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Scalable GMP compliant suspension culture system for human ES cells

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Abstract Suspension bioreactors are an attractive alternative to static culture of human embryonic stem cells (hESCs) for the generation of clinically relevant cell numbers in a controlled system. In this study, we have developed a scalable suspension culture system using serum-free defined media with spinner flasks for hESC expansion as cell aggregates. With optimized cell seeding density and splitting interval, we demonstrate prolonged passaging and expansion of several hESC lines with overall expansion, yield, viability and maintenance of pluripotency equivalent to adherent culture. Human ESCs maintained in suspension as aggregates can be passaged at least 20 times to achieve over 1×10^{13} fold calculated expansion with high undifferentiation rate and normal karyotype. Furthermore, the aggregates are able to differentiate to cardiomyocytes in a directed fashion. Finally, we show that the cells can be cryopreserved in serum-free medium and thawed into adherent or suspension cultures to continue passaging and expansion. We have successfully used this method under cGMP or cGMP-equivalent conditions to generate cell banks of several hESC lines. Taken together, our suspension culture system provides a powerful approach for scale-up expansion of hESCs under defined and serum-free conditions for clinical and research applications.

Introduction

Human embryonic stem cells (hESCs) derived from the inner cell mass of the blastocyst stage embryo possess great self-renewal capacity and the ability to differentiate to cell types representing the three germ layers. They represent an important source for cell therapies in regenerative medicine and the study of early human development. It is important to develop reproducible and scalable cell culture systems to support cell banking and to seed clinical scale hESC differentiation production processes.

Conventionally, hESCs are propagated and banked using adherent cell culture systems, which involve the use of feeder cells or matrices. To achieve the production of clinical-grade

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hESCs, recent research has focused on developing adherent culture systems with feeder-free, serum-free and defined media and depend upon the use of defined or tumor derived cell substrates in order to maintain hESCs in an undifferentiated and diploid state (Li et al., 2005; Ludwig et al., 2006; Melkoumian et al., 2010; Rodin et al., 2010; Villa-Diaz et al., 2010; Wang et al., 2007; Xu et al., 2001). Despite improvement in the reagents available for these culture systems, the scaling up of adherent cell culture systems continues to require the use of an artificial substrate, the handling large numbers of culture vessels and detachment of cells from solid phase during cell passaging and harvesting, making the process of scaling up labor-intensive and time-consuming, potentially affecting the reproducibility and quality of these cell cultures. An advantage of adapting hESCs to suspension culture is the elimination of feeder layers and tumor derived or synthetic matrices. Therefore, the development of robust suspension culture scale-up technologies will accelerate the entry of hESC therapeutics to clinical studies. While we and others have produced hESC banks under cGLP and cGMP conditions using adherent cell culture techniques (Crook et al., 2007), the development of reproducible, scalable, serum-free, and matrix-free suspension culture systems represents significant progress towards controllable automation systems for industrial manufacturing of clinical-grade hESC-based therapeutics.

It has been previously demonstrated that hESCs are extremely vulnerable to apoptosis after single cell dissociation (Amit et al., 2000; Reubinoff et al., 2000; Thomson et al., 1998) and are therefore often manually passaged as mechanically fragmented colonies or as small cell clumps to increase cell survival rate and plating efficiency. Recently it was demonstrated that ROCK inhibitors significantly increase the survival of hESCs upon single cell dissociation (Harb et al., 2008; Watanabe et al., 2007), allowing the use of enzymatic passaging methods. Furthermore, in the presence of a ROCK inhibitor, Y-27632, single cell-dissociated hESCs can efficiently form cell aggregates and grow in suspension (Watanabe et al., 2007). Utilizing ROCK inhibitors, several recent studies showed that hESCs can be maintained, serial passaged, and expanded in an undifferentiated state in suspension culture (Amit et al., 2010; Amit et al., 2011; Krawetz et al., 2010; Olmer et al., 2010; Singh et al., 2010; Steiner et al., 2010; Zweigerdt et al., 2011). These studies described various types of suspension systems, including low-attachment static plates, rotated dishes or Erlenmeyer flasks, and spinner flasks. Although the studies using plate, Petri dish, or Erlenmeyer flask systems appeared promising systems, the scalability was limited. Those utilizing spinner flasks are preferred for their potential scalability as closed bioreactor systems. However, the long-term scalable expansion of hESC lines using spinner flasks has not been demonstrated.

Despite the success of hESC suspension culture with spinner flasks from previous studies, the cultures were reported to yield either low expansion rates or low overall cell yield (Krawetz et al., 2010; Singh et al., 2010). Using a spinner flask for hESC suspension culture, Singh et al. showed a 2-fold expansion over 7 days when cells were seeded at 1×10^6 cells/ml. In contrast, with a much lower cell seeding density, 1.8×10^4 cells/ml, Krawetz et al. reported a significant expansion rate but a low cell yield, approximately 5×10^5 cells/ml over 6 days. To establish a scalable and efficient suspension culture system for large-scale hESC production, it will be necessary to further optimize culture conditions to achieve a high expansion rate with a high yield of hESCs.

The objective of this study was to develop a scalable hESC suspension culture system that can be applied to GMP production of hESCs. We showed that undifferentiated hESCs can propagate in aggregate form in suspension culture with addition of a ROCK inhibitor and retain characteristics of undifferentiated hESCs. We then compared different defined serum-free media conditions and determined seeding density and cell splitting interval for the suspension culture to optimize cell expansion rate, undifferentiation rate, and cell yields. With the optimized culture conditions, we demonstrated that the hESC suspension cultures can be maintained over extended culture times (up to 21 passages) in spinner flasks and provide cell expansion rates and yields comparable to that seen in adherent cell cultures. Cells harvested from this suspension system can be cryopreserved as single cell suspensions using defined serum-free freezing medium. Cryopreserved cells retained high viability after thaw and could be plated, passaged, and expanded in adherent or suspension cultures. Using this system we practically generated 3 master cell banks under GLP and GMP conditions, demonstrating the feasibility of our suspension culture system for mass hESC production.

Results

Adaptation of hESCs to suspension culture

In order to reduce the potential for MEF contamination of hESC suspension culture and to achieve fully defined cell culture conditions, we adapted all hESC lines to MEF-free and serum-free hESC adherent culture prior to adaptation to suspension culture. Before initiation of suspension cultures, hESCs maintained on MEF were adapted to culture conditions using a well-defined matrix, CELLstart, with a serum-free and defined medium, StemPro hESC SFM. Three cell lines, HES-2, H1, and H9, were adapted to these culture conditions and appeared to grow as a monolayer (Supplementary Figure 1A). Cells cultured under these conditions expressed pluripotency markers, Tra-1-60, Tra-1-81, SSEA-4, and Oct-4 (Supplementary Figure 1B). Human ES cells grown in this defined culture system were able to form teratomas containing 3 germ layers of various cell lineages after injection into immunocompromised mice (data not shown). Cells were maintained on CELLstart cultures with StemPro hESC SFM for over 3 passages before final adaptation to suspension conditions.

Initial experiments to adapt adherent hESCs to suspension culture focused on determining whether undifferentiated hESCs could survive as aggregates in suspension culture. Previous studies have shown that ROCK inhibitor increases hESC survival after dissociation to single cells and allows single hESCs to survive in suspension in the form of aggregates (Watanabe et al., 2007). Therefore, cells were pretreated with a ROCK inhibitor, Y-27632, for 1 h before single cell dissociation. To achieve a better adaptation in suspension, StemPro hESC SFM used for adherent culture of parent hESCs was used as a base medium for suspension culture. In this experiment,

dissociated single cells were seeded into this medium containing Y-27632 in 6-well plates with 70 rpm of orbital shaking to prevent cell attachment. Consistent with reports from other groups, we found that hESCs were able to form cell aggregates and continued to grow in size in the presence of Y-27632 (Figure 1A), while cultures without Y-27632 remained as single cells and failed to expand (Figure 1B). This result indicates that hESCs from adherent cultures maintained on CELLstart can survive and proliferate as cell aggregates in suspension with addition of Y-27632.

