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Original Article

Common culture conditions for maintenance and cardiomyocyte differentiation of the human embryonic stem cell lines, BG01 and HUES-7

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ABSTRACT Development of generic differentiation protocols that function in a range of independently-derived human embryonic stem cell (hESC) lines remains challenging due to considerable diversity in culture methods practiced between lines. Maintenance of BG01 and HUES-7 has routinely been on mouse embryonic fibroblast (MEF) feeder layers using manual- and trypsin-passaging, respectively. We adapted both lines to trypsin-passaging on feeders or on Matrigel in feeder-free conditions and assessed proliferation and cardiac differentiation. On feeders, undifferentiated proliferation of BG01 and HUES-7 was supported by all three media tested (BG-SK, HUES-C and HUES-nL), although incidence of karyotypic instability increased in both lines in BG-SK. On Matrigel, KSR-containing conditioned medium (CM) promoted undifferentiated cell proliferation, while differentiation occurred in CM containing Plasmanate or ES-screened Fetal Bovine Serum (FBS) and in unconditioned medium containing 100 ng/ml bFGF. Matrigel cultures were advantageous for transfection but detrimental to embryoid body (EB) formation. However, transfer of hESCs from Matrigel back to feeders and culturing to confluence was found to rescue EB formation. EBs formed efficiently when hESCs on feeders were treated with collagenase, harvested by scraping and then cultured in suspension in CM. Subsequent culture in FBS-containing medium produced spontaneously contracting EBs, for which the mean beat rate was 37.2 $\pm$ 2.3 and 41.1 $\pm$ 3.1 beats / min for BG01-EBs and HUES-7-EBs, respectively. Derived cardiomyocytes expressed cardiac genes and responded to pharmacological stimulation. Therefore the same culture and differentiation conditions functioned in two independently-derived hESC lines. Similar studies in other lines may facilitate development of universal protocols.

KEY WORDS: *embryoid body, differentiation, cardiomyocyte, transfection*

Introduction

Human embryonic stem cells (hESCs) potentially offer new routes to study early development, model genetic disorders, screen novel drugs and develop cell-based therapeutics. Realization of these applications would be greatly facilitated both by derivation of a range of hESC lines that represent a cross-section of genetic / haplotype diversity within the human population and by development of generic differentiation protocols that function between lines.

Although current estimates suggest that approximately 200 hESC lines have been derived worldwide (Brimble *et al.*, 2004), only a proportion are readily available to the scientific community and over 70% of studies have focused on the Wisconsin lines, H-1, -7, -9, -13 and -14 (Thomson *et al.*, 1998; reviewed in Allegrucci *et al.*, 2005; Goh *et al.*, 2005). Few reports describe direct comparisons of independently-derived hESC lines (Abeyta *et al.*, 2004; Amit *et al.*, 2004; Bhattacharya *et al.*, 2004), likely a consequence of the labor-intensive nature of maintaining multiple lines that apparently require different culture media, passaging methods, culture substrates and feeder cell types / densities (Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Hovatta *et al.*, 2003; Mitalipova *et al.*, 2003; Cowan *et al.*, 2004; Stojkovic *et al.*, 2004). Since these varying culture parameters may influence differentiation capacity, it is challenging to extrapolate the broad applicabil-

Abbreviations used in this paper: EB, embryoid body; hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast.

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ity of specific differentiation protocols and thus each must be tested empirically. For example, hESC-derived cardiomyocytes have been produced in sufficient numbers to permit characterization by two strategies: by spontaneous differentiation via embryoid bodies (EBs) from collagenase-passaged H-1, -7, -9 and -14 hESCs (Kehat *et al.*, 2001; Xu *et al.*, 2002; He *et al.*, 2003; Reppel *et al.*, 2004; Xue *et al.*, 2005) and by co-culture induced differentiation of manually-passaged HES-2, -3 and -4 colonies with END-2 visceral endoderm-like cells (Mummery *et al.*, 2003; Passier *et al.*, 2005). However, the effectiveness of either strategy on other independently-derived hESC lines is unknown.

We reasoned that using common hESC culture conditions would simplify maintenance of multiple lines and may facilitate translation of specific differentiation protocols between lines. Thus we selected BG01 and HUES-7, which were independently-derived at BresaGen (Mitalipova *et al.*, 2003) and Harvard University (Cowan *et al.*, 2004) using different embryo culture, inner cell mass isolation, hESC culture and hESC passaging methods. Here we report that the same media can support undifferentiated BG01 and HUES-7 proliferation and that cultures of both lines can be trypsin-passaged either on feeder layers or on Matrigel in feeder-free conditions. We demonstrate the utility of Matrigel cultures through transient and stable transfection. Finally, we evaluate efficiency of EB formation in various conditions and, by scoring the appearance of spontaneously beating areas, show that timing of cardiomyocyte differentiation is similar between the two lines.

Results

Effect of MEF feeder density on BG01 morphology

First we investigated the morphology of manually-dissected BG01 colonies on mitomycin C-mitotically inactivated mouse embryonic fibroblasts (MEFs) seeded at 0.6, 2, 6 or >12 x 10^4 cells / cm^2, since these densities have been used for culture of H9 (Vallier *et al.*, 2004), all Wisconsin lines (H-1, -7, -9, -13 and -14; Thomson *et al.*, 1998), HUES 1-17 (Cowan *et al.*, 2004) and BG01-03 (Mitalipova *et al.*, 2003), respectively. The characteristics typically described for undifferentiated hESC cultures, including relatively small size, uniform morphology with the high nuclear to cytoplasmic ratio and surface marker profile of SSEA-1-, SSEA-4+, TRA-1-61+, TRA-1-81+ (Thomson *et al.*, 1998; Draper *et al.*, 2002) were observed for BG01 cells on 6 x 10^4 MEFs / cm^2 (Figs. 1A, 2). At higher MEF densities the feeder layer was prone to detaching as a single sheet from the gelatinased plastic, thereby trapping the hESCs. Within 1 passage BG01 cells maintained on MEFs seeded at or less than 2 x 10^4 / cm^2 became enlarged and appeared more differentiated (Fig. 1B,C). Thus, on the basis of these data using BG01 and previous reports using HUES 1-17 (Cowan *et al.*, 2004), we used 6 x 10^4 MEFs / cm^2 as the standard density both for co-cultures with hESCs and for preparation of MEF conditioned hESC medium (CM) for feeder-free hESC culture.

