Chapter 12

Highly Efficient Directed Differentiation of Human Induced Pluripotent Stem Cells into Cardiomyocytes

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Abstract

Human-induced pluripotent stem cell (hiPSC)-derived cardiomyocytes are a novel source of cells for patient-specific cardiotoxicity drug testing, drug discovery, disease modeling, and regenerative medicine. We describe a versatile and cost-effective protocol for in vitro cardiac differentiation that is effective for a wide variety of hiPSC and human embryonic stem cell (hESC) lines. This highly optimized protocol produces contracting human embryoid bodies (hEB) with a near total efficiency of $94.7 \pm 2.4\%$ in less than 9 days, and minimizes the variability in cardiac differentiation commonly observed between various hiPSC and hESC lines. The contracting hEB derived using these methods contain high percentages of pure functional cardiomyocytes, highly reproducible electrophysiological profiles, and pharmacologic responsiveness to known cardioactive drugs.

Key words Human embryonic stem cell, Induced pluripotent stem cell, Heart, Cardiac, Cardiomyocyte, Differentiation, Forced aggregation

1 Introduction

The cardiac differentiation of human pluripotent stem cells provides a source of cardiomyocytes with great promise in drug screening, the study of the otherwise inaccessible biology of human cardiac development, and for cell replacement strategies. The advent of human-induced pluripotent stem cells (hiPSC), derived via somatic cell reprogramming, allows the production of pluripotent stem cells from patients with a known history—either carrying a specific genetic disease or from healthy patients. The cardiac differentiation of these cell lines thus allows for cardiac disease modeling along with patient-specific cardiotoxicity testing and novel drug discovery. In the future the use of non-diseased or disease-corrected hiPSC may provide a promising source of cells for regenerative medicine.

Once suitable pluripotent cell sources are available, the next step is to further refine cardiac differentiation. Three major techniques

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are commonly used for the differentiation of hESC or hiPSC to the cardiac lineage. They include differentiation involving cultivation of hPSC with a stromal cell layer possessing lineage inductive properties (1, 2), differentiation of cells in a monolayer (3, 4), and mechanical or enzymatic manipulation to form spherical clusters of cells termed human embryoid bodies (hEB) (5–7). Each of these techniques includes variables that influence the reproducibility of differentiation. These variables include the quality and culture method of the pluripotent stem cell line, contamination with murine embryonic fibroblast (MEF) feeders, the size of the pluripotent cell clumps used for hEB formation, and variation in the secreted factors produced from stromal cell coculture.

We describe here a highly efficient method for the generation of cardiac progenitors and cardiomyocytes from hESC and hiPSC (Fig. 1).

This protocol is further optimized from our earlier reports (8, 9), and is composed of four major steps: (1) culture of hESC/hiPSC in a rigorous and controlled manner in conditioned medium, (2) reproducible formation of hEB from a known number of cells using forced aggregation, (3) exposure to FBS for cardiac induction, and (4) adhesion and formation of contracting cardiomyocytes.

One of the most important problems with reproducibly differentiating hESC/hiPSC is that each cell line has been derived using different methods and often cultured using different conditions. Common culture conditions involve the growth of hESC/hiPSC as colonies, either on a MEF feeder layer or with feeder-free approaches on Matrigel/Geltrex or synthetic matrices. Colonies inherently have cells that are at different densities, which, due to contact inhibition, will usually be in a different part of their growth phase. To eliminate the variability in growth rate we developed a monolayer growth method (10). Using this method, cells are passaged as single cells every 3 days, and seeded at a known density, allowing tight control of growth rate prior to differentiation. Additionally, differentiation experiments are set up from cells that have been passaged on the previous day (11). We have shown that this technique substantially enhances differentiation by ensuring that only live and exponentially growing cells are incorporated into hEB. The differentiation of hESC/hiPSC as a hEB allows the formation of multidimensional cell-cell interactions more closely mimicking in vivo conditions whilst also producing cardiomyocytes electrically coupled in three dimensions. Simple methods for hEB formation, such as scraping colonies and suspending them in 20% FBS (5), produce hEB of a wide variety of sizes which results in poor yields of beating hEB. Here we use a forced aggregation method, first demonstrated for hESC differentiation (12), to form a known number of single cells into an embryoid body. We first described this for cardiac differentiation in 2007 (8) using a chemically defined medium (CDM) for the 96-well V-bottom plate