Characterization of hESC aggregates maintained in suspension culture

We next examined whether hESCs in these cell aggregates retain characteristics of undifferentiated hESCs. The size distributions of H9 cell aggregates at days 2 and 3 were approximately 100–120 and 150–200 μm, respectively. Cell aggregates harvested from day 2 or 3 suspension culture were assessed for expression of the pluripotency markers Nanog, Oct-4, E-cadherin, and Tra-1-60 and analyzed by confocal fluorescent imaging (Figure 1C). The results of z-stack scanning showed that cells inside aggregates showed nuclear staining for Oct-4 and Nanog and surface staining for E-cadherin and Tra-1-60, indicating hESCs in aggregates remained undifferentiated. Cell aggregates harvested from day 4 suspension culture were re-plated onto MEF to examine if they retained growth characteristics of hESCs in adherent culture. Our results showed that these cell aggregates could be successfully seeded onto MEF and formed typical compact hESC colonies with well-defined edges (Figure 1D). The colonies grown on MEF also displayed strong staining of alkaline phosphatase (Figure 1E), indicative of undifferentiated hESCs. Taken

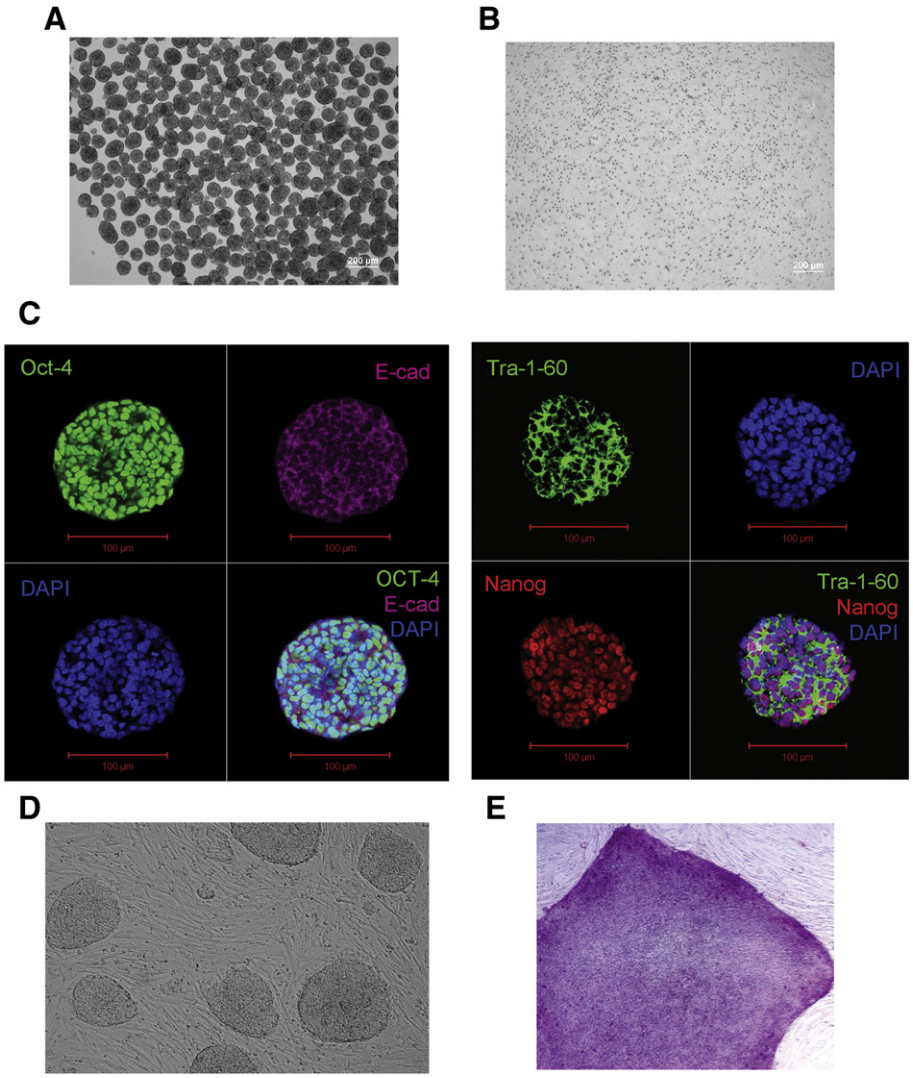


Figure 1 Growth of hESCs in suspension. (A) H9 hESCs grown on CELLstart were single cell-dissociated and cultured in suspension in low-attachment plates containing StemPro hESC SFM. In the presence of Y-27632, hESCs formed cell aggregates, (B) while cells stayed in single cells in the absence of Y-27632 after 3 days of culture. (C) Day 2 cell aggregates were whole-mount stained with Oct-4, E-cadherin, Tra-1-60, and Nanog and were imaged by confocal microscopy. (D) The cell aggregates formed colonies with well-defined edges as cultured back on MEF. (E) The colonies were shown positive for alkaline phosphatase staining.

together, hESCs grown in aggregates in suspension culture retain the characteristics of undifferentiated hESCs.

Selection of media for suspension culture

After determining that hESCs survive as aggregates in the presence of ROCK inhibitor, we assessed different media formulations suitable for clinical applications, including mTeSR complete medium and StemPro hESC SFM supplemented with 8 or 40 ng/ml bFGF. We assessed 2 different hESC lines, HES-2 and H9, in these media conditions for cell viability, aggregate size and homogeneity of aggregates. Our goal was to identify a media formulation that can support cell growth in small and homogeneous aggregates with high viability and undifferentiated rate.