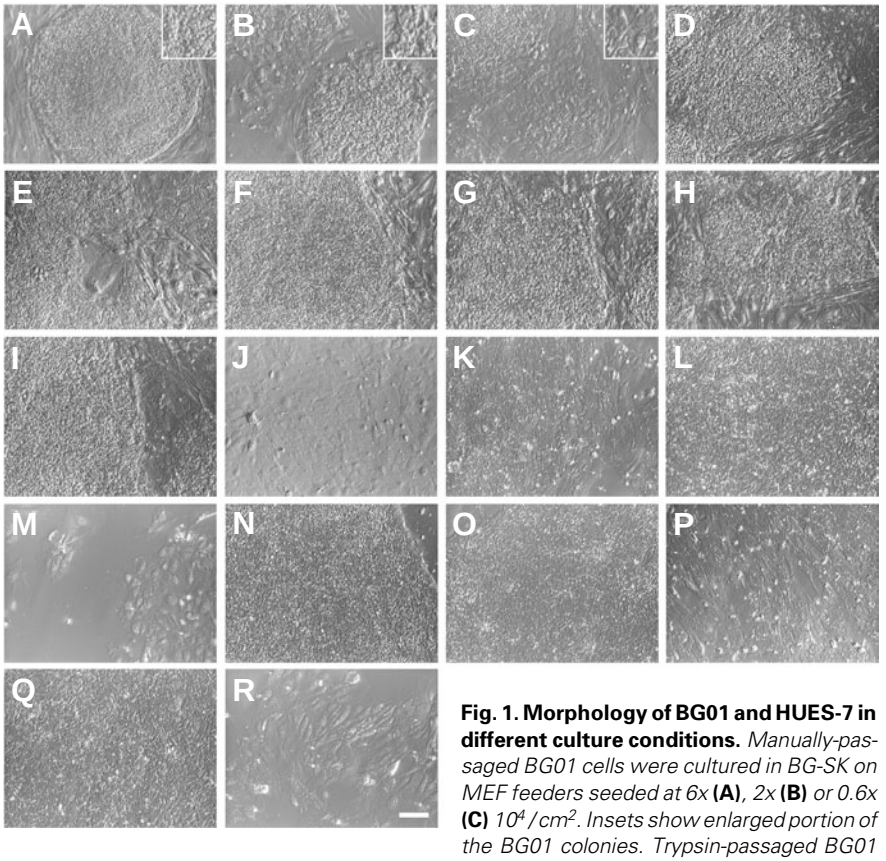

Fig. 1. Morphology of BG01 and HUES-7 in different culture conditions. *Manually-passaged BG01 cells were cultured in BG-SK on MEF feeders seeded at 6x* **(A)**, *2x* **(B)** *or 0.6x* **(C)** *10^4 / cm^2. Insets show enlarged portion of the BG01 colonies. Trypsin-passaged BG01* **(D, E or F)** *and HUES-7* **(G, H or I)** *were maintained on feeders seeded at 6 x 10^4 cells / cm^2 in BG-SK, HUES-C or HUES-nL, respectively. On Matrigel, cultures of BG01 in BG-SK CM* **(J)**, *HUES-C CM* **(K)** *BG-K CM* **(L)** *or BG-K-100* **(M)** *passaged with trypsin or in BG-K CM with collagenase* **(N)** *are shown. Undifferentiated proliferation of trypsin-passaged BG01 was supported when the DMEM base of BG-K was replaced with KO-DMEM* **(O)**. *Proliferation of trypsin-passaged HUES-7 was tested in HUES-C CM* **(P)** *BG-K* **(Q)** *or BG-K-100* **(R)**. *Morphology on Matrigel in HUES-C CM and HUES-nL CM or BG-K-4 and BG-K-100 were similar, thus images for only HUES-C CM and BG-K-100 for each hESC line are shown. Bar, 100 μm. All brightfield images were taken using Hoffman objective lenses.*

Common passage method and culture medium for BG01 and HUES-7

Manual dissection is commonly used for propagation of a range of hESC lines, including BG01 (Brimble *et al.*, 2004; Mitalipova *et al.*, 2005). In agreement with these reports, we manually-passaged BG01 52 times in the supplier-recommended medium, which we term BG-SK (see Table 1). Most dissected colony pieces attached after passaging and spread out to form colonies of undifferentiated hESCs that were SSEA1-, SSEA4+, TRA-1-60+ and TRA-1-81+ (Figs. 1A, 2). However, the labour-intensiveness of manual passaging prompted us to test the effect of various enzymatic passaging methods and culture media on undifferentiated BG01 and HUES-7 proliferation in feeder-dependent and feeder-free conditions (Table 2).

Passaging of BG01 grown in BG-SK on feeders was not successful with either Collagenase IV (1mg / ml) or Dispase (10mg / ml). Even with long enzyme incubations (up to 30 min at 37ºC) and scraping or pipetting, BG01 cells did not

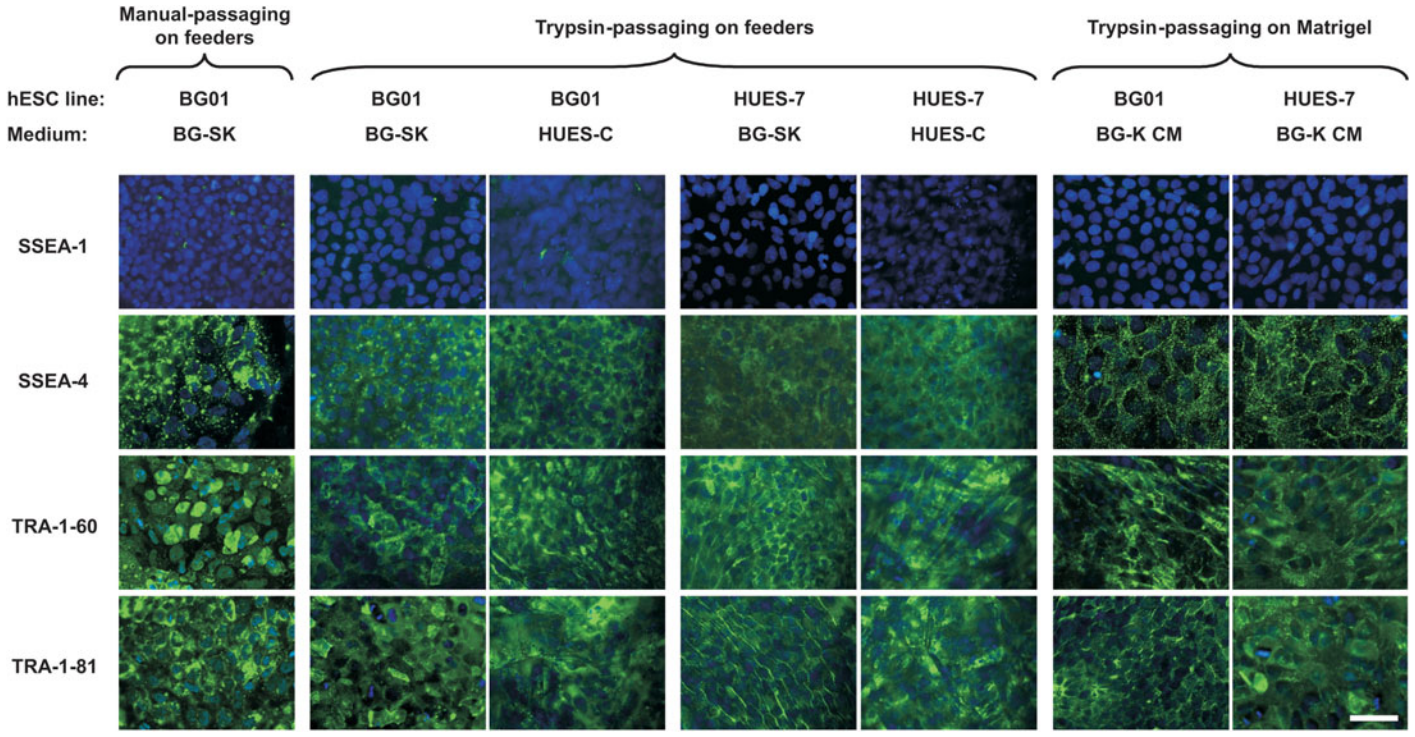

Fig. 2. Surface marker expression. *Representative immunofluorescence (green, FITC; blue, DAPI) images are shown for manually-passaged BG01 on feeders in BG-SK, trypsin-passaged BG01 or HUES-7 on feeders in BG-SK or HUES-C and trypsin-passaged BG01 or HUES-7 on Matrigel in BG-K CM. Antibody reactivity of BG01 or HUES-7 on feeders in HUES-C and HUES-nL were indistinguishable, thus images for only HUES-C for each hESC line are shown. Bar, 50 μm.*

detach readily from the MEF feeders. Moreover, most collagenase and dispase-dissociated clusters of BG01 differentiated when transferred to fresh feeders and could not be rescued by further passaging with either enzyme. In contrast, BG01 could be successfully passaged with trypsin (Fig. 1D-F, Table 2), allowing direct comparison with HUES-7, a hESC line adapted to trypsin-passaging soon after derivation (Cowan *et al.*, 2004).

