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Improved Human Embryonic Stem Cell Embryoid Body Homogeneity and Cardiomyocyte Differentiation from a Novel V-96 Plate Aggregation System Highlights Interline Variability

PAUL W. BURRIDGE,[a,b] DAVID ANDERSON,[b] HELEN PRIDDLE,[a,c] MARIA D. BARBADILLO MUÑOZ,[a,b] SARAH CHAMBERLAIN,[c] CINZIA ALLEGRUCCI,[a,b] LORRAINE E. YOUNG,[a,b] CHRIS DENNING[a,b]

[a]Wolfson Centre for Stem Cells, Tissue Engineering and Modelling, School of Human Development, [b]Institute of Genetics, and [c]Nottingham University Research and Treatment Unit, University of Nottingham, Queen's Medical Centre, Nottingham, United Kingdom

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ABSTRACT

Although all human ESC (hESC) lines have similar morphology, express key pluripotency markers, and can differentiate toward primitive germ layers in vitro, the lineage-specific developmental potential may vary between individual lines. In the current study, four hESC lines were cultured in the same feeder-free conditions to provide a standardized platform for interline analysis. A high-throughput, forced-aggregation system involving centrifugation of defined numbers of hESCs in V-96 plates (V-96FA) was developed to examine formation, growth, and subsequent cardiomyocyte differentiation from >22,000 EBs. Homogeneity of EBs formed by V-96FA in mouse embryo fibroblast-conditioned medium was significantly improved compared with formation in mass culture ($p < .02$; Levene's test). V-96FA EB formation was successful in all four lines, although significant differences in EB growth were observed during the first 6 days of differentiation ($p = .044$ to .001; one-way analysis of variance [ANOVA]). Cardiomyocyte differentiation potential also varied; 9.5% ± 0.9%, 6.6% ± 2.4%, 5.2% ± 3.1%, and 1.6% ± 1.0% beating EBs were identified for HUES-7, NOTT2, NOTT1, and BG01, respectively ($p = .008$; one-way ANOVA). Formation of HUES-7 V-96FA EBs in defined medium containing activin A and basic fibroblast growth factor resulted in 23.6% ± 3.6% beating EBs, representing a 13.1-fold increase relative to mass culture (1.8% ± 0.7%), consistent with an observed 14.8-fold increase in *MYH6* (αMHC) expression by real-time polymerase chain reaction. In contrast, no beating areas were derived from NOTT1-EBs and BG01-EBs formed in defined medium. Thus, the V-96FA system highlighted interline variability in EB growth and cardiomyocyte differentiation but, under the test conditions described, identified HUES-7 as a line that can respond to cardiomyogenic stimulation. STEM CELLS *2007;25:929–938*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Numerous differentiated lineages have now been derived from hESCs [1], supporting the notion that these cells hold promise as tools for scientific research and as cell-based therapeutics. It has also been suggested that approximately 150 hESC lines will be required to match the majority of HLA isotypes in the U.K. population for transplantation [2]. However, considerably more hESC lines will be required if differentiation toward specific lineages is favored or restricted by the inherent or culture-induced properties of individual lines. It will therefore be important to evaluate lineage-specific differentiation potential by direct interline comparison, preferably under parallel, standardized conditions.

Comparison of hESC lines has begun through several organizations, including the International Stem Cell Initiative, the NIH Stem Cell Unit, and the American Type Culture Collection [3]. These initiatives are investigating variability in parameters such as expression of surface markers and relative RNA levels in undifferentiated hESCs or at early stages of differentiation. Other interline comparisons indicate that there are differences in population doubling times [3], transcriptome [3], hematopoietic differentiation [4], and teratoma formation [5, 6]. However, the wide range of feeder cells, culture media, additives, and passage methods used between lines confounds interpretation of true interline differences. Consequently, it is challenging to draw conclusions as to whether variability is due to inherent genetic variation or environmental interference.

In an effort to limit the influence of culture-induced differences in interline comparison, we recently developed standardized conditions for culture and cardiomyocyte differentiation of two independently derived hESC lines, HUES-7 and BG01 [7]. These conditions have now been extended to NOTT1 and NOTT2, which we recently derived. However, the differentiation strategy we originally used [7] involved formation of embryoid bodies (EBs) from clumps of hESC colonies harvested using collagenase treatment, and

Correspondence: Chris Denning, B.Sc., Ph.D., University of Nottingham–Institute of Genetics, Queen's Medical Centre, Nottingham NG7 2UH, U.K. Telephone: 44(0)115-82-30385; Fax: 44(0)115-970-9906; e-mail: chris.denning@nottingham.ac.uk Received September 22, 2006; accepted for publication December 12, 2006; first published online in STEM CELLS *EXPRESS* December 21, 2006. ©AlphaMed Press 1066-5099/2007/$30.00/0 doi: 10.1634/stemcells.2006-0598

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this resulted in a high degree of heterogeneity in EB size, morphology, and formation efficiency within each experiment. This confounds the ability to meaningfully test the efficacy of new factors/protocols on differentiation.

In this report, we have adapted an approach proposed by Ng et al. [8] to develop a high-throughput, forced-aggregation system that involves centrifugation of defined cell numbers in V-bottom 96-well plates (termed V-96FA). More than 22,000 EBs were generated either from 1,000, 3,000, or 10,000 trypsin-passaged HUES-7, BG01, NOTT1, and NOTT2 hESCs, which were routinely maintained in the same feeder-free conditions on Matrigel. V-96FA resulted in significantly greater reproducibility in EB size and gross morphology during early differentiation and, in some cases, in more beating areas at late stages of differentiation. Interestingly, significant interline variability was observed in EB size and cardiomyocyte differentiation. Moreover, although V-96FA formation of HUES-7 EBs in defined medium supplemented with activin A and basic fibroblast growth factor (bFGF) could dramatically enhance cardiomyogenesis, these conditions failed to produce beating outgrowths in the other lines tested. These data suggest that derivation of hESC lines with redundancy in HLA coverage will likely be required to ensure delivery of the full spectrum of clinically relevant lineages suitable for transplantation.

Materials and Methods

Materials and General Culture

Culture reagents were purchased from Invitrogen (Paisley, U.K., http://www.invitrogen.com), and chemicals were from Sigma-Aldrich (Poole, U.K., http://www.sigmaaldrich.com) unless otherwise specified. Culture was carried out at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed daily for hESC cultures and every 3–4 days during differentiation.

hESC Culture

HUES-7p11 cells were kindly donated by Harvard University [9] and expanded using trypsin passaging on mouse embryo fibroblast (MEF) feeders for cryopreservation at p17, as recommended by the supplier. Aliquots of HUES-7 were thawed to Matrigel-coated flasks and cultured using trypsin passaging in feeder-free conditions in conditioned medium (CM); under these conditions, HUES-7 cells were routinely passaged by incubating with 0.05% trypsin for <1 minute at 37°C and then tapping the flasks to liberate single cells or preferably small clumps of cells. To prepare CM, MEFs (strain CD1, 13.5 days post coitum) were mitotically inactivated with mitomycin C (MMC) (10 μg/ml, 2.5 hours) and seeded at 6×10^4 cells per cm^2. The next day, inactivated MEFs in a T75 flask were incubated with 25 ml of Dulbecco's modified Eagle's medium-Ham's F-12 medium (DMEM-F12) supplemented with 15% KnockOut Serum Replacement, 100 μM β-mercaptoethanol (β-ME), 1% nonessential amino acids (NEAA), 2 mM GlutaMAX, and 4 ng/ml bFGF for 24 hours, at which time CM was harvested and made ready for use by supplementing with an additional 4 ng/ml bFGF. Each flask of inactivated MEFs was used to condition the medium for 7 consecutive days [7, 10].