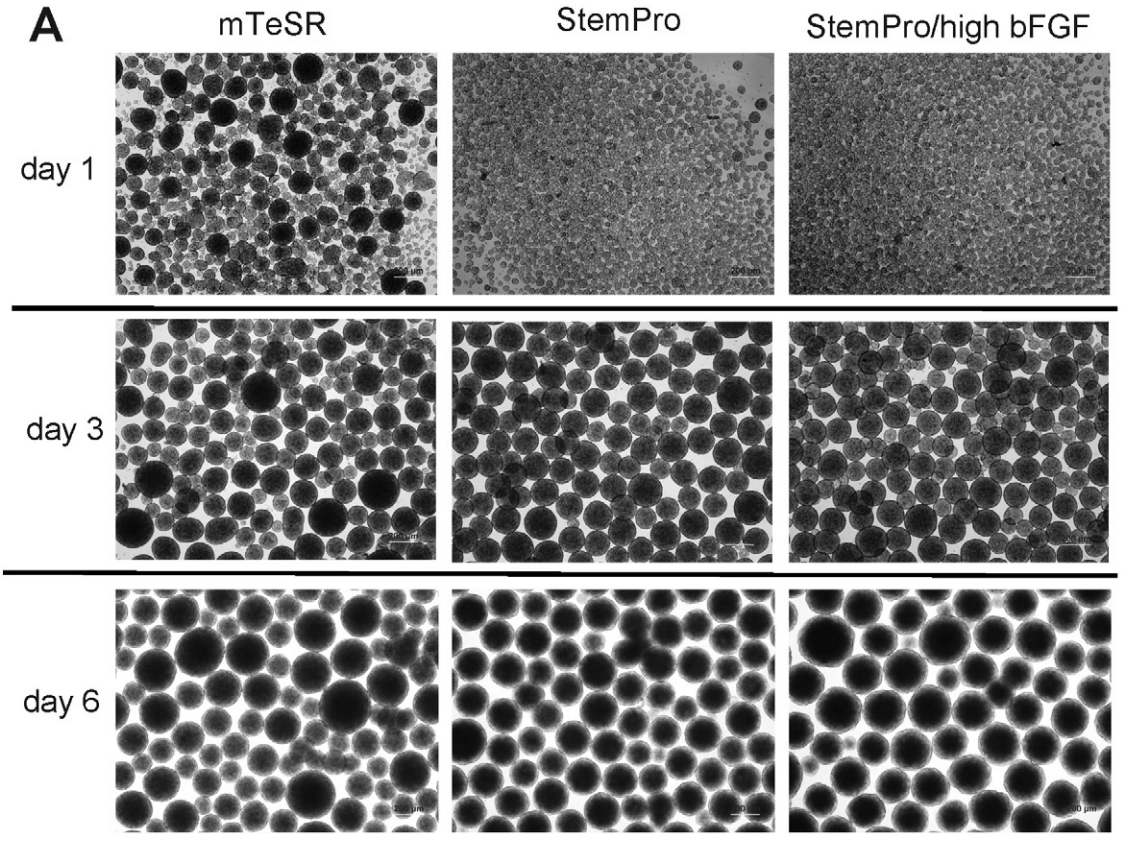
Prior to moving to spinner flasks for large-scale expansion, we tested these 3 conditions, mTeSR, and StemPro hESC SFM with 8, or 40 ng/ml bFGF, for growing hESCs in suspension in small scale of culture vessels. Single cell-dissociated HES-2 and H9 cells were seeded in suspension at 2×10^5 cells/ml into these 3 conditions containing Y-27632 in 6-well plates with orbital shaking. At day 1, cell aggregates were observed in all media conditions (Figure 2A and Supplementary Figure 2A). In general, cell aggregates generated in StemPro appeared more homogeneous, while cell aggregates generated in mTeSR were larger and heterogeneous in size. We also found that cells appeared to form more cell aggregates in StemPro media. At day 3, cell aggregates appeared more compact in all 3 media conditions. Cell aggregates formed in StemPro media were more uniform, and usually less than 200 μm in diameter, while many of cell aggregates formed in the mTeSR medium exceeded 200 μm (Figure 2A and Supplementary Figure 2A). Over time in culture, cell aggregates continued to gain in size. By day 6, HES-2 cell aggregates cultured in StemPro media started to show vacuolization, a sign of differentiation, but vacuolization was not observed in H9 cell aggregates in all media conditions (Supplementary Figure 2A). Flow cytometry analysis revealed that over 90% of the HES-2 cell population expressed pluripotency markers at day 3 but the positive population in all media conditions declined at day 6 (Supplementary Figure 2B), suggesting differentiation begins to occur between days 3 and 6. In contrast, in the H9 line, over 90% of the cell population retained expression of pluripotency markers in all media conditions at days 3 and 6 (Figure 2B). Viability of both hESC lines in these media conditions was found to be comparable (Figure 2C and Supplementary Figure 2C). At day 3, viability ranged from 90 to 98% which slightly decreased to 83–93% by day 6. For cell yields, HES-2 and H9 cultured with StemPro showed higher cell numbers at days 3 and 6, particularly with 40 ng/ml bFGF (Figure 2D and Supplementary Figure 2D). Taken together, these data suggest that StemPro hESC SFM with 40 ng/ml bFGF is superior in supporting both HES-2 and H9 hESCs to form small and homogeneous cell aggregates and to produce higher cell yields in suspension culture.

Determination of seeding density and cell split interval for suspension culture

As cell aggregates gain in size, it is more difficult for cytokines and nutrients to penetrate the aggregates which could result in increased differentiation and decreased proliferation. Consistent with this, our previous results have shown that expression of pluripotency markers of HES-2 cells decreased as the aggregates increased in size between days 3 and 6. Therefore we assessed passaging HES-2 and H9 cultures during this time period. Dissociated single cells were seeded in spinner flasks containing StemPro hESC SFM with 40 ng/ml bFGF and Y-27632 at 1.5 and 2.5×10^5 cells/ml. In spinner flasks (70 rpm), hESCs formed homogeneous cell aggregates. Cells were harvested from days 1 to 6 for analysis. At day 1, cell aggregates from low seeding density appeared slightly smaller and the number of aggregates also appeared lower. However, cell aggregates from days 1 to 4 did show compact sphere morphology (Figure 3A and Supplementary Figure 3A). At day 5, HES-2 cell aggregates from both seeding densities started to show evidence of vacuolization (Supplementary Figure 3A). A minor vacuolization was also noticed at the surface of some H9 cell aggregates (Figure 3A). Flow cytometric analysis of pluripotency markers revealed that positive populations for Tra-1-60, Tra-1-81, and OCT-4 significantly declined after day 4 in both HES-2 and H9 cells seeded at either cell density (Figure 3B and Supplementary Figure 3B). Despite the decline of the undifferentiated population after day 4, suspension cultures retained high viability, approximately 90% for 6 days (Figure 3C and Supplementary Figure 3C). However, cell growth rates started to decrease after day 4 (Figure 3D and Supplementary Figure 3D). At high cell seeding density, 2.5×10^5 cells/ml, the suspension cultures generated higher cell yields, approximately 1×10^6 cells/ml after 3 days in culture. Taken together, these results indicate that cells in suspension culture using spinner flasks begin differentiating and decreasing in expansion rate after day 4, and thus we determined that the optimal splitting interval is at day 3 or 4.