We next attempted to standardize the culture medium. The recommended medium for HUES-7 is HUES-C (see Table 1; Cowan *et al.*, 2004), which differs significantly from BG-SK medium. Duplicate cultures of BG01 and HUES-7 were grown in parallel on feeders for 5 trypsin-passages in BG-SK, HUES-C or HUES-nL (Figs. 1-3; see Table 1). Interestingly, the morphology of MEFs in HUES-C or HUES-nL was spindle-like with cells starting to retract within 30s of trypsin exposure, whereas, in BG-SK, MEFs were flatter and responded to trypsin only after >2 min (data not shown). Nevertheless, over 5 passages, BG01 and HUES-7 cells were phenotypically indistinguishable in the different media with respect to morphology (Fig. 1D-F, G-I) or surface marker expression (Fig. 2). Average population doubling (PD) intervals (Fig. 3A; hours / PD ± SEM) for BG01 were 80.7h±1.3 in BG-SK, 85.9h±0.2 in HUES-C and 91.5h±0.6 in HUES-nL. Average PD intervals for HUES-7 were 98.1h±2.2 in BG-SK, 98.5h±0.2 in HUES-C and 106h±0.3 in HUES-nL (Fig. 3B). The surprisingly long PD intervals calculated were likely a significant overestimate since substantial debris was observed in the medium after each passage. This indicated high hESC mortality and / or poor attachment post passage, which substantially reduced the theoretical plating densities. Despite similar phenotypic characteristics, genetic differences were observed in the different media. While BG01p50 and HUES-7p25 maintained normal karyotype in HUES-C and HUES-nL during the test period, clonal gains of chromosome X or trisomy 17 (BG01) and +12+17

TABLE 1

COMPOSITION OF CULTURE AND DIFFERENTIATION MEDIA TESTED

	Medium component											
Medium name	DMEM-F12 (%)	KO DMEM (%)	DMEM (%)	ES-FBS (%)	Plas. (%)	KSR (%)	FBS (%)	NEAA (%)	Gln (mM)	β-ME (μM)	bFGF h (ng/ml)	LIF (ng/ml)
BG-SK	78	-	-	15	-	5	-	1	2	100	4	-
BG-K	83	-	-	-	-	15		1	2	100	4	-
BG-K 100	83	-	-	-	-	15	-	1	2	100	100	-
HUES-C	-	78	-	-	10	10	-	1	2	55	10	12
HUES-nL	-	78	-	-	10	10	-	1	2	55	10	-
D-FBS	-	-	78	-	-	-	20	1	2	100	-	-
K-FBS	-	78	-	-	-	-	20	1	2	100	-	-

Abbreviations: KO-DMEM, KNOCKOUT-DMEM; ES-FBS, embryonic stem cell screened Fetal Bovine Serum; Plas., Plamanate; KSR, KNOCKOUT Serum Replacement; FBS, Fetal Bovine Serum; β-ME, β-mercaptoethanol, bFGF, basic fibroblast growth factor; NEAA, non-essential amino acids; Gln, glutamine; hLIF, human recombinant leukaemia inhibitory factor.

(HUES-7) were observed in duplicate cultures of the same passage number maintained in BG-SK.

Feeder-free culture and transfection of BG01 and HUES-7

In initial experiments we found that feeders appeared to confound hESC transfection (data not shown) since we were only able to demonstrate siRNA-induced gene knockdown in BG01 feeder-free cultures. In addition, generation of stable transgenic hESC lines would necessitate feeders resistant to one or more eukaryotic drug selection schemes, ideally requiring access to transgenic mice of the same strain used for feeder derivation. Thus, to simplify and facilitate genetic modification, we investigated proliferation and morphology of feeder-free BG01 and HUES-7 on Matrigel in CM.

Trypsin-passaged BG01 cells cultured in BG-SK CM assumed a differentiated morphology within the first passage on Matrigel (Fig. 1J). Similarly, decreased proliferation rate (Fig. 3C,D) and enlarged cell morphology (Fig. 1K,P) were observed in parallel cultures of BG01 and HUES-7 in HUES-C CM or HUES-nL CM. In contrast, parallel cultures of undifferentiated BG01 and HUES-7 could be maintained in BG-K CM, which contains 15% KSR but no ES-FBS. BG-K CM cultures displayed low mortality after passaging, with PD intervals of 37.9 ± 0.6 (BG01) and 29.4 ± 0.3 (HUES-7) hours averaged over 7 and 9 passages, respectively (Fig. 3C,D; Table 2). At confluence or high local density, morphology and surface markers were similar to hESCs on feeders (Figs. 1-2). In BG-K CM, BG01 and HUES-7 had a normal karyotype (manuscript in preparation). Unlike BG01 on feeders, BG01 on Matrigel could be passaged with Collagenase IV (Fig. 1N; Table 2), although this additionally required scraping to release hESCs from the Matrigel, resulting in substantial cell death following each passage, confounding culture expansion.

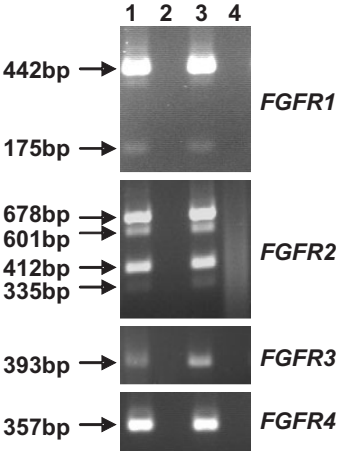

Fig. 4. Analysis of *FGFR1-4* gene expression. *RT-PCR analysis of undifferentiated BG01p48 (RT positive, lane 1; RT negative, lane 2) and HUES-7p28 (RT positive, lane 3; RT negative, lane 4). The product sizes indicate known alternative splice patterns.*

Recent reports have suggested unconditioned medium containing 40-100ng/ml bFGF can support undifferentiated proliferation of the Wisconsin hESC lines H-1, -7, -9 and -14 on Matrigel via BMP signalling pathway inhibition (Xu *et al.*, 2005a; Xu *et al.*, 2005b). Two independent experiments, each using duplicate cultures of both BG01 and HUES-7 set up in parallel, were initiated by seeding up to 14 x 10^4 trypsin-passaged hESCs / cm^2 in BG-K medium supplemented with bFGF at either 4ng/ml or 100ng/ml (BG-K-4 or -100, respectively) and in BG-K CM. Consistent with our observations above, cultures in BG-K CM showed limited mortality post passage and reached confluence in 2-3 days. However, substantial cell death was observed for BG01 and HUES-7 grown in BG-K-4 and -100 and while some of these cultures attained confluency within 3-8 days, they differentiated during the next 1-2 passages (Fig. 1M,R; Table 2). RT-PCR analysis of FGF receptors (*FGFR*) 1-4 in undifferentiated trypsin-passaged BG01 and HUES-7 maintained on Matrigel in BG-K CM revealed expression of all receptors in both lines (Fig. 4).

In contrast to hESCs on feeders, feeder-free hESC culture was permissive for transfection. Lipid-mediated transfection of BG01 on Matrigel with short-interfering (si)RNAs directed towards histone deacetylase 1 (*HDAC1*) or signal transducers and activators of transcription 3 (*STAT-3*) resulted in knockdown of RNA (by 59% or 68%, respectively) and protein, as confirmed by Western blot analysis (Fig. 5A,B). Simultaneous transfection with siRNAs to both targets induced knockdown of mRNA (by 44% *HDAC1*, 55% *STAT-3*; Fig. 5A). In addition, transient delivery of plasmid encoded short-hairpin (sh)RNAs targeted to DNA methyltransferase 1 (*DNMT1*) to BG01 reduced RNA levels by ~80% from days 1-3 after transfection (Fig. 5C). Finally, stable transfection of both BG01 and HUES-7 was effective using a CMV-chicken β-actin promoter to express a green