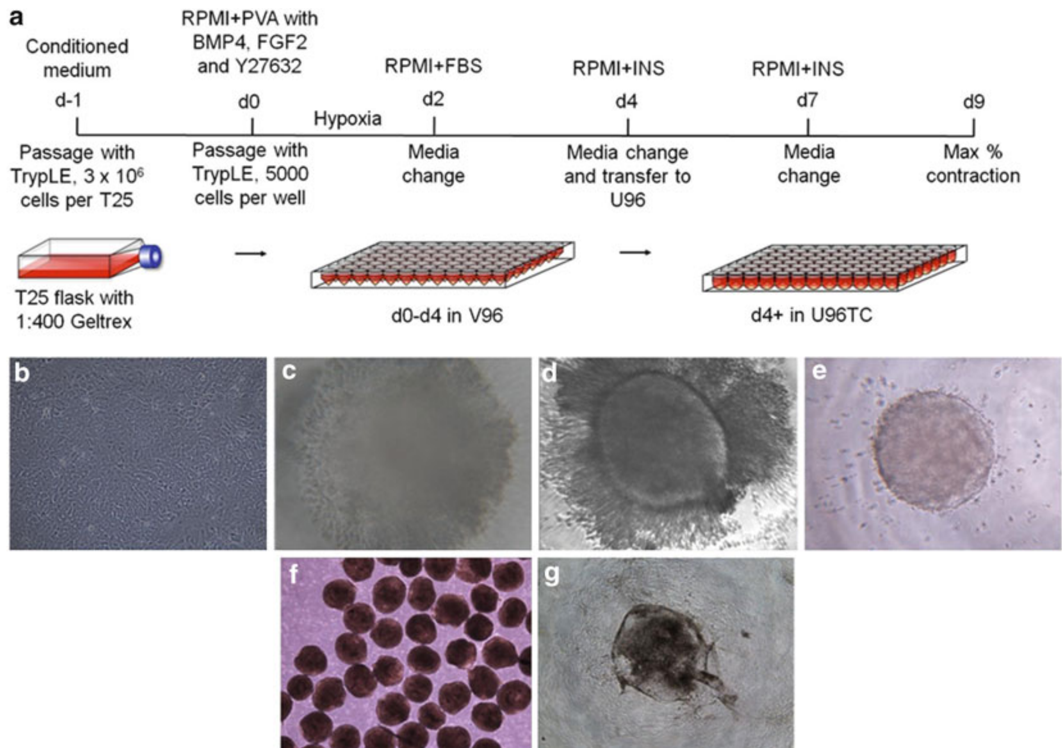

Fig. 1 Schematic of the forced aggregation directed cardiac differentiation method. (**a**) *Top row*: media used and timing of media changes; *middle*: physical tasks performed at each time point; *bottom*: cell format showing move from T25 flasks to V96 plates and U96 tissue culture-treated plates. (**b**) Growth of human pluripotent cells as a monolayer showing 100% confluence. (**c**) 5,000 cells per well of a V96 plate after 1 day. (**d**) On day 2 prior to first media change showing clear hEB formation. (**e**) Day 4 after media change and transfer to U96 plates showing removal of dead cells. (**f**) hEB on day 4 after transfer to bulk culture demonstrating uniformity of hEB size and shape. (**g**) Contracting hEB after 9 days of culture showing minimal surrounding fibroblasts