Serial passaging and expansion of suspension culture

In order to scale-up hESCs for large-scale banking, we assessed whether the suspension culture conditions described in previous sections were able to consistently maintain cell viability and expansion rate in spinner flasks over multiple passages. Y-27632-pretreated H9 cells cultured on CELLstart were dissociated to single cells and seeded at 2.5×10^5 cells/ml into a 125-ml spinner flask containing 60 ml of StemPro hESC SFM with 40 ng/ml bFGF and Y-27632. Half of the medium was replaced every day without further addition of Y-27632. After 3 to 4 days the suspension cultures were passaged by dissociating the aggregates to single cells using Accutase. This passaging process was repeated every 3 or 4 days to maintain the H9 suspension culture for a period of 64 days with a total of 21 passages. The cell number and viability from these suspension cultures were monitored on the day of passaging. The results indicate the cells in these cultures were able to maintain high and consistent viability with an average viability of $92.2\pm3.0\%$ (Figure 4A). In addition, the expansion rate was consistent throughout the 21 passages with an average expansion rate of 4.3 ± 0.4 per passage (Figure 4B). Based on this expansion rate, the culture achieved an overall calculated expansion of 1.5×10^{13} fold by the end of passage 21 (Figure 4C). We also tracked the undifferentiated state of the long-term suspension cultures during serial passaging by flow cytometric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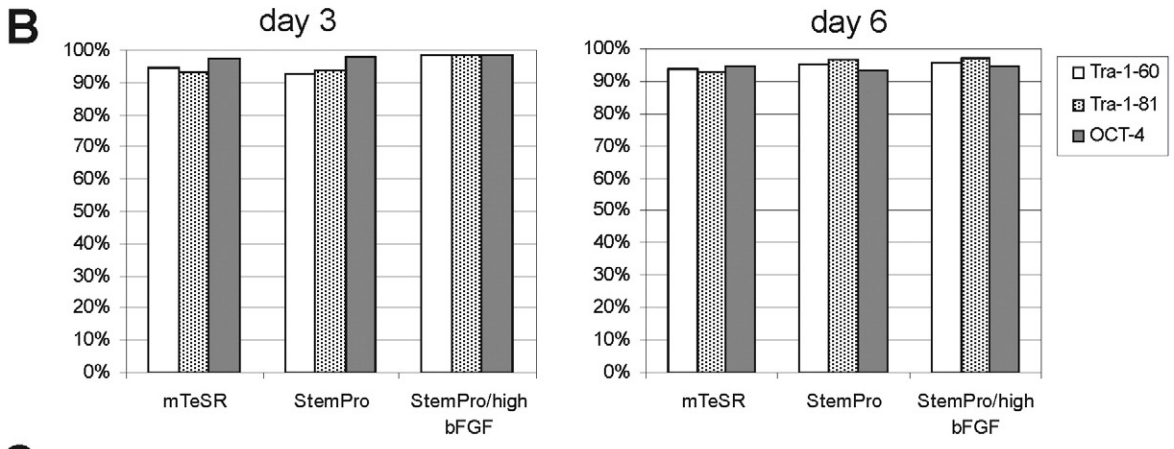
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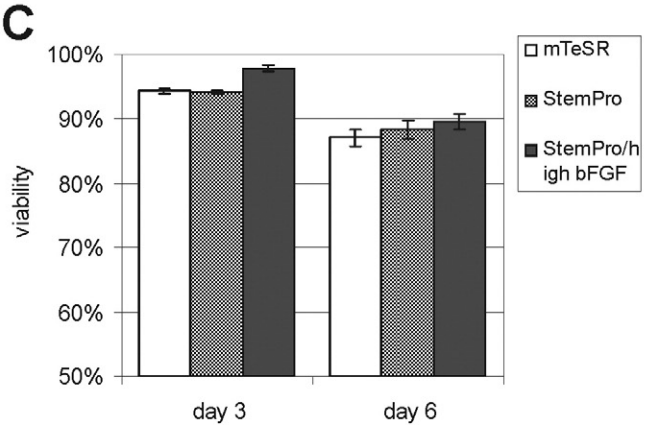
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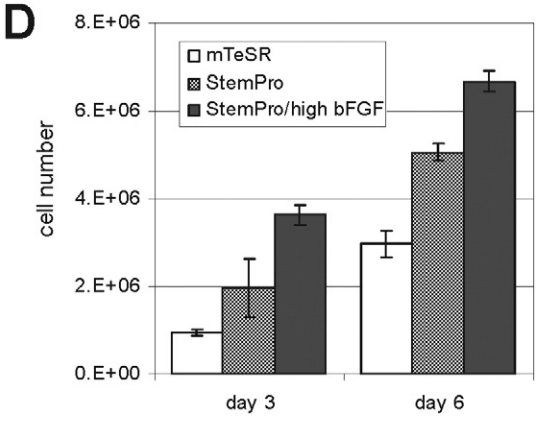
D
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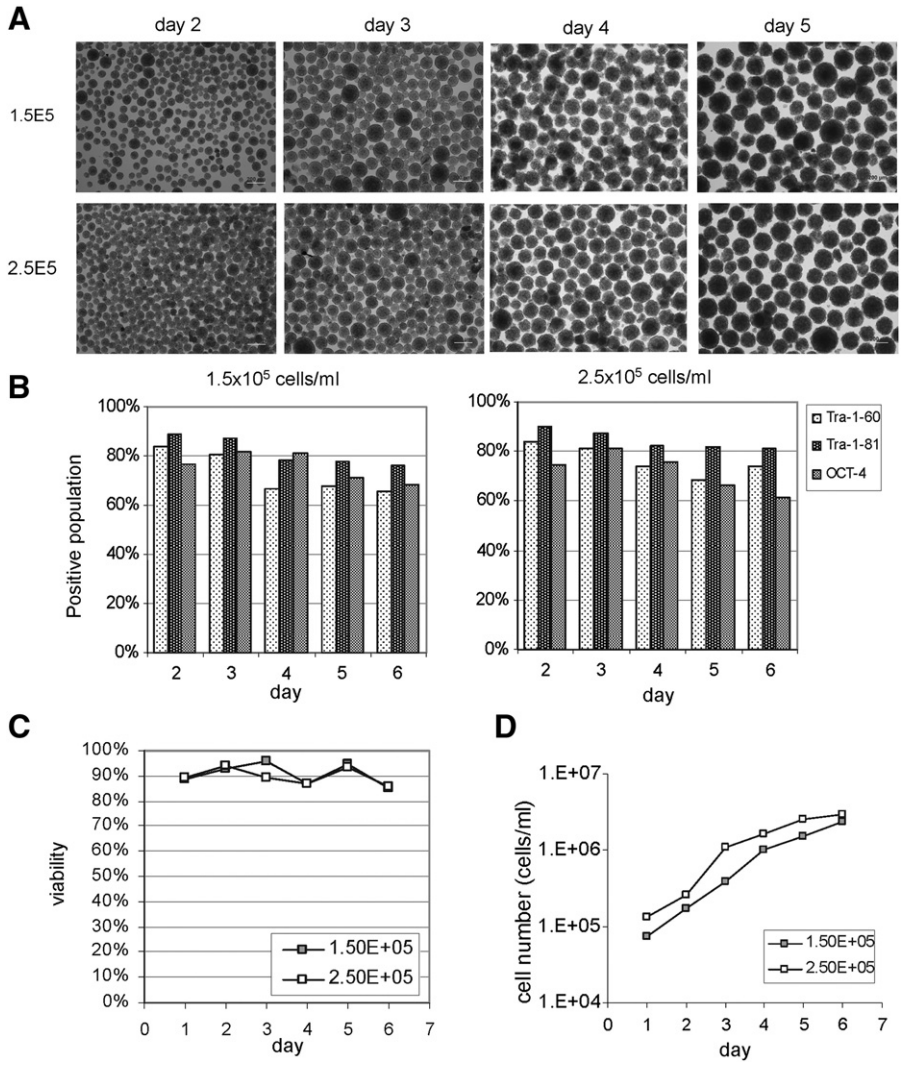
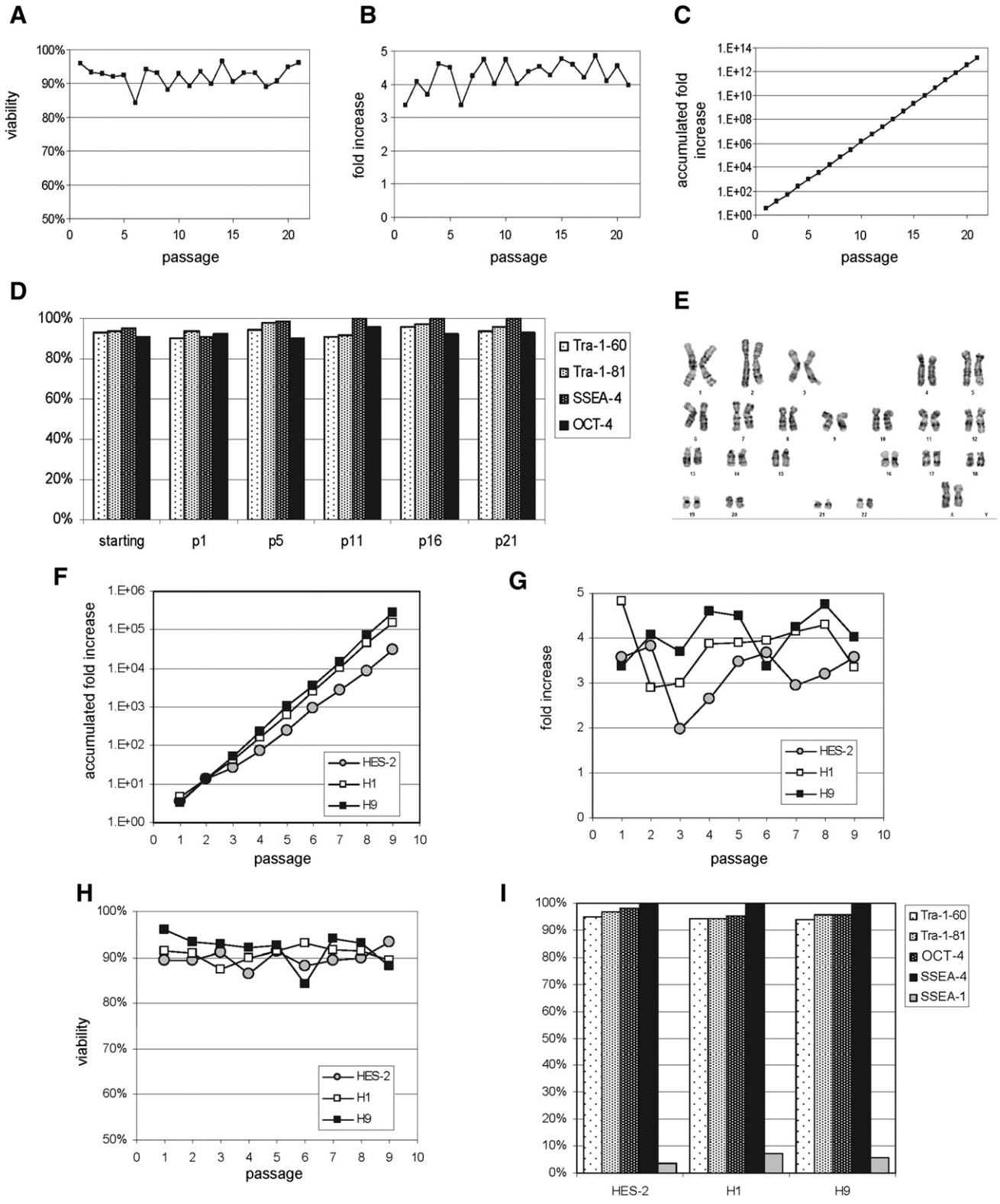


