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Original article

# Modulation of human embryonic stem cell-derived cardiomyocyte growth: A testbed for studying human cardiac hypertrophy?

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ABSTRACT

Human embryonic stem cell-derived cardiomyocytes (hESC-CM) are being developed for tissue repair and as a model system for cardiac physiology and pathophysiology. However, the signaling requirements of their growth have not yet been fully characterized. We showed that hESC-CM retain their capacity for increase in size in long-term culture. Exposing hESC-CM to hypertrophic stimuli such as equiaxial cyclic stretch, angiotensin II, and phenylephrine (PE) increased cell size and volume, percentage of hESC-CM with organized sarcomeres, levels of ANF, and cytoskeletal assembly. PE effects on cell size were separable from those on cell cycle. Changes in cell size by PE were completely inhibited by p38–MAPK, calcineurin/FKBP, and mTOR blockers. p38–MAPK and calcineurin were also implicated in basal cell growth. Inhibitors of ERK, JNK, and CaMK II partially reduced PE effects; PKG or GSK3β inhibitors had no effect. The role of p38–MAPK was confirmed by an additional pharmacological inhibitor and adenoviral infection of hESC-CM with a dominant-inhibitory form of p38–MAPK. Infection of hESC-CM with constitutively active upstream MAP2K3b resulted in an increased cell size, sarcomere and cytoskeletal assembly, elongation of the cells, and induction of ANF mRNA levels. siRNA knockdown of p38–MAPK inhibited PE-induced effects on cell size. These results reveal an important role for active protein kinase signaling in hESC-CM growth and hypertrophy, with potential implications for hESC-CM as a novel in vitro test system. This article is part of a special issue entitled, "Cardiovascular Stem Cells Revisited".



## 1. Introduction

Human embryonic stem cells (hESC) are presently the stem cell type with the greatest proven capacity for producing phenotypically authentic cardiomyocytes (hESC-CM). While their use for cardiac repair faces a number of logistical problems, they are widely held to have great promise as a potential human-based *in vitro* cardiomyocyte model system for the cardiac researcher and the pharmaceutical industry. This potential has been enhanced by the realization that both hESC and their close cousins, the induced pluripotent stem cells (iPSC), can be obtained with disease-specific genotypes [1]. hESC-CM are stable in long-term culture and show relative ease of genetic manipulation compared to adult primary cardiomyocytes. Based on their gene expression patterns and electrophysiological, morphological, and contractile properties, the majority of hESC-CM initially resemble human immature cardiomyocytes but have the capacity to develop in a number of respects [2–5]. Acute contractile and electrophysiological characteristics of hESC-CM show promise in terms of reflecting the adult human phenotype [4,6,7], and models of arrhythmia generation have already been described [8,9]. However, it is less obvious whether longer term responses of hypertrophy, proliferation, and apoptosis, important for both cardiac pathology studies and toxicology, would have similar fidelity.

In this study, we have focused on hypertrophic responses in hESC-CM. We have used canonical inducers of both pathological and physiological hypertrophy (phenylephrine, angiotensin II, and stretch) and quantitated the output in terms of a wide range of hypertrophic markers. Importantly, we have used high-content automated microscopy to gather a number of these measurements, pointing the way towards high-throughput assays. We have interrogated the mechanism underlying the hypertrophic changes, initially using a broad screen of small molecule inhibitors for some of the most widely known hypertrophic pathways. Selecting the most active stimulus/inhibitor combination, we have verified the result

*Abbreviations:* ANF, atrial natriuretic factor; bFGF, basic human fibroblast growth factor; CaMK II, $Ca^{2+}$/calmodulin-dependent kinase II; EB, embryoid body; ERK, extracellular signal-regulated kinases; GSK3, glycogen synthase kinase 3; HDACII, histone deacetylase; FKBP, FK506 binding protein; hESC, human embryonic stem cells; hESC-CM, human embryonic stem cell-derived cardiomyocytes; JNK, c-Jun N-terminal kinases; MAP2K4 and MAP2K3, MAPK kinase 4 and 3, respectively; MEF, mouse embryonic fibroblast; MHC, myosin heavy chains; MOI, multiplicity of infection; mTOR, mammalian target of rapamycin; p38–MAPK, p38 mitogen-activated protein kinase; PKG, protein kinase G; Ryr2, cardiac ryanodine receptor 2; and SERCA2, sarco/endoplasmic reticulum $Ca^{2\pm}$-ATPase.