BG01p24 cells were purchased from BresaGen (Athens, GA, http://www.bresagen.com) [11] and expanded using manual passaging on MEF feeders to p37, as recommended by the supplier. BG01 cells were transferred to feeder-free conditions in CM at p40 and cultured as for HUES-7. NOTT1 and NOTT2 were derived at the University of Nottingham with informed patient consent and in accordance with Human Fertilisation and Embryology Authority license R0141-2-a. Fresh IVF embryos (grade 2/3) were cultured for 5–6 days to the blastocyst stage in Vitrolife GIII sequential medium, as specified by the manufacturer. The zona pellucida was removed by treatment with acid Tyrode's, and the embryo was plated to MMC-inactivated MEFs (7.5×10^4 cells per cm^2). Cultures were maintained in 80% KnockOut DMEM, 20% ES Screened Hyclone fetal bovine serum (Perbio, Tattenhall, U.K., http://www.perbio.com), 1× GlutaMAX, 1% NEAA, 100 μM β-ME, 4 ng/ml bFGF, and 10 ng/ml human recombinant leukemia inhibitory factor (Chemicon, Temecula, CA, http://www.chemicon.com). After 5 days, hESC outgrowths were isolated and expanded using manual passaging on MEF feeders. NOTT1p15 and NOTT2p18 were transferred to feeder-free trypsin/Matrigel culture in CM and cultured as for HUES-7. Both NOTT lines expressed OCT-4, SSEA-4, TRA-1–60, and TRA-1–81 but were negative for SSEA-1 (data not shown).

Karyotype Assessment

Exponentially growing cultures of at least 1×10^5 cells were treated with 100 ng/ml colcemid (Karyomax) for 45 minutes and harvested with 0.05% trypsin-EDTA. Pelleted cells (200*g* for 4 minutes) were resuspended in 0.6% sodium citrate and incubated at 37°C for 20 minutes. Cells were then centrifuged (300*g* for 4 minutes) and fixed by resuspension in 16.7% glacial acetic acid in methanol before washing with two additional changes of fixative. Chromosome spreads were prepared by dropping cells onto glass slides, which were air-dried and heated to 70°C overnight. Chromosomes were G banded with trypsin and stained with Leishman's. For each culture, 30 metaphase spreads were examined; full analysis involving band-by-band comparison between chromosome homologues was performed on three spreads, and the presence of gross abnormalities was visually examined in 27 spreads, in accordance with the International System for Human Cytogenetic Nomenclature international guidelines [12].

Feeder-free, trypsin-passaged cultures of HUES-7 were maintained from p18 to p37. Normal karyotype (46,XY) was observed at p18 and p31. However, by p37, only 22 (73%) cells of the 30 cells analyzed per culture (duplicate cultures) were 46,XY, up to seven (23%) cells were 47,XY,+12, and the remaining spreads showed nonclonal random gain or loss of chromosomes. Therefore, differentiation of HUES-7 was initiated from p20 to p28 cultures. Feeder-free, trypsin-passaged cultures of BG01 were maintained from p40 to p55 (46,XY at p40 and p52), NOTT1 from p15 to p33 (46,XX at p23 and p33), and NOTT2 from p18 to p28 (46,XY at p26).

Differentiation and Cardiomyocyte Analysis

Mass cultures of HUES-7 EBs and BG01 EBs were prepared as described previously [7]. Forced aggregation of EBs in untreated V-96-well plates (Nunc, Rochester, NY, http://www.nuncbrand.com), termed V-96FA, and subsequent evaluation of EB size and efficiency of cardiomyocyte differentiation is described in Figure 1A. V-96FA EBs were initiated from feeder-free, trypsin-passaged cultures of HUES-7 p20–28, BG01 p48–55, NOTT1 p26–31, and NOTT2 p24–28. The media tested for their ability to support V-96FA EB formation and growth were as follows: (a) CM, (b) unconditioned medium (UCM) was prepared as for CM but without MEF conditioning, (c) DMEM supplemented with 20% fetal bovine serum [FBS], 100 μM β-ME, 1% NEAA, and 2 mM GlutaMAX (D-FBS), (d) defined medium supplemented with bovine serum albumin (CDM-BSA), and (e) defined medium supplemented with polyvinyl alcohol (CDM-PVA) (both CDM-BSA and CDM-PVA as described by Vallier et al. [13] [L. Vallier, M. Alexander, and R.A. Pedersen, personal communication]) and containing 1:1 mix of Iscove's modified Dulbecco's medium (F12 supplemented with 1× lipid, 450 μM monothioglycerol, 7 μg/ml insulin [Roche Diagnostics, Basel, Switzerland, http://www.roche-applied-science.com], and 15 μg/ml transferrin [Roche] plus 5 mg/ml bovine serum albumin fraction V [CDM-BSA] or 1 mg/ml polyvinyl alcohol). CDM-PVA was used either with or without 10 ng/ml activin A and 12 ng/ml bFGF (both from Peprotech, London, http://www.peprotech.com).

Numbers of viable cells present within HUES-7-derived EBs at d2, 4, and 6 of differentiation were measured using an MTT CellTitre 96 nonradioactive cell proliferation assay (Promega, Southampton, U.K., http://www.promega.com) since this system has been previously used to define cell numbers in mouse EBs [14]. HUES-7 EBs were transferred in 100 μl of medium to a flat-bottom 96-well plate on the appropriate day, and 15 μl of MTT dye solution

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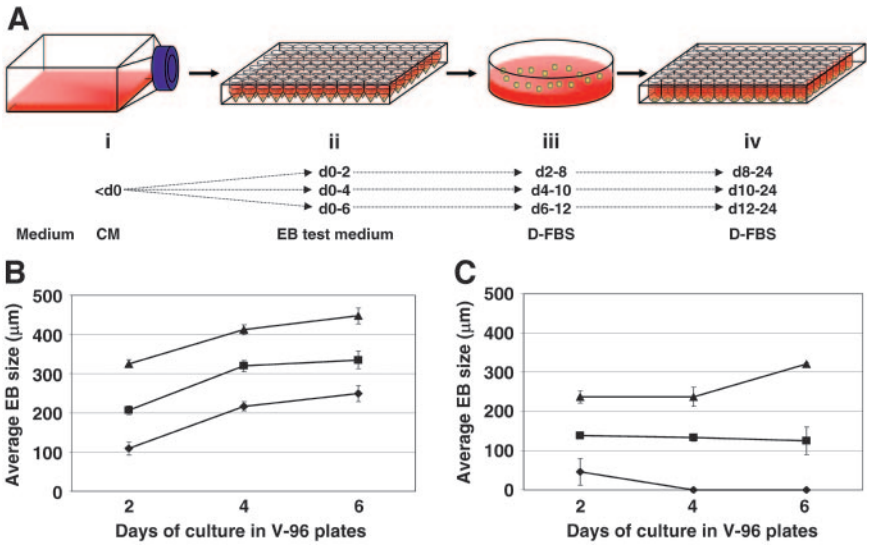