Figure 3 Determination of cell seeding density and cell split interval. H9 hESCs at density 1.5×10^5 and 2.5×10^5 cells/ml were seeded in 125 ml spinner flasks containing StemPro with 40 ng/ml bFGF with agitation rate 70 rpm. Cell aggregates were harvested from days 1 to 6. (A) Morphology of cell aggregates from days 2 to 5. (B) Expression of pluripotency markers, Tra-1-60, Tra-1-80, and Oct-4, from days 2 to 6 of cell aggregates were analyzed by flow cytometry. (C) Cell viability and (D) vital cell numbers were compared from days 1 to 6.

analysis. Cells collected from the initial CELLstart-adapted adherent culture and the suspension cultures at passages 1, 5, 11, 16, and 21 showed that over 90% cell populations expressed the pluripotency markers, Tra-1-60, Tra-1-81, SSEA-4, and OCT-4 (Figure 4D), indicating the cells in the suspension culture system retained appropriate expression patterns. Cytogenetic analysis of the end culture revealed that the cells maintained a normal karyotype (Figure 4E). To assess if other hESC lines can be stably maintained in suspension culture with spinner flasks, HES-2 and H1 lines were propagated under the conditions described above for 9 passages. A repeat of H9 suspension culture was also included in this set of experiment to test reproducibility of this culture system. The results from this experiment showed that all 3 hESC lines can be stably maintained in suspension culture with an average expansion rate of 3.2 ± 0.6, 3.8 ± 0.6, and 4.1 ± 0.5 fold per passage for HES-2, H1, and H9, respectively (Figure 4F and G). High viability was consistently observed in these cultures with $89.8\pm2.0\%$, $90.7\pm1.6\%$, and $91.8\pm3.5\%$ for HES-2, H1, and H9, respectively (Figure 4H).

Figure 2 Comparison of medium conditions for suspension cultures. Serum-free and defined media, mTeSR1 and StemPro hESC SFM, were compared for growing hESCs in suspension cultures in the presence of Y-27632. Single cell-dissociated H9 cells were seeded in 6-well plates containing mTeSR1 ($n=3$), StemPro ($n=3$), and StemPro with 40 ng/ml bFGF ($n=3$) with orbital shaking. (A) Morphology of cell aggregates formed at days 1, 3, and 6. (B) Flow cytometry analysis of pluripotent markers, Tra-1-60, Tra-1-81, and Oct-4, (C) cell viability and (D) cell numbers were compared at days 3 and 6. Error bars represent mean $\pm$ SD.

After 9 passages in suspension culture, over 90% of the cell populations were positive for Tra-1-60, Tra-1-81, SSEA-4, and OCT-4 staining and less than 7% were positive for differentiated marker SSEA-1 as analyzed by flow cytometry (Figure 4I). Cytogenetic analysis showed the 3 cell lines all maintained normal karyotypes (data not shown). To demonstrate the utility of the suspension culture, we expanded the HES-2, H1, and H9 lines in these suspension cultures to generate 3 large-scale cell banks under cGLP or cGMP conditions. The suspension cultures were initially expanded in 125 ml spinner flasks and then were scaled up to 4 or 5×500 ml spinner flasks. After 3 to 4 passages in



suspension cultures, cells expanded in 500 ml spinner flasks were used to generate 200 to 400 vial cell banks, with 4×10^6 cells per vial. These results demonstrate that all hESC lines tested can be serially passaged and expanded in suspension culture with spinner flasks and can be practically scaled up to generate cell banks under cGMP and cGLP conditions.

Expansion rate and population doubling time of hES in suspension and adherent cultures

The expansion rates of hESC lines, H9, H1 and HES-2, grown in suspension were compared with adherent cultures on MEF and CELLstart. In this study, suspension cultures were routinely passaged every 3 or 4 days as described in the previous sections. Cells cultured on MEF were passaged every 5 or 6 days after reaching about 70% confluence, and cells on CELLstart were passaged every 3 or 4 days after reaching about 80–90% confluence. Cell lines maintained in both adherent conditions were passaged at a ratio of 1:4. Cell cultures were carried for a period of 22–28 days and viable cell counts were determined every passage to determine the expansion rate. The accumulative fold of expansion was plotted against the passage number for all three lines used in this study (Figure 5A–C). The results indicated that the expansion rate of suspension culture was higher than for cells cultured on MEF (Figure 5A and C) and was similar to cells cultured on CELLstart (Figure 5A–C). Generally, suspension cultures and adherent cultures on CELLstart displayed better cell viability at passaging. The viability of HES-2, H1, and H9 in suspension cultures was 89.3±1.7%, 90.7±1.6%, and 91.8±3.5%, respectively and on CELLstart adherent cultures was 93.6±2.0%, 90.6±8.0%, and 93.9±2.5%, respectively.

In contrast, viability of HES-2 and H9 on MEF adherent cultures was 81.5±9.5% and 85.5±5.0%, respectively. The population doubling time was also assessed for H9 and HES-2 lines that were cultured in both suspension and adherent cultures. To determine the doubling time, the viable cell number was determined each day for 6 days starting from the day after the cells were seeded. The cell number was then plotted against the days of culture and the doubling time was calculated using the exponential regression method. The calculated population doubling time for HES-2 in suspension and on MEF was 30.9 and 29.4 h, respectively; and for H9 in suspension, on MEF, and CELLstart was 29.3, 26.1, and 27.2 h, respectively. The similar doubling times of suspension and adherent cultures indicate that cells in suspension retain normal population doubling time. Taken together, these results demonstrate that the hESC suspension culture displays comparable growth characteristics with adherent cultures.

Characterization of pluripotency and differentiation of hESCs cultured in suspension

The pluripotency of hESCs derived from suspension culture was examined by assessing embryoid body (EB) and teratoma formation. For EB formation assessment, H9 cell aggregates were directly differentiated in EB formulation medium. At day 6, EBs in suspension started to show complicated and heterogeneous structures, indicative of differentiation (Figure 6A). Gene expression analysis of EBs plated from adherent cultures revealed that expression of Pou5f1 (OCT-4) and Nanog decreased to undetectable levels within 6 days. Expression of *T* (*brachyury*), *Sox17*, *Sox1*, and *Tubb3* (*beta-Tubulin III*), representing genes of three germ layers, were detected at different stages of EB differentiation (Figure 6B). For teratoma formation, 2.5 million hESCs of each H1, H9, and HES-2 derived from suspension cultures were injected into immunocompromised mice and were able to generate teratomas containing cell lineages of 3 germ layers, including neuroepithelium, muscular cells and glandular epithelium (Figure 6C). These results demonstrated that hESCs cultured in suspension maintain pluripotency.