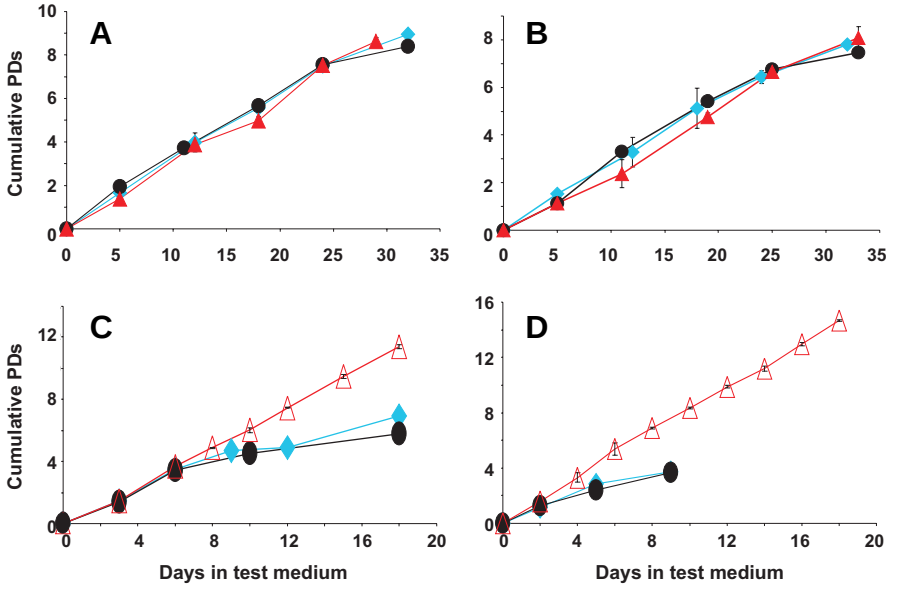

Fig. 3. Growth curves of BG01 and HUES-7. *BG01 and HUES-7 were serially trypsin-passaged on feeders* **(A,B)** *or Matrigel* **(C,)** *in different media. BG-SK is represented by solid red triangles; BG-K, open red triangles; HUES-C, solid blue diamonds; HUES-nL, solid black circles. CM was used for Matrigel cultures.*

fluorescent protein-internal ribosome entry site-puromycin N acetyltransferase (*pcag-gfp-ires-pac*) construct (Fig. 5D).

Differentiation via embryoid body formation

We next assessed whether hESCs from successful culture methods could differentiate via embryoid bodies (EBs). Efficiency of EB formation was scored qualitatively (size) and quantitatively (numbers) after culture of hESCs in suspension for 3-6 days in various media (Fig. 6A; Table 3). Initial experiments with HUES-7 indicated BG-K CM and HUES-C CM were comparable at inducing EB formation (Tables 1 and 3). Efficiency of formation was also comparable in two other media, D-FBS and K-FBS (Table 1), when evaluated on manually-passaged BG01 or trypsin-passaged BG01 and HUES-7 (Table 3). However, with the exception of manually-passaged BG01, where EBs readily formed in all media tested, EB formation efficiency was superior using BG-K CM or HUES-C CM (Table 3). Therefore, for simplicity and standardization, we elected to use BG-K CM for suspension culture.

When BG01 and HUES-7 maintained routinely by trypsin-passaging on feeders were harvested for EBs by using trypsin and then grown in suspension with BG-K CM, ~20 small EBs formed per 10^6 hESCs harvested. Size and numbers (~50-100 EBs / 10^6) markedly increased by harvesting trypsin-passaged BG01 and HUES-7 on feeders with collagenase + scraping, hence this method was selected for EB formation (Table 3).

Surprisingly, EB formation from trypsin-passaged BG01 and HUES-7 on Matrigel was poor. We were unable to generate EBs from either hESC line harvested with trypsin and observed only limited formation (0-20 EBs / 10^6 hESCs) using collagenase + scraping with BG-K CM (Table 3). However, formation of ~50-100 well-formed EBs / 10^6 cells was restored by transferring of Matrigel hESCs back to feeder culture (Matrigel‡feeder cultures), growing to confluence and harvesting with collagenase + scraping (Table 3), thus providing a simple and effective route to transfect hESCs in feeder-free conditions and yet retain the ability to produce EBs (Fig. 5D).

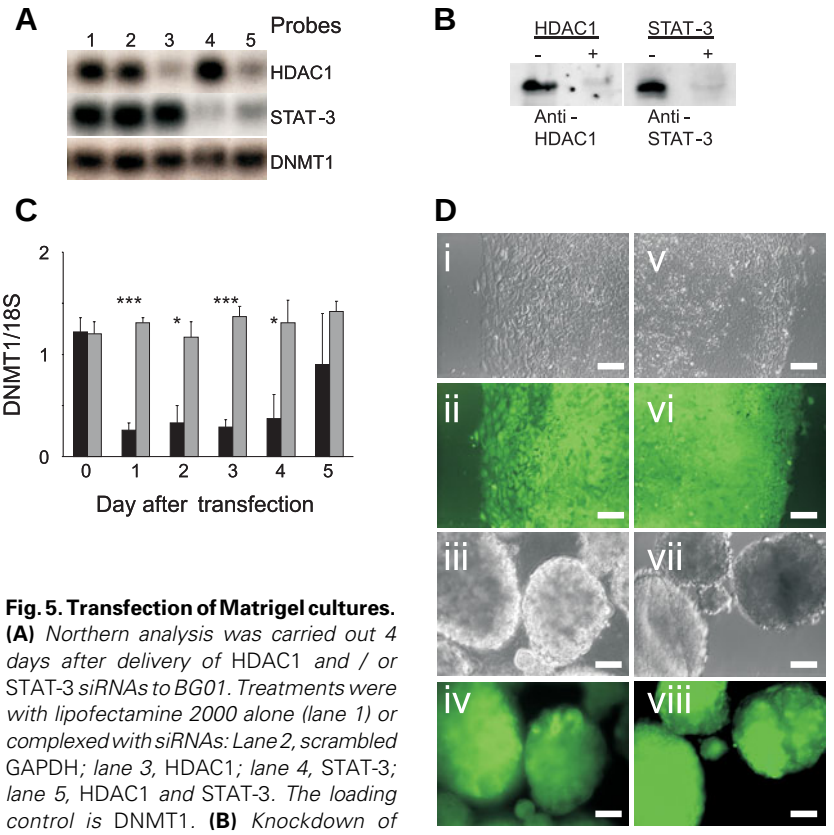

Fig. 5. Transfection of Matrigel cultures. **(A)** *Northern analysis was carried out 4 days after delivery of* HDAC1 *and / or* STAT-3 *siRNAs to BG01. Treatments were with lipofectamine 2000 alone (lane 1) or complexed with siRNAs: Lane 2, scrambled* GAPDH*; lane 3,* HDAC1*; lane 4,* STAT-3*; lane 5,* HDAC1 *and* STAT-3*. The loading control is* DNMT1*.* **(B)** *Knockdown of hDAC1 and STAT-3 proteins was confirmed by Western analysis: +, siRNA treated samples; -, control samples.* **(C)** *Semi-quantitative RT-PCR of* DNMT1 *relative to* 18S *after transient transfection of BG01 with a plasmid expressing* DNMT1 *shRNA (black bars) or no shRNA (empty vector; grey bars) from the human* U6 *promoter: * P<0.05, *** P<0.001; n=3.* **(D)** *Brightfield and fluorescence images of BG01 (i-ii) or HUES-7 (v-vi) and derived BG01-EBs (iii and iv) or HUES-7-EBs (vii-viii) after stable transfection with a* pcag-gfp-ires-pac *construct. Bar, 100 μm. All brightfield images have been taken using Hoffman objective lenses.*

Cardiomyocyte differentiation from BG01 and HUES-7

To determine whether a functional cell lineage could be produced from the EBs generated above, we scored the appearance of spontaneously beating outgrowths, which are indicative of cardiomyocyte differentiation. 3-6 day old BG01-EBs and HUES-7-EBs were cultured in D-FBS or K-FBS to promote further differentiation. Initial studies indicated D-FBS and K-FBS were comparable for cardiac differentiation and D-FBS was subsequently used (Table 3). Although EBs were cultured in suspension in non-tissue culture treated wells, they appeared to produce matrix and attached to the plastic. Initially they were forced back into suspension by gentle pipetting but after 8 days EBs were left to attach.