Figure 1. Developing the V-96FA system. **(A):** Schematic of the V-96FA procedure. (i): All human ESC (hESC) lines were cultured using trypsin passaging in feeder-free conditions on Matrigel in CM. (ii): On day 0 (d0) of differentiation, EB formation was initiated by seeding untreated V-96 plates in triplicate with either 1,000, 3,000, or 10,000 cells per well in different test media and centrifuging at 950*g* (~2,800 rpm) for 5 minutes at room temperature. To allow EBs to grow, V-96 plates were incubated for either 2, 4, or 6d (indicated by dotted arrows). (iii): On the specified day, growth was assessed by transferring all EBs from a V-96 plate to an untreated 90-mm dish in D-FBS and calculating the diameter of 20 randomly selected EBs per experiment by averaging the smallest and largest cross-sectional dimension of each body. (EBs were measured in 90-mm dishes, as optical distortion occurs in V-96 plates.) EBs were maintained in suspension for 6d to allow further differentiation; if necessary, EBs were detached from the culture dish by gentle pipetting. (iv): EBs were transferred to each well (one EB per well) of an untreated U-96-well plate in D-FBS. On d24 of differentiation, the percentage of beating EBs was calculated relative to 96 (i.e., the number of V-96 wells originally seeded with hESCs). CM **(B)** or defined medium supplemented with polyvinyl alcohol **(C)** V-96FA HUES-7 EBs were formed from 1,000 (diamonds), 3,000 (squares), or 10,000 (triangles) cells, and size was calculated on d2, 4, or 6 of differentiation. Abbreviations: CM, conditioned medium; d, day; D-FBS, Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum.

was added. Plates were incubated for 2 hours at 37°C before adding 100 μl of stop solution and solubilizing overnight at 37°C. Absorbance was determined at 570 nm using a reference wavelength of 650 nm in a μQuant plate reader (BioTek, Winooski, VT, http://www.biotek.com). Cell numbers per EB were calculated from a standard curve using a known number of cells; all readings taken were in the linear range of the assay.

Real-time TaqMan polymerase chain reaction (PCR) was used to determine relative expression of myosin heavy chain 6 (*MYH6*, encodes αMHC). Reverse transcription was carried out using Superscript II (Invitrogen) with 400 ng of RNA according to the manufacturers' instructions. The resulting cDNA was diluted to a final volume of 100 μl. TaqMan PCR of samples was carried out using Applied Biosystems Assay on Demand (Foster City, CA, http://www.appliedbiosystems.com) primers/probe sets to *MYH6* (part number Hs00411908_m1) and *HPRT* (internal control; Hs99999909_m1) in conjunction with TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems) and 2 μl of cDNA in a total reaction volume of 20 μl. Cycle conditions were one cycle of 95°C for 5 minutes followed by 50 cycles of 95°C for 10 seconds, 60°C for 1 minute. Two independent PCRs, each in triplicate, were run, and relative quantification was performed using the Applied Biosystems 7500 Fast Real-time PCR System and software.

To confirm the presence of cardiomyocytes in beating areas derived from HUES-7 EBs in defined medium supplemented with polyvinyl alcohol, 10 ng/ml activin A, and 12 ng/ml bFGF (CDM-PVA+AF), nonquantitative RT-PCR and immunostaining were used. cDNA was prepared as above. PCR cycle parameters were as follows: one cycle of 94°C for 5 minutes; 35–40 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 68°C for 1 minute; and one cycle of 68°C for 10 minutes using High Fidelity PCR Master Mix (Roche). Sequences were as follows: *HPRT1* forward (F), 5′-TTGACACTGG CAAAACAATG CAGA-3′, and reverse (R), 5′-TGGCGATGTC AATAGGACTC CAGA-3′, Gene ID 3251; *POU5F1* F, 5′-GAAGGTATTC AGCCAAAC-3′, and R, 5′-CTTAATCCAA AAACCCTGG-3′, Gene ID 5460; *GATA4* F, 5′-AAAGAGGGGA TCCAAACCAG AAAA-3′, and R, 5′-CAGATCCTCG GTGCTAGAAA CACA-3′, Gene ID 2626; *NKX2.5* F, 5′-AGGACCCTAG AGCCGAAAA G-3′, and R, 5′-GCCGAAGTTC ACGAAGTTGT-3′, Gene ID 1482; *TBX5* F, 5′-TGTGGCTAAA ATTCCACGAA-3′, and R, 5′-TTCTGGAAGG AGACGAGCTG-3′, Gene ID 6910; *MYH6* F, 5′-ATGACCGATG CCCAGATGGC TGA-3′, and R, 5′-TCACTCCTCT TCTTGCCCCGG TA-3′, Gene ID 4624; *MYH7* F, 5′-AGCTGGCCCA GCGGCTGCAG G-3′, and R, 5′-CTCCATCTTC TCGGCCTCCA GCT-3′, Gene ID 4625; *MYL2* F, 5′-CCAACTCCAA CGTGTTCTCC ATGT-3′, and R, 5′-CATCAATTTC TTCATTTTTC ACGTTCA-3′, Gene ID 4633; *SLC8A1* F, 5′-CCTTCTTCCT TGAGATTGGA GAGC-3′, and R, 5′-TCCTCTTCCT CTTTGCTGGT CAGT-3′, Gene ID 6546; *NEB* F, 5′-ATGATGAATCA CGTGCTGGCT AAA-3′, and R, 5′-TGACTTTCTT CTGGGAATCC TTGG-3′, Gene ID 4703.

Prior to immunostaining, beating areas were disaggregated as described previously [15, 16]. Briefly, beating areas were manually dissected, washed in phosphate-buffered saline (PBS), and then incubated for 30 minutes at room temperature in buffer 1 (120 mM NaCl, 5.4 mM KCl, 5 mM $MgSO_4$, 5 mM sodium pyruvate, 20 mM taurine, 10 mM HEPES, 20 mM glucose, pH 6.9), for 45 minutes at 37°C in buffer 2 (120 mM NaCl, 5.4 mM KCl, 5 mM $MgSO_4$, 5 mM sodium pyruvate, 20 mM taurine, 10 mM HEPES, 0.3 mM $CaCl_2$, 20 mM glucose, 1 mg/ml collagenase B, pH 6.9) and for 1 hour at room temperature in buffer 3 (85 mM KCl, 5 mM $MgSO_4$, 5 mM sodium pyruvate, 20 mM taurine, 1 mM EGTA, 5 mM creatine, 30 mM K_2HPO_4, 20 mM glucose, 1 mg/ml Na_2ATP, pH 7.2). Finally, cell clusters were dissociated by repeated pipetting through a P1000 tip, and the liberated cells were seeded to glass coverslips in D-FBS. Following attachment, cells were fixed with 4% paraformaldehyde (15 minutes at room temperature), permeabilized with Triton X-100 (0.1%; 8 minutes at room temperature), and then incubated with the mouse monoclonal anti-α-actinin (1:800) or anti-tropomyosin (1:50) for 1 hour at room temperature. Incubation with the secondary antibodies, Cy3 goat anti-mouse IgG (1:250), and fluorescein isothiocyanate goat anti-mouse IgG (1:100; both Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com), was performed for 1 hour at