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using overexpression of upstream activators or dominant-negative constructs and downregulation using siRNA. Our results form a basis for the use of hESC-CM as a hypertrophic model system for cardiac research and drug discovery/toxicology.

## 2. Materials and methods

### 2.1. Differentiation and isolation of human embryonic stem cell-derived cardiomyocytes

Cardiomyocytes were derived from human ESC line H7, which was grown on Matrigel (BD Sciences)-coated plates with daily changes of mouse embryonic fibroblast (MEF)-conditioned medium, supplemented with 8 ng/ml recombinant basic human fibroblast growth factor (bFGF, Invitrogen) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). MEFs were isolated from 13 dpc MF-1 strain mouse embryos and treated with mitomycin C (0.01 mg/ml, Sigma) at passage 4. MEF-CM was prepared from mitotically inactive MEFs by daily feeding/collecting hESC medium containing 80% KnockOut DMEM (KO-DMEM), 20% KOSR, 1 mM L-glutamine, 10 mM non-essential amino acids, antibiotics, 0.1 mM β-mercaptoethanol, and 4 ng/ml bFGF (all from Invitrogen) for up to a week (150 ml/$18.8\times10^6$ cells/T225 flask). Human ESC were differentiated via embryoid bodies (EBs) by mechanically breaking up the colonies after 3–10 min of collagenase IV (Invitrogen) treatment to remove spontaneously differentiated cells, followed by culturing in suspension culture in low adherence plates for 4 days in differentiation medium (hESC medium in which 20% KOSR was replaced by non-heat-inactivated foetal calf serum) [6,10]. The EBs were plated out onto gelatine (0.5%)-coated plastic dishes, and spontaneously beating areas, which appeared from day 9 after EB formation, were microdissected from EB outgrowths at around day 30 (range 25–40 days). In some experiments, cells were isolated from beating clusters at other time points after differentiation. Differentiated hESC in T175 flasks or 10-cm culture dishes were removed from the surface by treatment with trypsin-EDTA (Sigma-Aldrich) for 5 min and collagenase IV for 10 min, counted and plated onto 96-well plates coated with 0.5% gelatin. These were grouped either as 15 to 40 days (early), 41 to 60 days (intermediate) and 61–180 days (late) after differentiation. For high-content measurements, cells were generated from dense hESC monolayers, which were treated with human recombinant Activin A (100 ng/ml, R&D Systems) (day 0–1), and bone morphogenetic protein 4 (BMP4, 10 ng/ml, R&D Systems) (days 1–5) in RMPI-B27 medium (Sigma) [11]; spontaneously beating areas appeared within 1–2 weeks after BMP4 withdrawal. Following dissociation of clusters or monolayers into single cells, cells were seeded onto gelatinized dishes and subjected to treatments after overnight attachment in differentiation medium.

### 2.2. Use of phenylephrine, angiotensin II and cyclic mechanical stretch

To determine the effect of hypertrophic G-protein-coupled receptor agonists, hESC-CM were incubated in differentiation medium containing 10 μM α-adrenergic phenylephrine or 1 μM angiotensin II (both Sigma) for 48 h. In separate sets of experiment, cultures of isolated hESC-CM were exposed to cyclic equiaxial mechanical stretch in the presence of normal medium. Frequency of cyclic stretch was 0.5 Hz with pulsation of 10–25% elongation of cells for 24 h. Cells were stretched by applying a cyclic vacuum suction under Bioflex plates with computer-controlled equipment (FX-2000; Flexcell International). Control cultures remained on the plate without stretch.