aggregation step followed by culture in 20% FBS. In this iteration of our differentiation methodology for hEB formation and mesoderm induction, we use a simplified version of the CDM (13) medium, based on RPMI 1640, along with the growth factors BMP4 and FGF2. The mechanism of action of BMP4 and FGF2 synergy has recently been investigated, which highlighted a role for the FGF2-induced maintenance of *NANOG* (14). We have recently demonstrated that during the initial d0–d2 stage, low level of Y27632 (ROCK inhibitor) (15), high levels of polyvinyl alcohol (PVA), and exposure to physiological oxygen (5%) levels combine to enhance cardiac differentiation. The protocol described here contains fetal bovine serum (FBS) during the d2–d4 stage, which provided the greatest reproducibility of cardiomyocyte generation, whilst the inclusion of L-ascorbic acid-2-phosphate further increased the final number of cardiomyocytes. After the d2–d4 exposure to

FBS, hEB are maintained in a simple CDM. By day 9 of differentiation, 94.7 ± 2.4% of hEB contracted, and each beating hEB routinely contained 60–90% pure cardiomyocytes as assessed by cardiac troponin I expression (TNNI3). hEB produced using this method will continue to contract for over 3 months.

2 Materials

2.1 Cells

1. E13.5 DR4 × CF1 mouse embryonic fibroblasts, passage 2, frozen at 3×10^6 cells per vial.
2. hESC (such as H9 (WA09), H7 (WA07), HES2 (ES02), or HES3 (ES03), supplied by WiCell Research Institute) (16, 17).
3. Viral construct-integrated hiPSC (such as iPS(IMR90)-1 or iPS(IMR90)-4, supplied by WiCell Research Institute) (18).
4. Nonviral, nonintegrated hiPSC (such as Gibco Episomal hiPSC line, derived from neonatal $CD34^+$ cord blood, Invitrogen) (9).

2.2 Reagents

1. Dulbecco's phosphate-buffered saline (D-PBS), pH 7.4, without $CaCl_2$ and $MgCl_2$.
2. TrypLE Express, store at RT, there is no need to warm to 37°C before use.
3. Geltrex Reduced Growth Factor Basement Membrane Matrix, store at −20°C, thaw at 4°C overnight, and subsequently store at 4°C.
4. 0.1% gelatin (1 g/L in distilled H_2O and autoclaved), store at 4°C.
5. DMEM, high glucose.
6. DMEM/F-12 (1×), liquid, 1:1 (with 2.5 mM glutamine).
7. RPMI 1640 (with 2.5 mM glutamine).
8. Fetal bovine serum.
9. KnockOut Serum Replacement.
10. Nonessential amino acids.
11. 2-Mercaptoethanol.
12. Chemically defined lipid concentrate.
13. L-ascorbic acid 2- phosphate (6.4 mg/mL in UltraPure water, store at 4°C for up to 3 weeks) (see Note 1).
14. UltraPure DNase/RNase-Free Distilled Water.
15. Human recombinant insulin, liquid.
16. 1-thioglycerol dilute to 150 mM in UltraPure water (13 μL in 987 μL), make fresh or store at −20°C and use once.
17. Polyvinyl alcohol (see Note 2).

18. Human serum albumin.
19. Recombinant human bone morphogenic protein 4 (BMP4), reconstitute to 100 ng/μL in 4 mM HCl with 0.1% HSA (1 mg/mL). Make aliquots and store at −20°C.
20. Recombinant human fibroblast growth factor 2 (FGF2), reconstitute to 100 ng/μL in D-PBS with 0.1% HSA (1 mg/mL). Make aliquots and store at −20°C.
21. Y27632 (ROCK inhibitor), 10 mM stocks in DMSO, store at −20°C.
22. Mitomycin C.
23. Hemocytometer.