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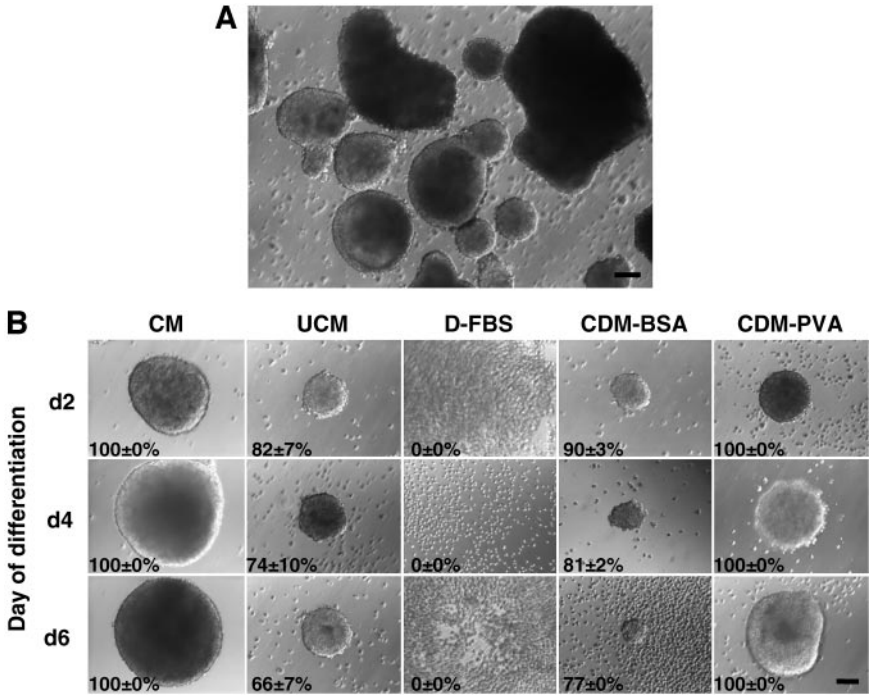

Figure 2. HUES-7-EB formation by mass culture or forced aggregation. Representative images are shown for mass culture EBs prepared by collagenase treatment + scraping of HUES-7 cells on mouse embryo fibroblast feeders and then culturing in suspension in CM until d6 of differentiation **(A)** and V-96FA EBs prepared from 10,000 feeder-free HUES-7 cells cultured in different media until d2, 4, or 6 of differentiation **(B)**. Media tested were as follows: CM, UCM, D-FBS, CDM-BSA, and CDM-PVA. Percentages were calculated from the number of wells in each V-96 plate that contained EBs (±SEM; two experiments). Scale bar = 100 μm. Abbreviations: CDM-BSA, defined medium supplemented with bovine serum albumin; CDM-PVA, defined medium supplemented with polyvinyl alcohol; CM, conditioned medium; d, day; D-FBS, Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (see Materials and Methods); UCM, unconditioned medium.

room temperature. Samples were mounted in Vectorshield Hardset containing 4,6-diamidino-2-phenylindole (Vector Laboratories, Peterborough, U.K., http://www.vectorlabs.com) and visualized on a Leica SP2 confocal microscope (Heerbrugg, Switzerland, http://www.leica.com).

H&E Staining

EBs were washed in twice in PBS, fixed in 4% paraformaldehyde in PBS for 15 minutes, and then washed twice in PBS. EBs were set into 1% agarose in double distilled H_2O and processed overnight using a Shandon Excelsior tissue processor (Thermo Scientific, Runcorn, U.K., http://www.thermo.com). Processed agarose blocks were embedded in paraffin wax (Tissue-Tek II embedding wax, Zoeterwoude, The Netherlands, http://www.tissue-tek.com) using a Tissue-Tek III thermal/dispensing/cryo-consol, sectioned using a microtome at 5 μm, and affixed to SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany, http://www.menzel.de). Paraffin sections were dewaxed using xylene, rehydrated through an ethanol/H_2O gradient and stained with Harris' Hematoxylin (VWR, West Chester, PA, http://www.vwr.com) and Eosin Yellowish (VWR), before dehydrating and mounting using DePeX mounting medium (VWR). Samples were visualized using a Leica DMRB upright microscope and captured using Improvision 4.0.2 software.

Results

Establishing a High-Throughput, Forced-Aggregation System

To establish a benchmark from which to gauge alternative strategies of EB formation, we first evaluated the degree of heterogeneity in size of EBs generated by a commonly used mass culture protocol [7]. HUES-7 or BG01 cells grown on MEFs were harvested using collagenase plus scraping and incubated in suspension culture in CM for 6 days, as previously described [7]. Considerable variability in gross morphology was observed between EBs within each mass culture (Fig. 2A). Moreover, the diameter (calculated by averaging the smallest and largest cross sectional dimension of each body) for 20 randomly selected EBs per experiment ranged from 100 to 1,200 μm and from 175 to 1,150 μm for HUES-7 and BG01 (Table 1; supplemental online Fig. 1).

These observations prompted us to evaluate methods to generate EBs directly from trypsin-passaged hESCs cultured on Matrigel. This would enable each EB to be seeded from a defined number of hESCs maintained in feeder-free conditions and therefore potentially reduce variability in EB size, precisely specify the numbers of EBs generated in each experiment and eliminate MEF contamination. Incubation of hanging drop cultures seeded with between 300 and 10,000 HUES-7 or BG01 cells for up to 6 days failed to produce EBs (Table 1). As an alternative, we investigated EB formation using forced aggregation by centrifugation. Both hESC lines formed EBs efficiently when ultralow-attachment U-96 plates (ULA U-96) were seeded with 3,000 or 10,000 cells per well in CM, centrifuged at 950*g* for 5 minutes and incubated for 6 days (Table 1). However, these plates are prohibitively expensive for analysis of large numbers of EBs, and we sought to evaluate alternative plasticware.

Centrifugation of HUES-7 or BG01 cells in untreated (i.e., not treated for cell culture) U-96 plates resulted in erratic and/or poor EB formation (Table 1). In contrast, forced aggregation by centrifuging untreated V-96 plates (termed V-96FA) seeded with 3,000 or 10,000 hESCs/well in CM and then incubating for 6 days produced EBs at a similar efficiency and of similar size to those formed in ULA U-96 plates (Table 1). Furthermore, the

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Table 1. Formation of EBs from HUES-7 and BG01 by mass culture or forced aggregation

	Undefined input cell number (mass culture): collagenase from MEFs	Defined input cell number (cells per hanging drop or cells per well)						
		Hanging drop (0.3–10,000)[a]	ULA (U-96)		Untreated (U-96)		Untreated (V-96)[b]	
			3,000	10,000	3,000	10,000	3,000	10,000
HUES-7								
% EB formation[c]	NA	0	82 ± 18	100 ± 0	0	56 ± 43	93 ± 6	100 ± 0
Average EB diameter (mm)	318 ± 27 (100–1,200)[d]	NA	389 ± 23	445 ± 15	NA	ND	377 ± 16 (250–450[d])	476 ± 15 (400–575[d])
BG01								
% EB formation	NA	0	91 ± 2	100 ± 0	0	0	90 ± 4	100 ± 0
Average EB diameter (mm)	333 ± 28 (175–1150[d])	NA	257 ± 16	401 ± 27	NA	NA	285 ± 11 (250–325[d])	387 ± 9 (325–450[d])

All EB size measurements were taken after incubation for 6 days in CM and represent averages (± SEM) from at least two independent experiments, with 20 EBs measured per experiment.
[a]Hanging drop cultures (30 μl) were seeded with 300, 1,000, 3,000, or 10,000 cells.
[b]Without centrifugation, formation efficiency was poor (data not shown).
[c]% EB formation was calculated as the number of wells containing EBs on day 6 of differentiation.
[d]Numbers in parentheses indicate the range of EB sizes (minimum to maximum) observed within the experimental groups; Levene's test for equality of variance indicated significant differences between HUES-7 EBs formed by mass culture EBs and by V-96FA from 3,000 ($p < .001$) or 10,000 ($p < .001$) cells or BG01 EBs formed by mass culture and by V-96FA from 3,000 ($p < .001$) or 10,000 ($p = .02$) cells.
Abbreviations: CM, conditioned medium; MEF, mouse embryo fibroblast; NA, not applicable; ND, not done; ULA, ultralow-attachment U-bottomed 96-well plates.

diverse range of sizes observed between EBs formed in mass culture was significantly reduced by V-96FA (Table 1; supplemental online Fig. 1; $p < .02$, Levene's test for equality of variances).