Directed cardiomyocyte differentiation from cell aggregates

We next tested if hESC aggregates derived from the suspension culture can be applied to directed differentiation. H9 cell aggregates from day 3 or 4 suspension culture were directly induced to cardiomyocyte differentiation in a spinner flask using a serum-free and stage specific induction method (Yang et al., 2008). At day 9 post-induction, contracting EBs were observed. At day 19, flow cytometric analysis of differentiated EBs showed that approximately 27% of the cell population was positive for the cardiac-specific marker, Troponin T (Figure 6D). By immunohistostaining EB sections, positive staining for sarcomeric myosin heavy chain (sMHC) and β myosin heavy chain (βMHC) were observed (Figure 6E). Gene expression analysis of the induced cells showed the expression of a broad spectrum of cardiac-specific genes, *Isl-1*, *Nkx2.5*, *MYH6*, *Tnnt2*, *Myl2*, and *Myl7* (Figure 6F). These

Figure 4 Serial passaging and expansion of suspension cultures. H9 hESCs at seeding density 2.5×10^5 cells/ml were cultured in 125 ml spinner flasks with 70 rpm agitation. Cells were passaged every 3–4 days for 21 passages. (A) Cells retained high viability, over 90% in average and (B) consistent fold increase, approximately 4 folds in average at very passage. (C) The expansion rate was calculated by fold increase of cell number at every passage. The accumulated fold increase of the cell suspension culture stably reached over 1×10^{13} folds over 21 passages. (D) At passages 1, 5, 11, 16, and 21, the undifferentiation rates of cells from suspension cultures were analyzed by flow cytometry for pluripotency markers, Tra-1-60, Tra-1-81, SSEA-4, and Oct-4. (E) The chromosomal stability of the end culture was shown normal as analyzed by G-banding karyotyping. With the same culture conditions, HES-2, H1, and a repeat of H9 were cultured in suspension with 125 or 500 ml spinner flasks for 9 passages. (F) The calculated cell expansion rates reached over 50,000 folds for HES-2 and over 100,000 for H1 and H9. (G) Fold increase of cell number at every passage ranged from 2 to 4.8 folds. (H) Cell viability was approximately 90% at passaging. (I) The undifferentiation rates of the suspension cultures were analyzed by flow cytometry of pluripotent markers, Tra-1-60, Tra-1-81, SSEA-4, and Oct-4, and a differentiated marker SSEA-1 staining.

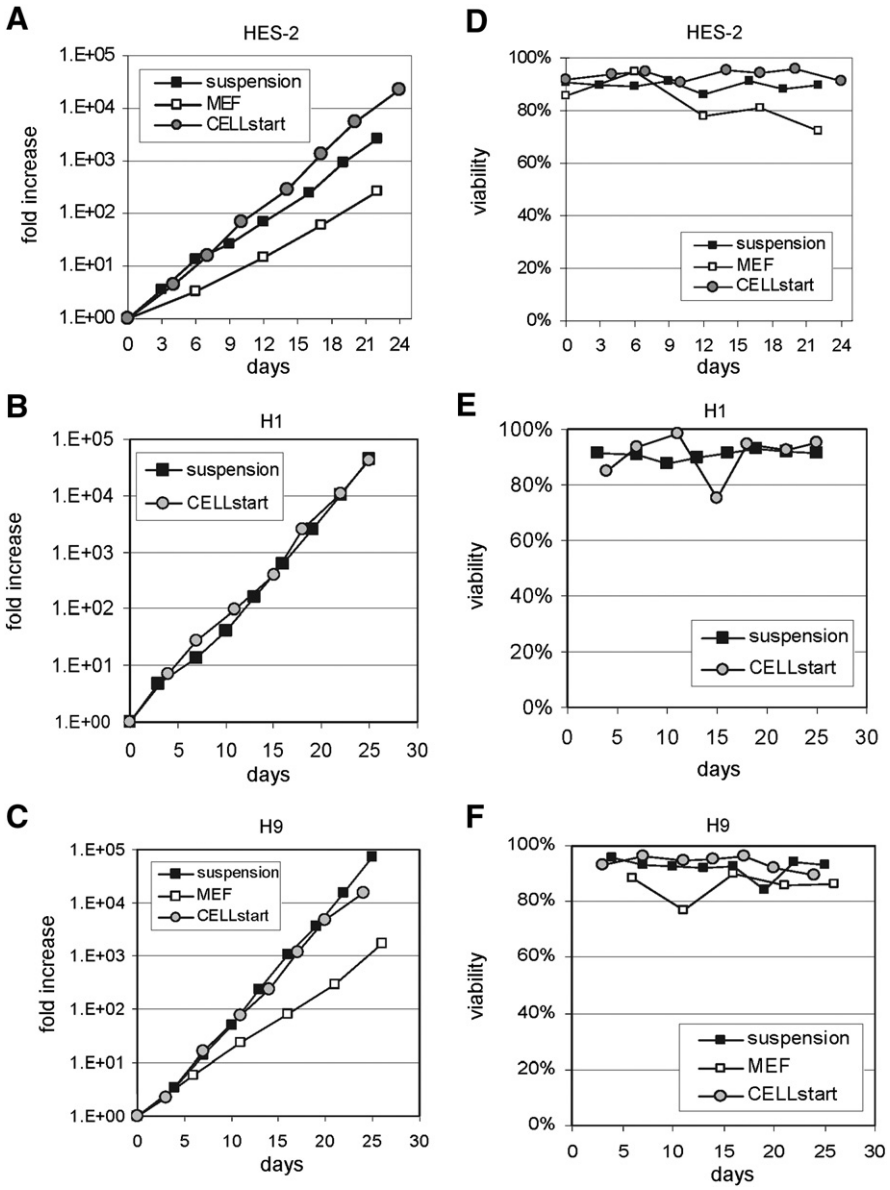


Figure 5 Comparison of expansion rates of hESC cultured on feeder layer, CELLstart and in suspension. The expansion rates and cell viability of HES-2, H1 and H9 cells cultured on MEF, CELLstart and in suspension were compared for 22–28 days of time periods. The accumulative fold of expansion and cell viability was plotted against the days of cultures for all three lines used in this study: (A–C) calculated fold increase of HES-2 (A), H1 (B), and H9 (C) cells grown on MEF, CELLstart, or in suspension culture at every passage; (D–F) cell viability of HES-2 (D), H1 (E), and H9 (F) cells grown on MEF, CELLstart, or in suspension culture at passaging.

results demonstrated that hESC aggregates generated from these suspension cultures can be potentially used for directed differentiation.

Cryopreservation of hESC suspension culture

An objective of our hESC production system is to use serum-free and defined conditions through all phases of adherent culture, suspension culture and cryopreservation. It has been reported that inclusion of Y-27632 in cryopreservation medium enhances survival rate and plating efficiency of hESCs at thaw (Li et al., 2009; 2008; Martin-Ibanez et al., 2008; Mollamohammadi et al., 2009). We therefore added Y-27632 into StemPro hESC SFM containing 10% DMSO as cryopreservation medium. Human ES cell aggregates from suspension culture were single cell dissociated and cryopreserved in the freezing medium. Upon thaw, HES-2, H1, and H9 cells showed a viability of 86.0±3.0%, 92.1±2.0%, and 88.7±3.8%, respectively. The thawed cells were plated at 1.5×10^6 cells/well of 6-well plates (approximately 1.56×10^5 cell/cm^2) on MEF and CELLstart, and at 2.5×10^5 cells/ml in spinner flasks for suspension culture. Cells thawed on MEF and CELLstart culture reached 70–90% confluence in 4–7 days (Figure 7A and B),

and cells thawed into suspension were also able to form and grow in cell aggregates (Figure 7C). Cells thawed and seeded back into adherent and suspension cultures were passaged and over 80 or 90% (82–99%) of the cell population expressed pluripotency markers after 3 passages (Figure 7D). Cryopreserved cells thawed directly into suspension culture expanded 1.7 fold at the first passage followed by 2.6 to 4.5 fold increase at passages 2 to 5 (Figure 7E). These cultures also retained high percentages of cells expressing pluripotency markers as analyzed by flow cytometry (Figure 7D). These results demonstrate that hESCs maintained in suspension cultures can be cryopreserved in serum-free conditions in the presence of Y-27632, and can be thawed back into either adherent or suspension cultures for serial passaging and expansion.

