMEF microtissue and (C) S100A1 siRNA transfected CMEF microtissue (results from 1 experiment representative of 3 separate experiments).]
Previously highlighted a correlation between S100A1 protein expression and inotropy response (Bennett et al., 2014). Furthermore, adenoviral mediated over-expression of S100A1 improved β, adrenergic mediated contractile reserve in CMs from failing human hearts, suggesting that this protein may play multiple roles in regulating cardiomyocyte physiology (Brinks et al., 2011).

In adult CM, Ca2+ induced Ca2+ release (CICR) from the sarcoplasmic reticulum contributes almost 70% of the total Ca2+ release. In contrast, immature CMs demonstrate Ca2+ transients that are smaller and slower (Robertson et al., 2013), with most cation influx of Ca2+ through the cell membrane (Liu et al., 2007). This results in abnormal diffusion of Ca2+ into the cell and reduces the synchrony in contraction necessary for large force generation. There is also consensus that intracellular Ca2+ stores are smaller than in adult CM and less reliant upon ryanodine receptors for Ca2+ release. Ca2+ handling and response to compounds that modify Ca2+ handling varies greatly between immature and mature CMs. The differences in the amplitude of the Ca2+ transient between CMEF and CM microtissues, that were not evident for the edge transient (Figs. 3G and H), could represent a difference in the excitation–contraction coupling process between the two microtissues, potentially due to differences in Ca2+ handling and storage. Indeed, Ca2+ currents have been recorded in both endothelial cells (Tran et al., 2000) and cardiac fibroblasts (Li et al., 2009), therefore Ca2+ transients in these cell types could contribute to the increase in Ca2+ transient amplitude in the CMEF microtissue. The increased Ca2+ transient amplitude, response to Ca2+-dyshomeostatic compounds (for example levosimendan and caffeine) and increased expression of the Ca2+-handling protein S100A1 suggests a dimensional co-culture with stem cell-derived CMs have the potential to highly impact cardiac research and analysis of drug-induced cardiovascular toxicity.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org.

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