Induction of beating EBs was most effective from preparations of EBs that had formed efficiently (Table 3). Therefore, we used collagenase to harvest BG01 and HUES-7 cultured on feeders to prepare 1752 BG01-EBs and 3429 HUES-7-EBs (numbers scored at differentiation day 16). Beating was first detected on d13 in BG01-EBs and on d14 in HUES-7-EBs, while the median day of appearance was d19 for both lines (Fig. 6B). By d36 cumulative totals of 35 (BG01) and 59 (HUES-7) beating EBs were recorded (Fig. 6B). Beating EBs from both lines could still be detected on d50, the longest time assessed. The cumulative totals represent 2% (BG01) and 1.7% (HUES-7) relative to total EBs scored on d16. This is a conservative calculation because total number of EBs consistently decreased during extended culture mostly due to degradation. For example, during one experiment using HUES-7, beating was detected in 21 EBs, which represented 7% relative to total EBs (299) scored on d16 but by d21 the total number of EBs had decreased to only 83 and thus the relative proportion beating increased by ~4-fold to 25%.

The beat rate of BG01-EBs and HUES-7-EBs derived from cultures on feeders was similar at 37.2 ± 2.3 (SEM, n=33) and 41.1 ± 3.1 (SEM, n=49) beats / min, respectively (P=0.37). Although beating areas were rarely detected for EBs produced directly from

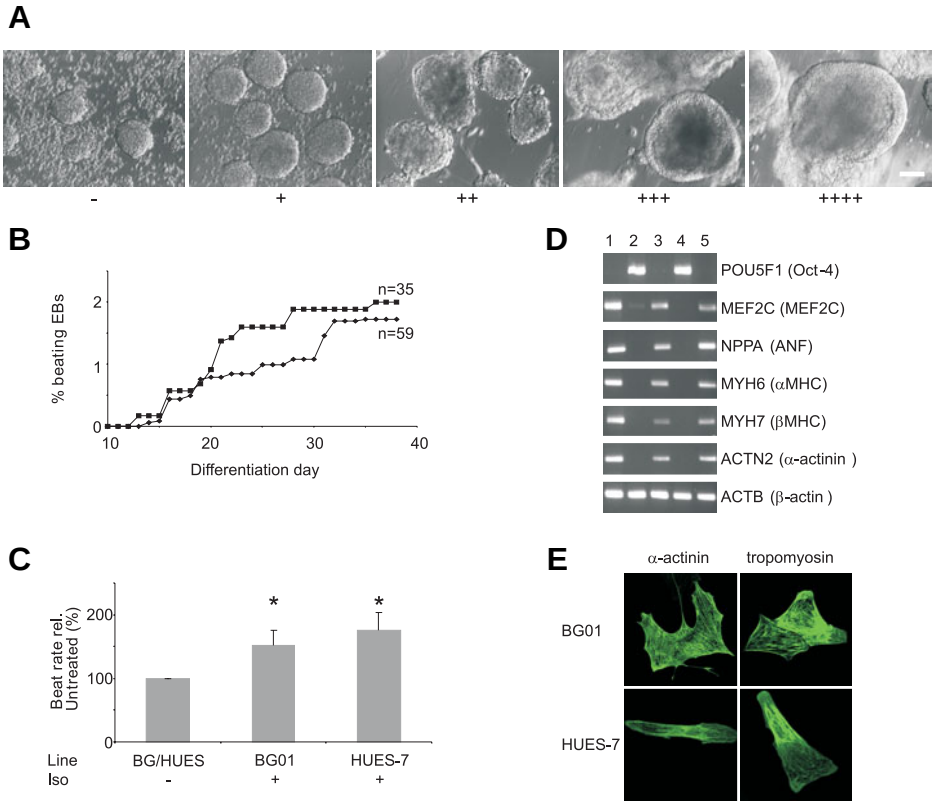

Fig. 6. Embryoid body (EB) and cardiomyocyte formation from BG01 and HUES-7. (A) *Representative images of EB size described in the scoring system used in Table 3. Bar, 100 μm. All brightfield images have been taken using Hoffman objective lenses.* **(B)** *Cumulative appearance of 35 beating BG01-EBs (squares) and 59 beating HUES-7-EBs (diamonds) relative to the total number of EBs scored at day 16 (1752 BG01-EBs and 3429 HUES-7-EBs).* **(C)** *Response of relative beat rate to 10^{-6} M isoprenaline (Iso), * P<0.05.* **(D)** *RT-PCR analysis of adult human heart RNA from Ambion (lane 1), undifferentiated BG01 (lane 2), undifferentiated HUES-7 (lane 4), beating BG01-EB at differentiation day 21 (lane 3) or beating HUES-7-EB at day 37 (lane 5).* **(E)** *Antibody reactivity of cells from dissociated beating BG01-EBs and HUES-7-EBs.*

Matrigel cultures, beat rates of 30.1 ± 12.8, n=4 (BG01-EBs) and 19.3 ± 2.4, n=9 (HUES-7-EBs) were recorded, which, for HUES-7-EBs, was significantly slower (P=0.005) than beating EBs derived from feeder cultures. Beat rate of EBs derived from hESCs cultured on feeders was significantly increased (P<0.05) by isoprenaline, a β-adrenoceptor agonist, to 152 ± 24% (SEM; n=4) and 175% ± 29% (n=5) for BG01-EBs and HUES-7-EBs respectively (Fig. 6C).

Gene and protein expression were examined in beating BG01-EBs and HUES-7-EBs. RT-PCR analysis showed high levels of *POU5F1* (Oct-4) gene expression levels in undifferentiated BG01 and HUES-7 that were markedly reduced in the beating EBs (Fig. 6D). Genes encoding the cardiac transcription factor, myocyte enhancer factor (MEF2C) and the structural proteins, atrial naturetic factor (ANF), α- and β-myosin heavy chain (MHC) and α-actinin, were all upregulated in beating areas relative to undifferentiated hESCs (Fig. 6D). Antibody staining of cells from dissociated beating BG01-EBs and HUES-7-EBs demonstrated reactivity for tropomyosin and α-actinin, revealing sarcomeric striations, albeit disorganised (Fig. 6E).

Discussion

While there have been considerable efforts to characterize some hESC lines (primarily the Wisconsin lines [reviewed in Allegrucci *et al.*, 2005; Goh *et al.*, 2005; see e.g. Schatten *et al.*, 2005]), for most lines information regarding conditions permissive for culture, transfection and differentiation is lacking. We have provided the following evidence to show culture and differentiation of two independently-derived hESC lines could be standardized: 1) both BG01 and HUES-7 were maintained using trypsin-passaging on 6x10^4 MEFs / cm^2 in HUES-C and HUES-nL or on Matrigel in BG-K CM; 2) EB formation was effective when BG01 and HUES-7 on feeders were harvested by collagenase treatment + scraping and then cultured in suspension in BG-K CM; 3) cardiomyocyte differentiation was induced in BG01-EBs and HUES-7-EBs by extended culture in D-FBS.

Since MEF feeders support undifferentiated hESCs by providing growth substrate and unknown growth factors, feeder density may be a critical parameter. Notably while most hESC lines have been cultured on confluent feeder layers seeded at ≥ 6 x 10^4 cells / cm^2 (Reubinoff *et al.*, 2000; Mitalipova *et al.*, 2003; Cowan *et al.*, 2004; Stojkovic *et al.*, 2004), undifferentiated proliferation of Wisconsin lines was supported by at 0.6-2 x 10^4 MEFs / cm^2 (Thomson *et al.*, 1998; Vallier *et al.*, 2004; Schatten *et al.*, 2005) but densities of >2 x 10^4 MEFs / cm^2 were detrimental (Heng *et al.*, 2004a). We found 6 x 10^4 MEFs / cm^2 were required to efficiently support BG01, with differentiated morphology evident at lower densities and peeling of the co-cultures from the plates at higher densities. This is consistent with a report by Zeng *et al.* (2004) who demonstrated maintenance of BG01 on 10 x 10^4 and not 1 x 10^4 MEFs / cm^2 but contrasts with Brimble *et al.* (2004), where undifferentiated BG01 were maintained by 2 x 10^4 MEFs / cm^2. Since we used the same strain of mouse to derive MEFs as Brimble *et al.* (2004), these differences are likely explained by variation in media; BG01 was isolated in medium containing 20% FBS and LIF (Mitalipova *et al.*, 2003) and early cultures were maintained in BG-SK medium (originally recommended to us by BresaGen; used here for feeder density trials and used by Zeng *et al.* [2004]), whereas Brimble *et al.* (2004) suggested that BG-K CM (containing 20% KSR) minimized differentiation of BG01 cultured on feeders (see also: http://stemcells.nih.gov/research/protocols/BresaGen_hESC_manual_2.1.pdf).