Discussion

As pluripotent stem cells have moved forward to preclinical and clinical studies, a robust scalable system for hESC production under GMP or GLP conditions to provide sufficient quality cells is essential. In the current study, we have established a serum-free, feeder-free, and defined system for the expansion of hESCs in suspension culture by optimizing media conditions, cell seeding density, and cell passage interval. These hESCs can be cryopreserved in a serum-free defined medium and can be successfully thawed into either adherent or suspension culture. We demonstrate for the first time the feasibility of long-term serial passaging of hESC suspension culture in spinner flasks (over 20 passages) and practically use this culture system to scale up the production of hESCs under cGMP or cGLP conditions. Our study not only provides a scalable suspension culture method but also provides a strategy to optimize hESC suspension culture.

Conventionally, expansion of hESC adherent culture requires passaging the cells in small cell clumps, as cells which have been dissociated to single cells show poor viability and seeding efficiency (Amit et al., 2000; Reubinoff et al., 2000; Thomson et al., 1998). Recently, several reports have demonstrated that single cell-dissociated hESCs can survive in suspension by forming cell aggregates. (Amit et al., 2010; Krawetz et al., 2010; Olmer et al., 2010; Singh et al., 2010; Steiner et al., 2010). Unlike mES cells, which can form cell aggregates from single cells in suspension (Cormier et al., 2006; Kehoe et al., 2008; zur Nieden et al., 2007), hESCs require a ROCK inhibitor, Y-27632, to form cell aggregates and propagate. Formation of cell aggregates appears to be a critical step for hESCs to survive and expand in suspension and the ROCK inhibitor is a key factor for this step.

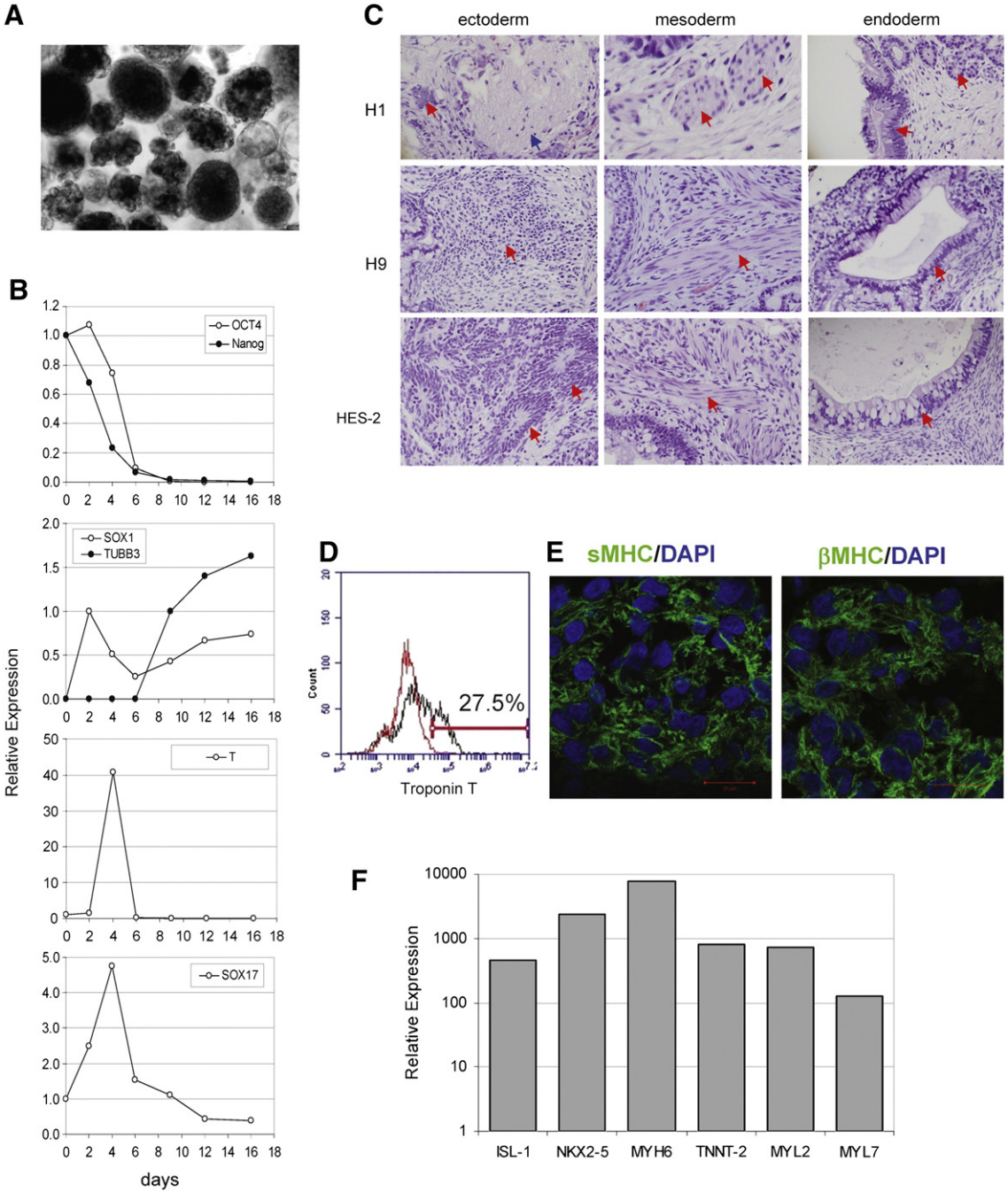
We have found that when hESC aggregates are maintained in suspension culture, it is critical to control the size of the aggregates to ensure efficient expansion and quality of hESCs. It is a reasonable speculation that nutrients and cytokines will be difficult to penetrate into cell aggregates to maintain hESC growth as cell aggregates gain in size. We selected culture conditions which can generate a great number of cell aggregates in small sizes at the initial stage to achieve a better expandability. We found passaging cells every 3–4 days provided the optimal expansion rate. In addition we found that cultures in StemPro media formed small and homogeneous aggregates and resulted in a better expansion rate than cultures in mTeSR media. At lower cell seeding density, 1.5×10^5 cells/ml, initial sizes of cell aggregates appeared slightly smaller than those of seeding at 2.5×10^5 cells/ml but we also noticed that aggregates appeared less at lower seeding density, resulting in a lower cell yield. Previous studies have shown great expansion rates of their hESC suspension cultures using much lower seeding density, while the cell yields were not comparable with adherent cell cultures (Krawetz et al., 2010; Olmer et al., 2010). In contrast, a higher seeding cell density results in higher cell yield but lower expansion rate (Singh et al., 2010). Therefore, it is crucial to optimize cell seeding density to ensure proper expansion rates and cell yields of hESC suspension culture. As the growth characteristics of hESC lines are varied, the passaging interval and cell seeding density may also need to be optimized for different lines.