2.3 Equipment

1. 6-well plates.
2. T25 and T175 flasks.
3. 15 and 50 mL conical tubes.
4. 2 mL serological pipettes.
5. 5, 10, 25 and 50 mL serological pipettes.
6. 150 mL PES media filters.
7. 250 and 500 mL and 1 L PES media filters.
8. 96-well V-bottom plates.
9. 96-well U-bottom tissue culture treated plates.
10. Reagent reservoirs.
11. Multichannel Pipette.
12. 8-channel aspirator.
13. P200 pipette tips without filters for aspirator.
14. Automatic cell counter.
15. Tissue culture incubator at 37°C, 5% CO_2, 85% relative humidity Hypoxic incubator or hypoxia chamber capable of 5% O_2, 5% CO_2.
16. Centrifuge.
17. Inverted tissue culture microscope with heated stage.
18. Cesium source irradiator capable of 5,000 cGy.

2.4 Reagents Setup

2.4.1 MEF Medium

DMEM, 10% (v/v) FBS, 1% (v/v) NEAA, 55 μM 2-mercaptoethanol. Filter sterilize and store at 4°C for up to 2 weeks (Table 1).

2.4.2 hESC Medium

DMEM/F-12, 15% (v/v) KSR, 1% (v/v) NEAA, 100 μM 2-mercaptoethanol. Filter sterilize, add 4 ng/mL FGF2, and store at 4°C for up to 2 weeks (Table 2).

Table 1
Mouse embryonic fibroblast medium formulation

MEF medium	100 mL	200 mL	562 mL
DMEM high glucose (with 4 mM L-glutamine)	89 mL	178 mL	500 mL
10% FBS	10 mL	20 mL	56.2 mL
1% NEAA (100×)	1 mL	2 mL	5.62 mL
55 µM 2-mercaptoethanol (55 mM)	100 µL	200 µL	562 µL
	Filter sterilize		

Table 2
hESC medium formulation

hESC medium	200 mL	595.6mL	1,074mL
DMEM/F-12 (with 2.5 mM L-glutamine)	167.6mL	500 mL	900 mL
15% KnockOut SR	30 mL	89.3mL	161 mL
1% NEAA (100×)	2 mL	5.97mL	10.61mL
100 µM 2-mercaptoethanol (55 mM)	364 µL	1.08mL	1.954mL
	Filter sterilize		
4 ng/mL FGF2 (100 ng/mL)	8 µL	23.8 µL	43 µL

2.4.3 Conditioned Medium (CM)

The above hESC medium conditioned (0.5 mL/cm^2) on irradiated MEF seeded at 6×10^4 cells/cm^2 for 22–26 h. Filter sterilize and add an additional 4 ng/mL FGF2 (see below for full protocol). Store at 4°C for up 1 week or make aliquots and store at −20°C for up to 6 months.

2.4.4 RPMI + PVA Medium

RPMI 1640, 4 mg/mL PVA, 1% (v/v) chemically defined lipid concentrate, 10 µg/mL human recombinant insulin, 450 µM 1-thioglycerol. Filter sterilize and make fresh before each use. Add growth factors immediately before use (Table 3).

2.4.5 RPMI + FBS Medium

RPMI 1640, 20% (v/v) FBS, 221 µM L-ascorbic acid 2- phosphate, 450 µM 1-thioglycerol. Filter sterilize and store at 4°C for up to 2 weeks (Table 4).

2.4.6 RPMI + INS Medium

RPMI 1640, 1% (v/v) chemically defined lipid concentrate, 10 µg/mL human recombinant insulin, 221 µM L-ascorbic acid 2- phosphate, 450 µM 1-thioglycerol. Filter sterilize and store at 4°C for up to 2 weeks (Table 5).