Although we found manual dissection of BG01 was successful, we adapted this line to trypsin-passaging on feeders and on Matrigel to facilitate comparison with an independently-derived line, HUES-7 (Cowan *et al.*, 2004). On feeders, proliferation rates

and morphology were similar between BG01 and HUES-7 in three different media. LIF was not advantageous, consistent with observations in HES-1 and HES-2 (Reubinoff *et al*., 2000) and H-1, -7 and -9 (Ginis *et al*., 2004). However, our calculated PD intervals for BG01 and HUES-7 on feeders were ~80-100 hours, significantly longer than previously reported for HUES-7 (48hrs; Cowan *et al*., 2004) or H-9 and clonal derivatives (35.3hrs; Amit *et al*., 2004) on feeders. Substantial cell death and / or poor attachment was observed in BG01 and HUES-7 post passage, possibly confounded by incomplete separation of hESCs and high density feeders. Cowan *et al*. (2004) calculated PD interval by counting HUES-7 periodically over 7 days without passaging, while Amit *et al*. (2004) achieved efficient separation of H-9 and feeders by exploiting the tolerance of this line to collagenase-passaging on low density MEFs. Furthermore, by transferring BG01 and HUES-7 to Matrigel, where post-passage mortality was greatly reduced, we subsequently recorded consistent PD intervals of ~30 and ~38hrs, respectively, similar to that described by Xu *et al*. (2001) for collagenase-passaged H-1, -7, -9 and -14 on Matrigel.

We observed a normal karyotype in trypsin-passaged BG01 and HUES-7 on feeders in HUES-C and HUES-nL during the test period, but clonal aneuploid populations were observed in both lines cultured in BG-SK. Interestingly, a recent report attributed karyotypic instability of BG01 cultured in BG-SK medium (albeit supplemented with LIF) to the bulk passaging methods, which employed Cell Dissociation Buffer (CDB) or combined collagenase / trypsin treatment (Mitalipova *et al*., 2005). However, our data suggest the culture medium may provide an alternative or additional route for instability, which will be an important consideration for future studies.

Of various conditions trialed on Matrigel, only BG-K CM sustained undifferentiated BG01 and HUES-7 proliferation. Since BG01 also maintained undifferentiated morphology when the DMEM base of BG-K was replaced with KO-DMEM (Fig. 1O), it is likely that components within ES-FBS or Plasmanate used to supplement BG-SK or HUES-C and HUES-nL can induce differentiation on Matrigel. We also found that unconditioned BG-K supplemented with 100 ng/ml of bFGF (FGF-2) did not support BG01 and HUES-7. This contrasts with recent reports where 40-100 ng/ml bFGF supported H-1, -7, -9 and -14 (Xu *et al*., 2005a; Xu *et al*., 2005b). The observed differences may relate to supplier of bFGF (Sigma vs Invitrogen) or passaging method (trypsin vs collagenase), a variable recently demonstrated to quantitatively influence the relative expression of *FGFR1-4* (Dvorak *et al*., 2005). Nevertheless, in the current study, all four receptors were expressed in both BG01 and HUES-7, suggesting the ability to respond to high concentrations of bFGF was not the limiting factor. Thus other genetic or epigenetic causes may be implicated, highlighting the importance of testing protocols on independently-derived lines.

Transfection using lipofection was successful when BG01 and HUES-7 were cultured on Matrigel but not on feeders, possibly suggesting that high density feeders sequester the transfection reagents. Consistent with this suggestion, H-7, -9 and -14 have been successful transfected on feeders when the hESC:feeder ratio was ~4:1 (Matin *et al*., 2004; Vallier *et al*., 2004), whereas in our BG01 transfections it was 1:4. Others have temporarily avoided the feeder layer by transducing hESCs in suspension with recombinant lentivirus (Pfeifer *et al*., 2002; Gropp *et al*., 2003] but the necessity of drug resistant feeders for stable selection of hESCs remains (Vallier *et al*., 2004).

Interestingly, we observed poor EB formation of BG01 and HUES-7 harvested directly from Matrigel, although this could be rescued by short-term transfer back to feeders. This implies expression of key adhesion factors is absent or altered when trypsin-passaged BG01 and HUES-7 are cultured on Matrigel and warrants further investigation. Consistent with this suggestion, the Wisconsin line, H1, has been shown to express high levels of the gap junction protein, connexin 43 and adhesion molecules such as integrins α6 and β1 (with moderate levels of α2 and low levels of α1, α2, α3 and β4) in feeder-dependent and feeder-free conditions (Xu *et al*., 2001), whereas a recent study showed only connexin 43 and integrin β5 were highly expressed in BG01 cells on feeders (Zeng *et al*., 2004). Further analysis is required to determine whether differences in adhesion protein gene expression relate to why Wisconsin lines formed EBs efficiently in KO-DMEM containing 20% FBS when directly collagenase-harvested from Matrigel (Xu *et al*., 2001) whereas similar harvesting conditions were not effective in BG01 and HUES-7.

Differentiation of BG01 and HUES-7 resulted in appearance of beating areas between days 13 and 36, with spontaneous contraction continuing to d50 in some BG01-EBs and HUES-7-EBs. Since derived cardiomyocytes also responded to isoprenaline, expression of β1-adrenoceptors is inferred. Analysis by RT-PCR and / or immunocytochemical also demonstrated expression of

TABLE 2

CULTURE METHODS TESTED FOR BG01 AND HUES-7

Culture substrate	Passage method	Culture medium (from/to)	No. passages tested
BG01			
Feeders	Manual dissection	BG-SK	52 (p24 to p76)
	Collagenase	BG-SK	D
	Dispase	BG-SK	D
	Trypsin	BG-SK	14 (p36 to p50) * K
		HUES-C	5 (p45 to p50) *
		HUES-nL	5 (p45 to p50) *
Matrigel	Collagenase	BG-K CM	28 (p41 to p69)
	Trypsin	BG-K CM	10 (p42 to p52) *
			25 (p39 to p64)
		BG-SK CM	D
		HUES-C CM	5 (p42 to p47) * D
		HUES-nL CM	4 (p42 to p46) * D
		BG-K-4	2 (p48 to p50) D
		BG-K-100	2 (p45 to p47) D
			2 (p48 to p50) D
HUES-7			
Feeders	Trypsin	BG-SK	5 (p20 to p25) * K
		HUES-C	13 (p12 to p25) *
		HUES-nL	5 (p20 to p25) *
Matrigel	Trypsin	BG-K CM	14 (p23 to p37) *
		HUES-C CM	3 (p23 to p26) * D
		HUES-nL CM	3 (p23 to p26) * D
		BG-K-4	2 (p31 to p33) D
		BG-K-100	2 (p27 to p30) D
			2 (p31 to p33) D

* denotes samples featured in growth curves in Fig. 3. Also indicated are: D, sample differentiated; K, high proportion of cells with abnormal karyotype: +X or +17 (BG01) and +12+17 (HUES-7).

cardiac transcription factors and structural proteins. These observations are consistent with those of others using HES-2 (Mummery *et al.*, 2003) and H-1, -7, -9 and -14 (Kehat *et al.*, 2001; Xu *et al.*, 2002; He *et al.*, 2003; Reppel *et al.*, 2004; Xue *et al.*, 2005). However, unlike these reports where relatively high numbers of beating outgrowths (8-70%) were identified, the relative proportion of beating BG01-EBs and HUES-7-EBs ranged from 2% (relative to total EBs counted early in differentiation) to ~8% (relative to total EBs counted later in differentiation). While differences may relate to variation in passaging method and / or genetics between these lines and BG01 or HUES-7, in contrast to these reports, we did not induce cardiac differentiation with batch selected serum (He *et al.*, 2003; Reppel *et al.*, 2004), 5-aza-deoxycytidine (Xu *et al.*, 2002) ascorbic acid (Passier *et al.*, 2005) or by co-culture with visceral endoderm-like cells (Passier *et al.*, 2005). These and other cardiac inducers such as growth factors (e.g. members of the TGF-b1 superfamily), oxytocin, erythropoietin and DMSO (Heng *et al.*, 2004b), may facilitate cardiomyogenesis in BG01 and HUES-7.