Evaluating the Effect of Media, Cell Number, and Day of Differentiation on V-96FA EBs

Successful formation of EBs in CM via the V-96FA system allowed the effectiveness of different media to be tested. As an initial screen, percentage EB formation was compared on d2, 4, or 6 after V-96FA with 10,000 HUES-7 cells in CM, UCM, or D-FBS, which contains 20% serum (Fig. 2B). CDM-BSA and CDM-PVA were also tested, since these semidefined and defined media, respectively, were recently reported to support undifferentiated proliferation of the hESC lines H9 and HSF-6 [13] (L. Vallier, M. Alexander, and R.A. Pedersen, personal communication). Consistent with our observations above, high efficiency formation (100%) in CM was observed on d2, 4, and 6 of differentiation (Fig. 2B). Efficiency was the same in CDM-PVA but lower in UCM (66%–82%), CDM-BSA (77%–90%), and D-FBS (0%). Therefore, subsequent experiments focused on formation, growth, and cardiomyocyte differentiation from EBs produced by V-96FA in CM and the defined medium, CDM-PVA.

The utility of the V-96FA system was developed further by investigating the kinetics of HUES-7 EB growth in relation to input cell number, days of differentiation, and medium formulation. Plates were seeded in triplicate with 1,000, 3,000, or 10,000 HUES-7 cells in CM or CDM-PVA. After centrifugation, one plate at each density was cultured until d2, 4, or 6 of differentiation, at which time average EB size was calculated (Fig. 1A). Analysis of EB size indicated a high degree of reproducibility between independent experiments using CM or CDM-PVA (Fig. 1B, 1C), which was confirmed by evaluating viable cell numbers per EB (supplemental online Fig. 2A, 2B). These observations were also consistent with histological examination using H&E staining, which showed greater homogeneity in size and tissue morphology of V-96FA EBs formed in either medium than their mass culture counterparts formed in CM (Fig. 3). For EBs formed in CM from all cell seeding densities, both increasing input cell number and day of differentiation contributed to increasing size (Fig. 1B). Similarly, EBs initiated from 10,000 cells in CDM-PVA increased in size from d2 to d6 of differentiation (Fig. 1C). However, the size of CDM-PVA EBs formed from 3,000 cells remained static, whereas those from 1,000 cells declined, with no EBs detectable by d6 of differentiation (Figs. 1C, 3). These observations indicated that V-96FA provided a reproducible platform with which to evaluate the effect of different media on EB formation and growth, thus enabling comparison between different hESC lines.

Formation of V-96FA EBs in CM was efficient from HUES-7, BG01, NOTT1, and NOTT2, with similar overall growth profiles observed between lines (Fig. 4), demonstrating that the system has the potential to be applied generically. However, statistical analysis indicated several interline differences in EB size (supplemental online Table 1). For example, on d4 of differentiation, the size of EBs seeded from 1,000 hESCs varied between the lines ($p = .002$; one-way analysis of variance [ANOVA]), as did those from 3,000/d4 ($p = .001$) and 10,000/d4 ($p = .001$) or 10,000/d6 ($p = .042$). Similar observations were made for CDM-PVA EBs from the three lines examined (HUES-7, BG01, and NOTT1; compare Fig. 1C with supplemental online Fig. 3A, 3B), with significant differences in size observed from 10,000/d2 ($p = .031$), 3,000/d4 ($p = .038$), and 10,000/d6 ($p = .007$; supplemental online Table 1). Thus, clear differences in EB growth kinetics were observed between the lines.

Variable Cardiomyocyte Differentiation Between hESC Lines

The reproducibility of the V-96FA system and the ability to dictate EB size by altering input cell number and/or time in culture provided an opportunity to carry out systematic comparison of cardiomyocyte differentiation efficiency between the four hESC lines. As before, V-96FA EBs were formed from 1,000, 3,000, or 10,000 cells in CM and cultured until d2, 4, or 6 of differentiation. EBs were then switched to and maintained in D-FBS until d24 of differentiation (Fig. 1A). FBS was included during the extended differentiation period because absence of serum is reported to be detrimental to maintenance of primary cardiomyocytes [17].

For all four hESC lines, transfer of the V-96FA EBs produced from 1,000, 3,000, or 10,000 hESCs to D-FBS on d2 of differentiation yielded only limited numbers of beating EBs

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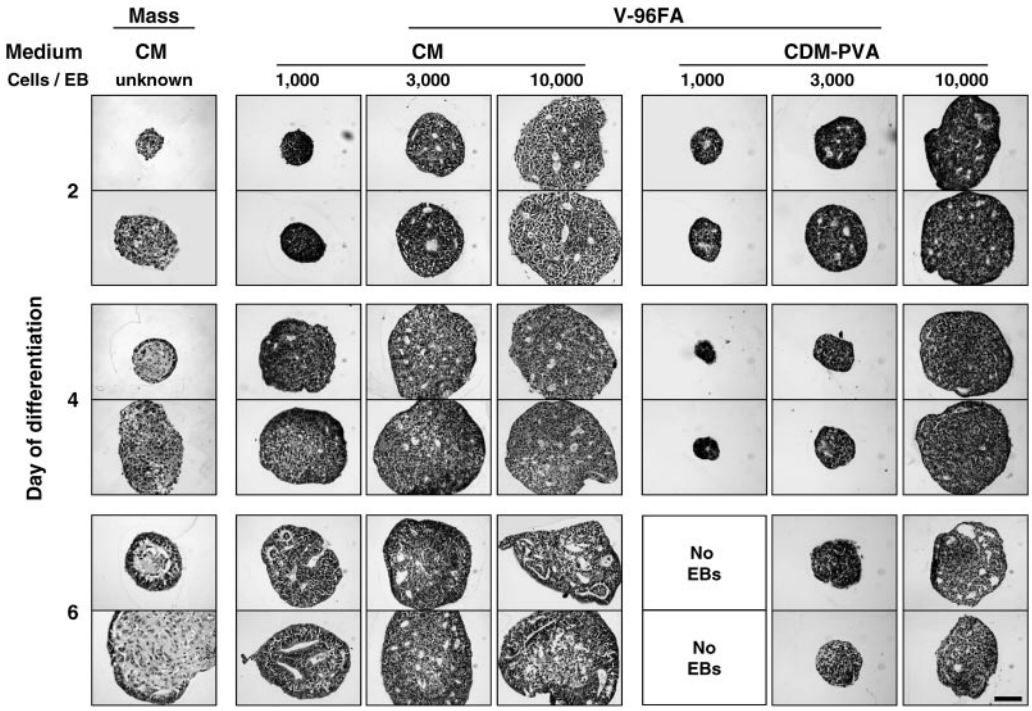

Figure 3. Sectioning and H&E staining of HUES-7-EBs. EBs were formed by mass culture of unknown numbers of cells per EB in CM or by V-96FA of 1,000, 3,000, and 10,000 cells per well in CM or CDM-PVA. EBs were incubated until d2, 4, or 6 of differentiation and then selected at random for processing. Representative images of sections from two separate EBs are shown. Scale bar = 100 μm. Abbreviations: CDM-PVA, defined medium supplemented with polyvinyl alcohol; CM, conditioned medium.