Table 3
RPMI + PVA medium formulation

RPMI + PVA medium	50 mL	100 mL	200 mL
RPMI 1640 (with 2 mM L-glutamine)	47.5 mL	95 mL	190 mL
4 mg/mL PVA (100 mg/mL)	2 mL	4 mL	8 mL
1× chemically defined lipid concentrate	500 µL	1 mL	2 mL
450 µM 1-thioglycerol (150 mM)	150 µL	300 µL	600 µL
10 µg/mL recombinant human insulin	50 µL	100 µL	200 µL
Filter sterilize			
25 ng/mL recombinant human BMP4 (100 ng/µL)	12.5 µL	25 µL	50 µL
5 ng/mL recombinant human FGF2 (100 ng/µL)	2.5 µL	5 µL	10 µL
1 µM Y27632 (10 mM)	5 µL	10 µL	20 µL

Table 4
RPMI + FBS medium formulation

RPMI + FBS	100	200 mL	635 mL
RPMI 1640 (with 2 mM L-glutamine)	77 mL	158 mL	500 mL
20% FBS	20 mL	40 mL	127 mL
64 µg/mL L-ascorbic acid 2- phosphate (64 mg/mL)	1 mL	2 mL	6.4 mL
450 µM 1-thioglycerol (150 mM)	300 µL	600 µL	1.9 mL
Filter sterilize			

Table 5
RPMI + INS medium formulation

RPMI + INS medium	100	200 mL	512 mL
RPMI 1640 (with 2 mM L-glutamine)	98 mL	196 mL	500 mL
1× chemically defined lipid concentrate	1 mL	2 mL	5.1 mL
10 µg/mL recombinant human insulin (10 mg/mL)	100 µL	200 µL	510 µL
64 µg/mL L-ascorbic acid 2- phosphate (64 mg/mL)	1 mL	2 mL	5.1 mL
450 µM 1-thioglycerol (150 mM)	300 µL	600 µL	1.5 mL
Filter sterilize			

3 Methods

3.1 Compatible Methods for Pluripotent hESC/hiPSC Growth

All cultures should be maintained at 37°C in a humidified incubator with 5% CO_2 and atmospheric O_2 unless otherwise stated. This method is not optimized for hESC/hiPSC cultured on MEF, as colonies, or in defined media such as mTeSR1 (StemCell Technologies) or Nutristem (Stemgent). If cells have been cultured using these methods, they must be adapted to the monolayer conditions outlined below for at least 2 weeks prior to differentiation.

3.1.1 Production of MEF Conditioned Medium in a Reproducible Manner

Thaw a vial of p2 MEF frozen at 3×10^6 cells per vial in a T175 flask in 35 mL of MEF medium and grow to high confluence (~3–5 days) changing medium on day 1 and then every 2 days. The day before MEF will reach confluence, treat two to three T175 flasks with 35 mL of 0.1% gelatin solution and incubate at 37°C overnight (see Note 3). Aspirate medium from MEF and wash cells with D-PBS to remove any dead cells, add 3 mL of room temperature TrypLE, and incubate at 37°C for 1 min. Tap flask gently to release cells, wash bottom of flask with 20 mL of MEF medium, and transfer cell suspension to a 50 mL conical tube. Irradiate cells with 5,000 cGy (see Note 4). Centrifuge at $200 \times g$ for 5 min at RT. Aspirate supernatant and resuspend cells in 20 mL MEF medium, and count the number of cells using an automated cell counter or hemocytometer. Aspirate gelatin solution from treated T175 flasks and plate at 6×10^4 cells/cm^2 (10.5×10^6 cells in a T175) and top up to 35 mL with MEF medium. Allow MEF to attach for 24 h, rinse with D-PBS, and replace with 0.5 mL/cm^2 (87.5 mL in a T175) of hESC media (see Note 5). Condition hESC media for 22–26 h. Remove conditioned medium, filter sterilize, and add an additional 4 ng/mL FGF2 (see Note 6). Replace media with fresh hESC media every day for 7 days. CM can be stored at 4°C for up to 1 week or at −20°C for at least 6 months (see Note 7).