In conclusion, we have identified standardized conditions for BG01 and HUES-7 culture and differentiation and initiated characterization of derived cardiomyocytes. Thus we routinely maintain undifferentiated BG01 and HUES-7 using trypsin-passaging on Matrigel in BG-K conditioned medium. Efficient formation of embryoid bodies is achieved by transferring hESCs transiently from Matrigel back to feeders before harvesting with collagenase. Further differentiation is achieved by suspension culture in BG-K conditioned medium for 6 days prior to long-term culture in D-FBS. Whether the protocols applied to these two lines with very diverse derivation and culture backgrounds can be further applied to other independently-derived lines should now be tested. Clearly however, standardization in a range of independently-derived hESC lines that recapitulate human genetic diversity will facilitate use of derived, differentiated lineages in drug screening, disease modeling and transplantation.

TABLE 3

EFFICIENCY OF EMBRYOID BODY FORMATION FROM BG01 AND HUES-7

hESC line & culture method	Harvesting method	Medium 1 (days 3-6 of differentiation)	Efficiency of EB formation [b]	Medium 2 (continued differentiation)	Beating areas observed
BG01					
Manual / feeders	Manual dissection	D-FBS	++++	D-FBS	Yes
		K-FBS	++++	K-FBS	Yes
		BG-K CM	++++	D-FBS	Yes
Trypsin / feeders	Collagenase + scraping	BG-K CM	++++	D-FBS	Yes
Trypsin / matrigel	Collagenase + scraping	D-FBS	-	n/a	-
		K-FBS	-	n/a	-
		BG-K CM	+	D-FBS	Rarely
Trypsin (M→F[a])	Collagenase + scraping	BG-K CM	++++	D-FBS	Yes
HUES-7					
Trypsin / feeders	Trypsin	D-FBS	++	D-FBS	Rarely
		K-FBS	++	D-FBS	No
		BG-K CM	+++	D-FBS	No
Trypsin / feeders	Collagenase + scraping	D-FBS	+++	D-FBS	Yes
		K-FBS	+++	D-FBS	Yes
		BG-K CM	++++	D-FBS	Yes
		HUES-C CM	++++	D-FBS	Yes
Trypsin / matrigel	Collagenase + scraping	D-FBS	-	n/a	-
		BG-K CM	+	D-FBS	Rarely
		HUES-C CM	+	D-FBS	Rarely
Trypsin (M→F[a])	Collagenase + scraping	D-FBS	+++	D-FBS	Yes
		BG-K CM	++++	D-FBS	Yes

[a] Matrigel cultures were transferred to feeders and grown to confluence before harvesting for EBs.

[b] Grading system reflects both size and number of EBs. For representative images, see Fig. 5.

Materials and Methods

Materials and general culture

All culture components were from Invitrogen and all chemicals were from Sigma unless otherwise specified. Culture was carried out at 37ºC in a humidified atmosphere containing 5% CO_2. Medium was changed daily for hESC culture.

Culture of BG01 and HUES-7

Manually-passaged BG01 (passage 24; p24) were purchased from BresaGen (Athens, GA; Mitalipova *et al.*, 2003) and HUES-7 (p11) were kindly gifted by Harvard University (Cowan *et al.*, 2004). Successful continued culture was by manual dissection (BG01) or trypsin-passaging (BG01 and HUES-7) on MEFs (strain CD1, 13.5dpc) and by collagenase-passaging (BG01) or trypsin-passaging (BG01 and HUES-7) feeder-free on Matrigel. MEF feeders were cultured in DMEM High Glucose supplemented with 10% Fetal Bovine Serum (FBS; Invitrogen E.U. Approved), 2mM glutamine and 1% non-essential amino acids. MEFs were mitotically inactivated for 2.5 hours with 10 μg/ml mitomycin C, seeded at densities specified in the text and used 24-48h later at p3. Tested media were 1) BG-SK (DMEM-F12 supplemented with 15% ES-screened Hyclone FBS [ES-FBS], 5% KNOCKOUT Serum Replacement [KSR], 100 μM β-mercaptoethanol [β-ME], 4ng/ml basic fibroblast growth factor [bFGF], 1% non-essential amino acids [NEAA], 2mM glutamine); 2) BG-K (as for BG-SK but supplemented with 15 % KSR and no FBS); 3) BG-K-100 (as for BG-K but supplemented with 100ng/ml bFGF); 4) HUES-C (KNOCKOUT DMEM supplemented with 10% Plasmanate, 10% KSR, 55 μM b-ME, 10ng/ml bFGF, 12ng/ml leukaemia inhibitory factor (LIF), 1% NEAA, 2mM glutamine); 5) HUES-nL (as for HUES-C but no LIF). For Matrigel culture, medium was conditioned on mitotically inactivated MEFs for 20-24 hours, harvested and supplemented with 4ng/ml (BG-SK, BG-K) or 5 ng/ml (HUES-C, HUES-nL) bFGF.

For MEF feeder culture, BG01 colonies of ~0.5-1.0 mm diameter with undifferentiated morphology were manually-dissected into 8 pieces using a Stem Cell ToolTM (Swemed Lab) under a stereomicroscope and seeded onto fresh MEFs. Alternatively BG01 or HUES-7 cultures on feeders were incubated with 0.5% Trypsin-EDTA for 1-2 min at 37ºC. After disaggregation by pipetting, large clumps of feeders were eliminated by centrifugation at 100 x g for 6s. For feeder-free culture on Matrix Growth Factor Reduced Matrigel (BD Biosciences) coated flasks, cells were passaged either by 1mg/ml Collagenase IV treatment (BG01) for 3 min at 37°C followed by gentle scraping of the colonies or by 0.05% Trypsin-EDTA treatment (BG01 and HUES-7) of the cell monolayer for 1 min at 37°C.

Population doublings (PDs) at each pas-

sage were calculated using the formula: Log10 (total cell counts/cells seeded) / Log10 (2). Proliferation rates over a minimum of 5 passages were calculated by hours in test culture/cumulative PDs.

Analysis of *FGFR1-4* was carried out by RT-PCR on undifferentiated trypsin-passaged BG01p48 and HUES-7p28 cells maintained on Matrigel in BG-K CM. RT was carried out on extracted RNA (RNAeasy kit, Qiagen) using Superscript II (Invitrogen) according to manufacturer's instructions. PCR cycle parameters were 94ºC 15 min for 1 cycle, [94ºC 30 sec, 60ºC 30 sec, 68ºC 1 min for 40 cycles], 68ºC 10 min for 1 cycle using High Fidelity PCR Master Mix (Roche). Primers were designed to map to exons known to be common to all isoforms, as indicated from the NCBI Entrez Gene database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene).
FGFR1, Gene ID 2260,
F = 5'- ATGTGGAGCTGGAAGTGCCTCCT-3' and
R = 5'-GGTTTGGTTTGGTGTTATCTGTTTCTT-3';
FGFR2, Gene ID 2263,
F = 5'-CTCCTTCAGTTTAGTTGAGGATACCA-3' and
R = 5'-CCACAACATCCAGGTGGTACGTGT-3';
FGFR3, Gene ID 2261,
F = 5'-GTCAAGGATGGCACAGGGCTGGT-3' and
R = 5'-CTTGATGCCTCCAATGCGGTGCT-3';
FGFR4, Gene ID 2264,
F = 5'-ATGCGGCTGCTGCTGGCCCTGT-3' and
R = 5'-GTCACCTGTAATCAAGGTGAGATTCT-3'.