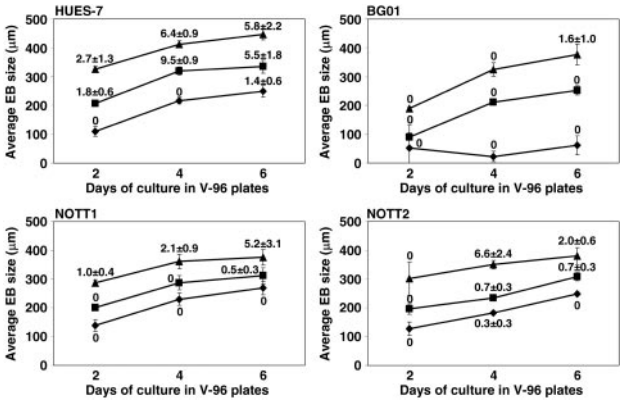

Figure 4. EB formation and cardiomyocyte differentiation between human ESC lines. EBs were formed by V-96FA from 1,000 (diamonds), 3,000 (squares), or 10,000 (triangles) cells in CM. On day (d)2, 4, or 6 of differentiation, EBs were transferred to Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, where they were measured and then cultured until d24, as described in Figure 1A. Each data point for EB size represents average (±SEM) of between 2 and 17 independent experiments (representing 40–340 EBs), and numbers represent average (±SEM) percentage of beating EBs scored from 2–21 independent experiments (representing 196–2,016 EBs).

(Fig. 4). Similarly, cardiomyocyte differentiation was consistently poor using 1,000 hESCs switched to D-FBS on d4 or 6. However, significant variability was observed between the lines in the percentage of beating areas produced by switching EBs formed from 3,000 cells/d4 ($p < .001$; one-way ANOVA), 10,000/d4 ($p = .003$), or 3,000/d6 ($p = .005$; Fig. 4; supplemental online Table 1). In the 3,000 and 10,000/d4 samples, these differences were also validated by showing good association between percentage of beating EBs and quantitative TaqMan PCR of *MYH6* [18], which encodes αMHC (Fig. 5A). Moreover, there was an interline difference in absolute efficiency of cardiomyogenesis (Fig. 4; HUES-7, 9.5% ± 0.9%; BG01, 1.6% ± 1.0%; NOTT1, 5.2% ± 3.1%; NOTT2, 6.6% ± 2.4%; $p = .008$, one-way ANOVA).

We also analyzed the percentage of beating areas identified on d24 of differentiation relative to EB size at time of transfer on d2, d4, or d6 from V-96 plates to D-FBS. Interestingly, of the five arbitrarily assigned EB size categories, all lines showed the highest percentages of beating EBs were obtained when size at transfer was 250–350 μm (Fig. 5B; HUES-7, 7.2% ± 0.9%; BG01, 1.1% ± 0.8%; NOTT1, 2.4% ± 1.1%; NOTT2, 3.1% ± 1.5%). However, interline differences were still evident in three categories (150–250 μm, $p = .016$; 250–350 μm, $p = .001$; 350–450 μm, $p = .025$; one-way ANOVA). Thus, together these data indicate that under the current test conditions, the cardiomyocyte differentiation potential of the four hESC lines varies significantly.

Growth Factor Induction of Cardiomyogenesis

Although cardiomyocyte differentiation was observed from EBs formed in CM for all the hESC lines, this medium is undefined and may contain factors that antagonize the effect of potentially cardiomyogenic agents. Therefore, CDM-PVA V-96FA EBs were generated with activin A and bFGF present or absent during the first 2–6d of differentiation, and the effect on subsequent cardiomyogenesis was evaluated; both growth factors have been proposed to support maintenance of undifferentiated hESCs [13] and during differentiation are involved in specification of cardiomyocyte/mesodermal fate [19, 20].

Size profiles of EBs were similar irrespective of whether CDM-PVA was supplemented with 10 ng/ml activin A and 12 ng/ml bFGF (CDM-PVA+AF), indicating that EB growth was not stimulated by addition of the factors (Fig. 6A, 6B; supplemental online Fig. 3). Formation of BG01 EBs or NOTT1 EBs in either medium failed to generate beating EBs. In contrast,

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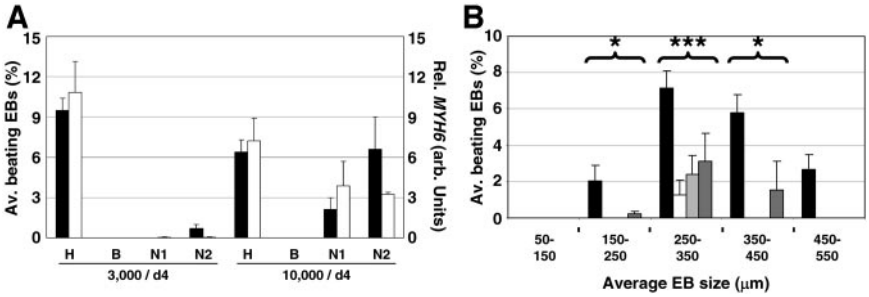

Figure 5. Analysis of beating EBs by real-time polymerase chain reaction (PCR) and by size category. **(A):** The percentage of wells containing beating areas (black bars) was shown to associate well with *MYH6* expression, as determined by TaqMan PCR relative to *HPRT* (white bars). **(B):** Average percentages of beating EBs (±SEM) were plotted versus arbitrarily assigned size categories that relate to EB size at time of transfer to Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum. Black bars, HUES-7; white bars, BG01; light gray bars, NOTT1; dark gray bars, NOTT2. *, interline difference of $p < .05$; ***, $p = .001$ (one-way analysis of variance). Abbreviations: Av., average; B, BG01; d, day; H, HUES-7; N1, NOTT1; N2, NOTT2.