3.1.2 Making Geltrex-Coated Flasks

Allow a bottle of growth factor-reduced Geltrex (see Note 8) to thaw at 4°C overnight (see Note 9). Add 125 μL of Geltrex to 50 mL of cold (4°C) DMEM/F-12 (a 1:400 dilution) and mix thoroughly, plate 5 mL into each T25 flask (see Note 10), allow to polymerize at 37°C for at least 1 h, and store flasks at 4°C for up to 2 weeks. Before use allow flasks to warm to RT and aspirate media; washing with D-PBS is not required. To transfer hESC/hiPSC grown on MEF to monolayer culture, treat one confluent well of hPSC from a 6-well plate with TrypLE, wash with DMEM/F-12, centrifuge, resuspend in conditioned medium, and transfer cells to Geltrex-coated flask. It will take approximately two to three passages for the MEF to be removed and for the cells to be suitable for forced aggregation differentiation.

Table 6
Mouse embryonic fibroblast medium formulation

Plating density	Time to confluence
1×10^6	4–5 days
1.25×10^6	3 days
1.5×10^6	2–3 days
2×10^6	2 days
2.5×10^6	1 day

3.1.3 Passaging hPSC on Geltrex for Reproducible Cell Growth

On the first day that cells are confluent, aspirate medium, wash cells with D-PBS, add 1 mL of room temperature TrypLE for 1 min at 37°C, and gently tap the side of the flask once to free cells from the surface. Add 10 mL per flask DMEM/F-12 and using a 10 mL pipette jet the medium against the surface, transfer to a 15 mL conical tube and centrifuge at $200 \times g$ for 5 min at RT, aspirate supernatant, and resuspend pellet in 5 mL of conditioned medium. Count cells using an automated cell counter or hemocytometer. Plate 1.25×10^6 cells into a Geltrex-coated flask and top up to 5 mL of conditioned medium (see Note 11). 1.25×10^6 cells/T25 will result in confluence in 3 days and growth to $4–5 \times 10^6$ cells (see Table 6 and Note 12). Change media every day with fresh conditioned medium.

3.2 Forced Aggregation, Directed Cardiac Differentiation Procedure

1. *Day 1*—The day before intended forced aggregation split confluent hESC/hiPSC to $2.5–3.5 \times 10^6$ cells per T25 flask (see Note 13).
2. *Day 0*—Aspirate medium, wash cells with D-PBS, and add 1 mL of room temperature TrypLE into each flask. Incubate at 37°C for 1 min, and tap the side of the flask once to free cells from the surface. Add 10 mL DMEM/F-12 per flask and using a 10 mL pipette jet the medium against the surface and transfer to a 15 mL conical tube.
3. Centrifuge at $200 \times g$ for 5 min at RT, resuspend pellet in 1 mL RPMI + PVA (including growth factors) per flask, pipette up and down to confirm cells are single cell, and count cells using an automated cell counter.
4. Add 5×10^5 cells (5,000 cells per well) per 10 mL of RPMI + PVA, mix well, and transfer to a sterile reagent reservoir.
5. Aliquot 100 μL of the cell suspension into each well of a 96-well V-bottom plate using a 12-channel pipette (see Note 14).

6. Place the plates at 5% O_2, 5% CO_2, and 37°C for 2 days; hEB should be visible after 24 h, usually surrounded by a small amount of dead cells.
7. *Day 2*—On day 2 of differentiation (48 h after plating) aspirate media using a Corning 8-channel aspirator held at 45° placing the pipette tip into the opposite corner of the well (approximately 15 μL of media will remain in the bottom of the V shape). Replace media with 100 μL of RPMI + FBS and place plates in a regular ambient O_2, 5% CO_2 incubator.
8. *Day 4*—On day 4 of differentiation aspirate media as above and replace with 100 μL of RPMI + INS (see Note 15). Set the 12-channel pipette to ~130 μL; in one motion suck up ~60 μL of media, then return it to the well to resuspend the hEB, then take up all the media/hEB, and transfer to a U96 tissue culture-treated plate.
9. *Day 7*—hEB will begin to contract on d7 or d8. All hEB that are going to contract will be beating by d9 and should be counted on this day for comparative analysis. Plates must be kept at 37°C to maintain contraction.
10. From day 7 onwards media should be changed every 2–3 days.