### 2.3. Small molecule inhibitors of hypertrophy

To determine the effect of protein kinase inhibition on growth in cell size and proliferation, selective small molecule p38 inhibitor SB202190 (1 μM, Sigma), PKG inhibitor KT5823 (1 μM), HDAC II inhibitor trichostatin A (0.25 μM), ERK inhibitor PD98059 (10 μM), JNK inhibitor SP600125 (1 μM), GSK3β inhibitor 1-azakenpaullone (10 μM), CaMK II inhibitor KN93 (10 μM), calcineurin inhibitor cyclosporine A (0.2 μM), mTOR inhibitor rapamycin (10 ng/ml), and calcineurin/FKBP inhibitor FK506 (0.1 μM) were administered to hESC-CM in the presence of absence of phenylephrine for 48 h. The effect of phenylephrine was also tested in the presence of cell cycle inhibitors: myosin II inhibitor blebbistatin (10 μM, for 48 h) and synthetic anti-tubulin agent nocodazole (50 ng/ml, for 6 h). DMSO was used as control and did not affect cell size.

### 2.4. Targeting of p38–MAPK by dominant negative p38–MAPK and constitutively active MAP2K3b adenoviruses and p38 siRNA knockdown

For further characterization of p38–MAPK effects, we over-expressed a dominant-negative form of p38α (p38αDN, a gift from Dr. Yibin Wang) or constitutively active MAP2K3b (a gift from Dr. Michael Marber) in hESC-CM. p38α DN was mutated in its dual phosphorylation site (from T-G-Y to A-G-F), causing lack of kinase activity. Cells were infected on day 1 in culture by adding titered adenovirus to the culture medium at a multiplicity of infection (MOI) of 4 or greater. The gene transfer efficiency of cultures was determined through parallel infections with GFP adenovirus (Ad-CMV-GFP). For siRNA knockdown, p38 siRNA (Ambion Silencer pre-designed MAPK14, s3586, Applied Biosystems) transfection was performed using Oligofectamine reagent (Invitrogen, 1 μl/well, final incubation volume 50 μl) per manufacturer's instructions. Scrambled siRNA and mock transfection were used as negative controls.

### 2.5. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and labeled with anti-cardiac specific troponin I (cTnI, Santa Cruz, 1:200 dilution), anti-Ki67 (proliferation marker, Abcam, 1:100), anti-p38–MAPK (A-12, Santa Cruz, 1:100), anti-atrial natriuretic factor (ANF, a marker of hypertrophy, Santa Cruz, 1:300), Rhodamine–phalloidin (Invitrogen, 1:500), mTOR (Abcam, 1:100), anti-sarcomeric myosin heavy chain (MF20, Hybridoma Bank, 1:200), and anti-myosin heavy chain α/β (MHC α/β, clone 3-48, Abcam, 1:200) primary antibodies. Primary antibodies were detected with FITC- (Abcam), Alexa 488- (Invitrogen), Alexa 546- (Invitrogen), and Cy5- (Abcam) conjugated secondary antibodies (all 1:400). DNA was visualized with DAPI (0.5 μg/ml; Sigma). Images were acquired on Zeiss Axio Observer Z1 fluorescence microscopy.

### 2.6. Plate imaging

Combinations of immunocytochemistry markers were used to further characterize detailed phenotypic properties of hESC-CM culture. The hESC-CM cultures were dissociated into individual cells before treatment and plated at low density (up to 5000 cells per well of a 96-well plate). Plates were scanned on ArrayScan™ VTi automated microscopy and image analysis system (Cellomics Inc., Pittsburgh, PA, USA) using modified Target Activation, Cell Cycle, Morphology Explorer and Compartmental Analysis BioApplication protocols. Using the system of automated highly sensitive fluorescence imaging microscope with 10× objective and suitable filter sets, the stained cells were identified with DAPI in fluorescence channel 1, cTnI- or MHC α/β-Alexa 488 in channel 2 and ANF-, and Ki67-Alexa546 in channel 3, respectively. The arbitrary value calculated from the standard deviation of the intensity of the pixels under the channel measuring DAPI reflected the content of the intact and fragmented DNA. The maximal ratio of the MHC-positive cells versus the whole differentiated hESC population was $45.4\pm3.5\%$ (from $n=15$ experiments, the average ratio was $20.4\pm3.3\%$, e.g., Fig. 1A). An approximate estimate showed that ~$4\times10^5$ initial