Immunocytochemistry

SSEA-1, SSEA-4, TRA-1-60 and TRA-1-81 surface markers of undifferentiated, paraformaldehyde-fixed hESCs were assessed by permeabilizing cells with Triton-X100 and then blocking with 4% goats serum in PBS for 30 min. hESCs preparations were incubated with 20 μg/ml of the relevant primary antibody (ES Cell Characterization kit, Chemicon, 1:50) for 1 hr and with 30 μg/ml FITC-conjugated goat anti-mouse IgG+IgM secondary antibody (Jackson Immuno Research Inc, 1:50) for 45 min. For cardiomyocyte characterization, beating EBs were dissociated by treatment with Cell Dissociation Buffer (CDB) for 15 min at 37ºC. Permeablized (Triton-X100), paraformaldehyde-fixed cells were blocked with 4% goats serum in PBS for 30 min, incubated with mouse anti-tropomyosin (1:50; Sigma T9283) or anti-α-actinin (1:800; Sigma A7811) for 1 hr and then incubated with FITC-conjugated goat anti-mouse IgG for 1 hr. hESC marker and cardiomyocyte preparations were mounted by using Vectashield (Vector Laboratories) with or without DAPI respectively and observed under an epifluorescence microscope.

hESC transfection and analysis

Lipofectamine 2000 was used for hESC transfection. Transient knock-down in BG01 using *STAT-3* and / or *HDAC1* siRNA or *DNMT1* shRNA was achieved by incubating 10 μl of 20 μM siRNA (*STAT-3* and *HDAC1* SmartPoolTM; Upstate Biologicals) or 2 μg *DNMT1* shRNA plasmid (gifted from Matsukura *et al*. [2003]) with 2 μl Lipofectamine 2000 according to manufacturers instructions (Invitrogen). Complexes were added to 0.15 x 10^6 BG01 cells in suspension in 2 ml BG-K-CM and seeded to a 35mm Matrigel-coated dish. For northern analysis, 20 μg RNA / sample was blotted, crosslinked and then hybridized at 42ºC in ultrahyb buffer (Ambion) for 16 hrs with *STAT-3, HDAC1* or *DNMT1* probes, which had been generated by RT-PCR.
STAT-3 primers
F = 5'-atggcccaatggaatcagctacagc-3' and
R = 5'-ggtgatcaggtgcagctcctcag-3', Accession NM_139276;
HDAC1 primers
F = 5'-atggcgcagacgcagggcacc-3 and
R = 5'-gcaacctaaccgatccccagatag -3', Accession NM_004964;
DNMT1 primers
F = 5'-gtgggggactgtgtctctgt-3' and
R = 5'-gaaagctgcatgtcctcaca-3', Accession NM_001379). For RT-PCR semi-quantitation of *DNMT1*, total RNA was extracted using RNAeasy kit, (Qiagen) and RT was carried out using Omniscript (Invitrogen) according to manufacturer's instructions. Multiplex PCR was performed using *DNMT1* primers together *18S* primers and competimer (2:8 ratio, Ambion). PCR cycle parameters were 94ºC 3 min for 1 cycle, [94ºC 1 min, 57ºC 1 min, 72ºC 1 min for 30 cycles], 72ºC 7 min for 1 cycle using Taq DNA polymerase (Roche). Western blot analysis of STAT-3 and HDAC1 was performed by sample separation (15mg/lane) on 12.5% SDS PAGE and blotting onto nitrocellulose membrane (PhastSystem, Amesham). Blots were blocked with 2% BSA blocking reagent in TBST-I (15 mM Tris-HCl, 150 mM NaCl, 0.2% Tween 20, pH7.5) for 1hr, incubated with 2 μg/ml diluted anti-STAT3 or anti-HDAC1 antibody (Upstate Biologicals) for 16hrs at 4°C and then incubated with 1:25,000 diluted donkey anti-rabbit IgG-HRP (Amersham, NA934) for 1hr. Band location was resolved by chemiluminescence (ECL Advance Kit, Amersham).

For stable transfection with *pcag-gfp-ires-pac* (gifted from Ying *et al*. [2003]), 0.15 x 10^6 BG01 or HUES-7 cells were seeded in 35mm Matrigel-coated dishes. The next day, 5 μg plasmid was complexed with 2 μl Lipofectamine 2000 according to manufacturer's instructions and added to BG01 or HUES-7 in 2mls BG-K-CM. After 48 hrs, puromycin selection (300 μg/ml) was initiated.

Cardiomyocyte differentiation and analysis

Assessment of beat rate (beats / min) of single beating BG01-EBs and HUES-7-EBs at rest or stimulated with 10^{-6}M isoprenaline was measured at 37ºC in D-FBS, which contains 1.8mM Ca^{2+} and 5.2mM K^+. RT-PCR analysis was performed on adult human heart RNA, undifferentiated BG01 and HUES-7 and derived beating EBs. RT was carried out on extracted RNA (RNAeasy kit, Qiagen) using Superscript II (Invitrogen) according to manufacturer's instructions. PCR cycle parameters were 94ºC 15 min for 1 cycle, [94ºC 30 sec, 60ºC 30 sec, 68ºC 1 min for 40 cycles], 68ºC 10 min for 1 cycle using High Fidelity PCR Master Mix (Roche). Primers were:
POUF1 (Oct-4)
F = 5'-ctatttgggaaggtattcagccaaac-3' and
R = 5'gaagaacttaatcccaaaaaccctgg-3', Accession BC020712;
MEF2C (MEF2C)
F = 5'-agattacgaggattatggatgaacgt-3'and
R = 5'-acctgcacttggaggtcgatgtg-3', Accession NM_002397;
NPPA (ANF)
F = 5'- ggaaccagaggggagagacagag -3' and
R= 5'-cgccctcagcttgctttttaggag-3', Accession X01471;
MYH6 (αMHC)
F = 5'- atgaccgatgcccagatggctga -3' and
R = 5'tcactcctcttcttgccccggta-3', Accession D00943;
MYH6 (βMHC)
F = 5'- agctggcccagcggctgcagg -3' and
R = 5'-ctccatcttctcggcctccagct -3', Accession X06976;
ACTN2 (α-actinin)
F = 5'-ccggcgtgcagtacaactacgtg-3' and
R = 5'-gtagtcaatgaggtcaggccggt-3', Accession BC051770;
ACTN2 (β-actin)
F = 5'-ggacttcgagcaagagatggcca-3' and
R = 5'-tcgtcatactcctgcttgctgatc-3', Accession X00351.

Karyotype assessment

Exponentially growing cultures of at least 1x10^5 cells were treated with 100ng/ml colcemid (Karyomax) for 45 min and harvested (including feeders where used) with 0.05% Trypsin-EDTA. Pelleted cells (200 x g for 4 min) were resuspended in 0.6% sodium citrate and incubated at 37ºC for 20 min. Cells were then recentrifuged (300 x g for 4 min) and fixed by resuspension in 16.7% glacial acetic acid in methanol and were washed with 2 further changes of fixative. Chromosome spreads were prepared by dropping cells onto glass slides, which were air dried and heated to 70ºC overnight. Chromosomes were G banded with trypsin (BD Difco) and stained with Leishmans. For each culture, 30 metaphase spreads

were examined; full analysis involving band by band comparison between chromosome homologues was performed on 3 spreads and presence of gross abnormalities visually examined in 27 spreads, in accordance with ISCN Human Cytogenetic Nomenclature international guidelines (Mitelman, 1995).

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