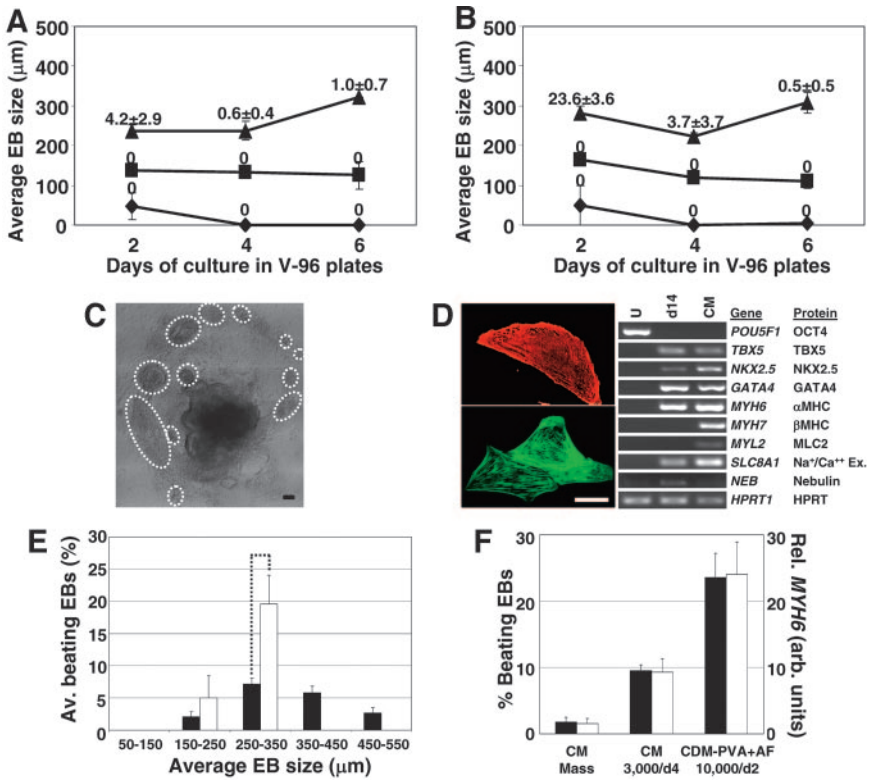

Figure 6. EB formation and cardiomyocyte differentiation from HUES-7 cells in defined media. EBs were formed by V-96FA from 1,000 (diamonds), 3,000 (squares), or 10,000 (triangles) cells in CDM-PVA **(A)** or CDM-PVA+AF **(B)**. On d2, 4, or 6 of differentiation, EBs were transferred to Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (D-FBS), where they were measured and cultured until d24, as described in Figure 1A. Each data point for EB size represents the average (±SEM) of 2–4 independent experiments (representing 40–80 EBs), whereas numbers represent average (±SEM) percentage of beating EBs observed from 2–9 independent experiments (representing 196–864 EBs). **(C):** A representative image of the location and morphology of beating outgrowths (dotted ellipses) arising from EBs initially formed in CDM-PVA+AF. Scale bar = 100 μm. **(D):** The presence of cardiomyocytes in the beating areas derived from CDM-PVA+AF EBs was confirmed by immunostaining to α-actinin (red) and tropomyosin (green; scale bar = 50 μm), as well as by reverse transcription polymerase chain reaction (PCR). **(E):** Average percentages of beating EBs (±SEM) were plotted versus arbitrarily assigned size categories that relate to EB size at time of transfer to D-FBS. EBs were formed in CM (black bars) or CDM-PVA+AF (white bars). Dashed line signifies $p = .001$ (t test). **(F):** Percentage of wells containing beating areas (black bars) relative to expression of *MYH6* (TaqMan PCR relative to *HPRT*; white bars) was determined for EBs that were initially formed by mass culture or by V-96FA from 3,000 cells in CM/d4 and 10,000 cells in CDM-PVA+AF/d2. Abbreviations: Arb., arbitrary; Av., average; CDM-PVA, defined medium supplemented with polyvinyl alcohol; CDM-PVA+AF, defined medium supplemented with polyvinyl alcohol, 10 ng/ml activin A, and 12 ng/ml basic fibroblast growth factor; CM, areas containing spontaneously contracting cells; d, day; d14, EBs at day 14 of differentiation; Rel., relative to expression of; U, undifferentiated HUES-7.

HUES-7 EBs formed in CDM-PVA with 10,000 cells and switched to D-FBS on d2 of differentiation produced 4.2% ± 2.9% beating areas by d24 of differentiation and this increased significantly to 23.6% ± 3.6% by formation in CDM-PVA+AF (Fig. 6A, 6B; $p = .01$, t test). The beating outgrowths from the CDM-PVA+AF HUES-7 EBs had a readily identifiable cellular morphology, even before the onset of beating, and were most commonly located in a horseshoe shape at the EB perimeter (Fig. 6C). To confirm the presence of cardiomyocytes in these outgrowths, beating areas were manually dissected and then

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disaggregated with collagenase to single cells or small clumps of cells. After replating, the cultures contained cells that continued to spontaneously contract and were immunoreactive to antibodies against α-actinin and tropomyosin (Fig. 6D). Analysis of the cultures by RT-PCR indicated the presence of transcripts for cardiac transcription factors (*GATA-4, TBX-5,* and *NKX2.5*) and structural/regulatory elements (*MYH6, MYH7*, *SLC8A1,* and *MYL2*) but not for *NEB,* which encodes the skeletal muscle protein nebulin (Fig. 6D) [18, 21]. Analysis by size category indicated that CDM-PVA+AF HUES-7 EBs of 250–350 μm at time of transfer to D-FBS produced the highest percentages (19.6% $\pm$ 4.5%) of beating EBs, a pattern similar to that seen for HUES-7 EBs formed in CM (Fig. 6E).

Formation of HUES-7 EBs in mass culture resulted in beating outgrowths arising from 1.8% $\pm$ 0.7% of total EBs analyzed on d24 of differentiation. This percentage increased to 9.5% $\pm$ 0.9% using V-96FA from 3,000 cells in CM with transfer to D-FBS on d4 and to 23.6% $\pm$ 3.6% using 10,000 cells in CDM-PVA+AF/d2. These data associated well with relative quantification by real-time PCR of *MYH6* transcripts (Fig. 6F), indicating that forced aggregation of HUES-7 in defined medium with addition of activin A and bFGF increased cardiomyogenesis by greater than 13-fold compared with mass culture in CM. Thus, the ability to form EBs in defined media makes the V-96FA system amenable to evaluating growth factor induction of cardiomyocyte differentiation.

Discussion

Although formation of cell lineages representing the three germ layers via in vitro differentiation and expression of various pluripotency markers, such as SSEA-4, TRA-1–60, OCT4, or NANOG, forms a useful umbrella under which to group the 300 or so hESC lines derived to date, the phenotype of each line is likely influenced by both inherent genetics and environmental parameters [3]. Indeed, the culture environment can have a profound effect on the molecular signature of hESCs; changing the serum component of the culture medium to KnockOut Serum Replacement altered the expression profile of 1,417 genes in the hESC line HS237 [22]. Therefore, we used standardized conditions to provide a common platform to initiate differentiation of independently-derived hESC lines to test generic applicability of differentiation protocols.

Most differentiation protocols for human EB formation initiate suspension cultures by harvesting hESC colonies directly from feeders using manual dissection or collagenase. However, consistent with other reports [8, 23], we found that this strategy resulted in a high degree of heterogeneity in EB size and limited cardiomyocyte differentiation in all lines tested, likely a consequence of the variability in cell numbers that seed each EB. In mouse ESCs, this issue has been solved by seeding hanging drop cultures with a defined number of trypsin-passaged cells (typically 300–700). This results in reproducible production of mouse EBs that are 100–200 μm in diameter by d3 of differentiation and has enabled a reliable system that recapitulates the early stages of development [24]. Moreover, after extended differentiation, many of the murine EBs develop beating outgrowths [19]. Formation of human EBs in hanging drops has been reported from manually dissected hESC colony pieces [23]. However, the size of starting material remains heterogeneous, and the labor-intensive nature of the system is not amenable to high-throughput development. Our attempts to form EBs in hanging drops seeded with defined numbers of trypsinized in HUES-7 and BG01 cells were unsuccessful, consistent with a previous report using HES-1 and HES-2 [25].

As an alternative approach, Ng et al. [8] developed a forced aggregation system to form EBs from defined numbers of HES-2, -3, and -4 by centrifuging at 500*g* for 4 minutes at 4°C in ultralow-attachment U-96-well plates. Although data relating to EB size were not reported, efficient blood formation required a minimum of 500 cells per well, and input cell number was critical for directing efficient differentiation to specific hematopoietic lineages [8]. Although this strategy provided valuable proof of concept that forced aggregation was a viable technique, there are several potential limitations with the study. First, ULA U-96 plates are difficult to obtain and prohibitively expensive (~$20 USD per plate) for high-throughput development. Second, the three lines used were all derived at the same institution, and so the generic utility of the strategy between independently derived hESC lines was unknown. Finally, hESCs were cultured on MEFs, and mixed populations of the two cell types were used in the aggregation experiments; however, it has been suggested that contaminating MEFs have the potential to interfere with differentiation by both competing for cell-cell interactions and insulating communication between the ESCs [24].