4 Notes

1. L-ascorbic acid-2-phosphate is used rather than L-ascorbic acid as stock solutions maintain 80% activity for up to 4 weeks at 4°C (information from Sigma-Aldrich).
2. We have noted some variability in the solubility of PVA. Some batches may be made into solution by simply adding 2 g of PVA to a cold 500 mL bottle of RPMI 1640 and placing at 4°C for 72 h, shaking to mix periodically. The RPMI/PVA mix may then be kept at 4°C until use. Other batches of PVA will not go into solution at 4°C even after 1 month at 4°C. If this is the case with your batch an alternative method is as follows: Make a 10% w/v solution of PVA in cold, sterile distilled water in a glass bottle by slowly adding the PVA (5 g per 50 mL) to the water, trying to prevent the formation of clumps. Mix thoroughly and heat to 85°C for 30 min with agitation; a hybridization oven is ideal for this. Store at 4°C (Information from Sigma-Aldrich).
3. Coating T175 flasks with gelatin is essential for the MEF to stay attached for 7 days whilst conditioning the hESC media.
4. If you do not have access to an irradiator, confluent flasks can be treated with 10 μg/mL mitomycin C (in 0.2 mL/cm^2 DMEM) for 2.5 h at 37°C, then washed three times with D-PBS, passaged and counted, and then seeded.

5. hESC media to be placed on MEF to make CM must contain FGF2 for MEF to secrete/remove desired factors.
6. It is not necessary to supplement the media with additional 2-mercaptoethanol
7. Freezing CM already supplemented with FGF2 does not affect the capacity for hESC/hiPSC growth or maintenance of pluripotency.
8. We use Geltrex instead of Matrigel (BD Biosciences) as it is supplied in narrower range of concentrations (12–18 mg/mL) reducing potential variation. No difference in performance was noted for either matrix.
9. Geltrex can be subsequently maintained at 4°C for at least 2 months; aliquoting and storage at −20°C is not necessary.
10. We use double the recommended volume of media to dilute the Geltrex to prevent drying out and allow even coverage. Geltrex comes at a concentration of 12–18 mg/mL, using a 1:400 dilution and double volume results in 6–9 μg/cm^2, and 0.5 mg per 6-well plate (8 μg/cm^2) of Matrigel is commonly used.
11. We did not find that the addition of Y27632 (ROCK inhibitor) either 1 h before and/or for 24 h after passaging enhanced cell survival when using conditioned medium and plating cells at these recommended densities.
12. These growth rates are based on consistent growth. Cells do not respond well to changes in seeding density or being overgrown (greater than 8×10^6 cells per T25 flask). Cells will begin to grow faster after approximately 12 passages and seeding density will have to be reduced to prevent over-confluent growth that negatively affects subsequent differentiation. This method of growing hESC as a monolayer is unconventional but has been demonstrated to be successful for the growth of hESC for over 40 passages without karyotypic abnormality. We commonly grow cells for up to 12 passages in this manner to minimize concerns of karyotypic abnormality.
13. Splitting cells the day before forced aggregation assures that all cells are actively growing. Splitting to $2.5–3.5 \times 10^6$ cells per flask assures that cells are not in the lag phase seen when splitting cells to 1.25×10^6.
14. Although both uncoated U- and V-bottom plates can be used for this procedure we have found that it is easier to change the media on V-bottom plates whilst minimizing the chance of aspirating the hEB. Plates must be new as used plates will induced the formation of multiple hEB
15. Both RPMI + INS and RPMI + FBS media work the d4 onwards step. RPMI + FBS may produce more robust results for some cell lines but increases the proportion of fibroblasts growing around the hEB.

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