We observed >90% EB formation using defined numbers of trypsin-passaged HUES-7 and BG01 cells maintained in feeder-free conditions in ULA U-96 plates, consistent with observations using HES-3 [8]. Importantly for high throughout development, aggregation was also efficient in untreated V-96 plates (~$1 USD per plate), and this method proved to be transferable to NOTT1 and NOTT2. In our laboratory, V-96FA has since been successful using defined numbers of trypsin-harvested H1 (routinely passaged with trypsin) and HUES-7 (routinely passaged with collagenase), both cultured in feeder-free conditions (unpublished observations).

Certain parameters influenced efficiency and reproducibility of EB formation. Rather than containing one discrete EB per well, multiple EBs were identified in a minority of wells when V-96 plates were centrifuged at 500*g* for 5 minutes. This issue was virtually eliminated when plates were subjected to 950*g*. Interestingly, EB formation occurred from HUES-7 and BG01 irrespective of the undifferentiated hESC culture density, whereas formation from NOTT1 (and H1) became erratic when density exceeded 2×10^5 cells per cm^2. The medium also influenced EB formation, with highest efficiencies occurring in CM and CDM-PVA, efficiency decreasing in UCM or CDM-BSA, and no formation in D-FBS. The underlying mechanisms responsible for the differences observed in EB formation between hESC lines or media are not known, but altered expression levels of key adhesion factors seems a likely target for future studies. Indeed, it has been demonstrated that over 60 cell adhesion-related genes were upregulated in the hESC line HS237 by switching the serum component of the medium to KnockOut Serum Replacement [22]. It is also notable that previous microarray studies have highlighted potential differences in expression profile of integrin subclasses in H1 and BG01 [10, 26]. However, from our data and that of Skottman et al. [22], it is plausible that differences in cell density or culture medium at time of cell harvesting could also account for some of the documented variability between hESC lines, and the potential influence of these factors will need to be considered when designing array studies to make future interline comparisons.

Since the standardized culture and V-96FA strategy described here functioned between all the lines investigated, we were able to make parallel, interline comparisons. Significant variability in EB formation, growth, and cardiomyocyte differentiation was observed between independently derived hESC lines but, notably, not between NOTT1 and NOTT2, which were both derived in our laboratory. Other studies, investigating gene expression, have also observed greater similarity between lines isolated within the same laboratory, implicating the derivation

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and culture history of each line as a source of variability. For example, four lines from Finland (FES21, FES22, FES29, and FES30) were more similar to each other than to the three from Sweden (HS181, HS235, and HS237) [27], HSF1 and HSF6 were more similar to each other than to H9 [28], and BG01/BG01-MEDII were more similar to each other than to H1 [29]. Nevertheless, the greatest percentage of beating areas were obtained from NOTT1 by seeding EBs with 10,000 cells and culturing in V-96 plates until d4 before transfer to D-FBS, whereas 10,000/d6 was most effective for NOTT2, suggesting that inherent genetic and/or epigenetic differences are also important. This is consistent with observations of Yoon et al. [23], who observed spontaneous beating in 0% of EBs derived from Miz-hES-1, -4, and -6 but ~22% in Miz-hES2.

The variability in EB size and in cardiomyocyte differentiation observed between the hESC lines may also relate to passage number of the lines at time of differentiation. HUES-7 cells were provided at p12 by Harvard University [9], and we derived NOTT1/2. Consequently, differentiation could be carried out at p20–30. Conversely, only higher passage BG01 cells could be purchased from BresaGen [11], and p45–55 cells were used for the differentiation studies. Although the cells that were used for differentiation were assessed as karyotypically normal by G-banding analysis, there is mounting evidence to suggest that extended culture is associated with more subtle changes, such as gene amplification (e.g., the proto-oncogene *MYC*) and differential methylation [30]. Because this argues for using the lowest passage hESCs possible, we did not attempt to culture HUES-7, NOTT1, and NOTT2 to ~p50 for comparison with the passage BG01 cells and cannot rule out the possibility that high passage number has a negative effect on cardiomyocyte differentiation. However, it is notable that increasing passage number of H9.2 hESCs has been associated with increasing cardiomyogenic potential [31].

Cardiomyocyte differentiation of the four lines examined here appeared to be most efficient when EBs of 250–350 μm were transferred to D-FBS. This is consistent with several other studies that indicate EB size or input cell numbers are important for influencing lineage specification. Erythroid or myeloid lineages were formed most effectively from forced aggregation of 1,000 cells or 1,000–5,000 HES-3 hESCs, respectively. Moreover, a study investigating cardiomyocyte differentiation in hanging drops seeded with between 32 and 2,000 mouse AB2.2 ESCs found that EBs formed from 565 cells produced the highest percentage of beating areas [32]. In this case, primitive endoderm within the mouse EBs influenced the onset of cardiomyogenesis in a concentration-dependent manner [32]. In line with these observations, cardiomyocyte differentiation of mouse P19 embryonal carcinoma cells and human ESCs (HES-2, -3, and -4) has been induced by coculture with END-2, which has characteristics of visceral endoderm [33–35]. In addition, microarray studies monitoring cardiomyogenesis in HES-2 have shown that induction of genes of the primary mesoderm and endodermal lineages is followed by expression of those in cardiomyocyte progenitor cells and fetal cardiomyocytes [36]. Thus, detailed time course analysis of mesodermal- and endodermal-related proteins within developing human EBs may yield clues of how to promote cardiomyogenesis, which could be evaluated using V-96FA in defined media.

The V-96FA system also provides a much improved, homogeneous platform with which to test the effect of different media and/or factors on EB formation, growth, and differentiation between lines. Activin A and bFGF support proliferation of undifferentiated H9 and HSF6 hESCs [13] and have been implicated in cardiogenesis from mouse and human ESCs [19, 20]. Our observations with HUES-7 indicate that EB formation in defined CDM-PVA medium supplemented with both growth factors instigates up to a 2.5-fold increase in beating areas compared with forced aggregation in CM and a 13.1-fold increase compared with mass culture in CM. It will now not only be necessary to identify whether activin A, bFGF, or a combination of both factors has the greatest effect on cardiomyogenesis, but also to determine the most effective concentrations. Interestingly, formation in defined conditions did not produce beating EBs from BG01 or NOTT1, and it will therefore be important to use this system to test the interline efficacy of other potential cardiomyogenic agents, such as ascorbic acid [35], 5-azacytidine [18, 23], and BMP-4 [20]. An additional challenge will be to eliminate serum from the differentiation medium to develop a defined medium that both enables cardiomyogenesis from hESCs and sustains long-term cardiomyocyte function.

In conclusion, we have coupled standardized culture with a high-throughput, V-96FA procedure to facilitate accurate comparison of cardiomyocyte differentiation potential between independently derived hESC lines. Since this strategy will also be amenable for derivation of other differentiated lineages, it should provide a useful platform to rapidly identify whether particular lineages are favored by specific hESC lines. Relating such information to both the original derivation conditions of each line and the data emerging from organizations such as the International Stem Cell Initiative should expedite production of a new generation of hESC lines that are derived and cultured using universally agreed-upon conditions.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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