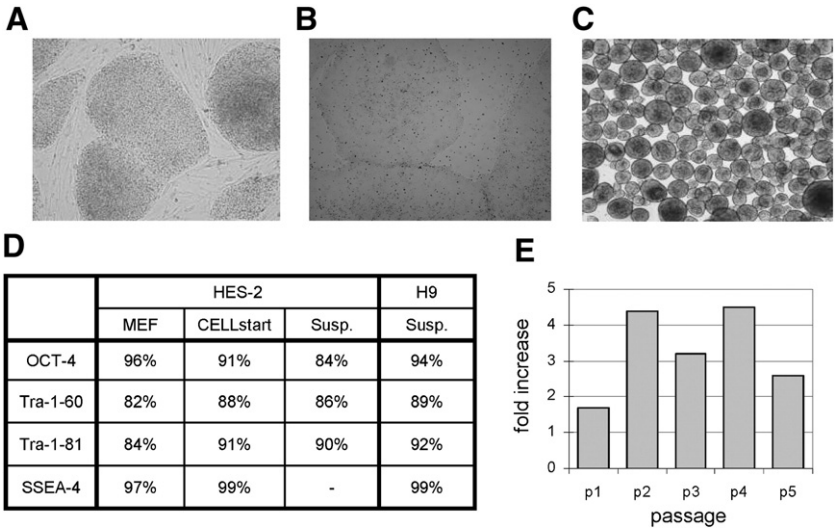
For large-scale manufacture of hESCs, it is crucial to validate a culture system by demonstrating a long-term culture which can be stably passaged and expanded to the level sufficient for making master cell bank to working cell bank, development cell bank, and differentiation. As shown by the work of Singh et al., the growth characteristics of hESCs in suspension cultures using static low-attachment plates, stirred Petri dishes, and spinner flask are varied, indicating that suspension cultures with different types of vessels need to be optimized individually. Though the long-term passaging of suspension cultures in small-scale vessels have been demonstrated, these culture conditions may not be suitable for long-term cultures with large-scale vessels. While two studies have shown serial passaging of suspension culture using spinner flasks with small volume for 3 to 5 passages, the suspension culture system we established is able to consistently expand hESCs for over 20 passages and scale up to 500 ml spinner flasks, demonstrating the feasibility of large-scale cell banking using the suspension culture system.

Maintenance of hESCs in suspension culture not only avoids MEF or matrix contamination but it also provides a system more amenable to scalable and controllable automation. It is interesting to note that the cell density achieved in the current suspension culture is approximately 1×10^6 cells/ml, similar to the cell density seen in the adherent culture. However, it is well accepted that suspension culture offers many advantages over the adherent culture, with the scalability being the most important factor. Since the spinner flask system used in this study is the most primitive form of bioreactor, it is reasonable to assume that the maximal cell density can be significantly increased when a more sophisticated bioreactor is used to provide better control of all process parameters, including the O_2 tension that has been reported to affect the growth of the hESCs. In addition, the cell density may also be increased using well established cell culture strategies, such as the fed-batch and perfusion systems that have been reported to increase cell density by 5 to 10 folds for numerous mammalian cell lines (Huang et al., 2010; Tao et al., 2011). Expanding hESCs in suspension culture using bioreactors also reduces the number of culture vessels for banking and significantly reduces labor for cell harvesting, dissociation, and passaging. We believe that the suspension culture system

presented here could be the foundation for the development of large-scale hESC culture system to address the demand of large-scale production of the hESCs for clinical applications, as the suspension culture can readily be scaled up 1000 to 10,000 folds in a single commercial size bioreactor.

The EB formation method has been broadly used to direct ES cell differentiation to tissue-specific cell lineages. Unlike mES cells, hESCs usually form EBs very inefficiently. Additionally, sizes and morphology of the formed EBs are highly heterogeneous. An efficient cell specific-differentiation protocol using EB method requires the initial EBs to be homogeneous to generate more controllable, predictable, and consistent results. The hESC suspension culture system we developed may provide a promising way to conquer the challenging step of EB formation. As we showed previously, hESC aggregates generated from our suspension culture system were homogeneous in size and shape. These undifferentiated cell aggregates can serve as starting material for EB formation. In this study, we demonstrated that these cell aggregates could be directly differentiated to cardiomyocytes in a spinner flask containing appropriate induction media. Therefore, our suspension culture system not only overcomes the difficulty of initial EB formation but also provides a promising scalable system for hESC differentiation.





	HES-2			H9
	MEF	CELLstart	Susp.	Susp.
OCT-4	96%	91%	84%	94%
Tra-1-60	82%	88%	86%	89%
Tra-1-81	84%	91%	90%	92%
SSEA-4	97%	99%	-	99%

Figure 7 Cryopreservation of hESC suspension culture. (A–C) hESCs harvested from suspension culture were cryopreserved in single cells with StemPro containing 10% DMSO and Y-27632. HES-2 cells were thawed on MEF (A), CELLstart (B) or in suspension (C) and were passaged for 3 times. H9 cells thawed directly into suspension culture were passaged and expanded for 5 times. (D) Flow cytometry analysis of pluripotency markers, Tra-1-60, Tra-1-81, SSEA-4, and Oct-4, for HES-2 cultures 3 passages post-thawed on MEF, CELLstart and in suspension, and for H9 cultures 5 passages post-thawed in suspension were shown in the tables. (E) Fold increase of H9 at every passage after thawed in suspension was shown in the chart.

Conclusions

We have developed procedures from cell adherent cultures, suspension cultures to cryopreservation under feeder-free, serum-free, and defined culture conditions. More importantly, hESCs can be maintained and consistently expanded in our suspension culture system for long-term culture while retaining normal karyotype, appropriate marker expression, and pluripotency. Additionally, the cryopreserved hESCs can be directly thawed and expanded in this suspension culture system. With these advantages, the suspension culture can serve as an alternative system for routine maintenance of hESCs. Furthermore, we have used the suspension culture system to produce 3 hESC banks under cGMP and cGLP conditions. Our cGMP scalable technology of hES suspension culture will provide an efficient and facile manufacturing system to generate sufficient clinical grade of hESCs with consistent and predictable characteristics for research and clinical applications.

Materials and methods

Human ES cell adherent cultures

MEF culture

hESC lines were cultured on mytomycin C-inactivated MEFs (Millipore) with DMEM supplemented with 20% knockout

Figure 6 Pluripotency of hESCs expanded in suspension. At the end of suspension culture, H9 cells were subjected to in vitro and in vivo differentiation analysis. Day 3 cell aggregates from the suspension culture were directly differentiated with serum-containing medium in a spinner flask for EB formation assay. (A) At day 6, cell aggregates differentiated into EBs with complexed structures. (B) Alternatively, day 1 EBs from suspension culture were plated on Matrigel-coated dishes with serum-containing medium for further differentiation. Cells differentiated in adherent cultures were harvested at days 2, 4, 6, 9, 12, and 16 and were analyzed by quantitative RT-PCR for the expression of pluripotency genes, Oct-4 and Nanog, and genes of 3 germ layers, SOX 1, TUBB3, T (Brachyury), and SOX17. (C) For teratoma assay, 2.5 million hESCs of each H1, H9, and HES-2 from the suspension cultures were injected into immuno-deficient mice. Teratoma cell mass was observed 8 weeks post cell injection. The tissue mass was dissected between 9 and 13 weeks after injection. H1 ectoderm: red arrow indicates ganglion cells, blue arrow indicates ganglio-neuronal tissue; mesoderm: arrow indicates smooth muscle cells; endoderm: arrow indicates intestinal-like glandular cells. H9 ectoderm: arrow indicates gangalio-neuronal tissue; mesoderm: arrow indicates smooth muscle cells; endoderm: arrow indicates intestinal-like glandular cells. HES-2 ectoderm: arrow indicates neuroblastic rosettes; mesoderm: arrow indicates smooth muscle cells; endoderm: arrow indicates intestinal-like glandular cells. Sections stained with hematoxylin and eosin. (D) The H9 cell aggregates were also subjected to directed cardiomyocyte differentiation in suspension in a spinner flask under serum-free conditions. EBs at day 19 were harvested and dissociated for flow cytometry analysis of cardiac Troponin T positive cell population. (E) The EBs were also sectioned and immunohistochemistry was carried out with staining of sMHC and βMHC. Green fluorescence indicated positive cells. Blue fluorescence is DAPI staining for nuclei. (F) Expression of cardiac-specific genes, ISL-1, Nxk2.5, MYH6, TNN-2, MYL2, and MYL7 of the EBs were analyzed by quantitative RT-PCR.

serum replacement, 1 mM L-glutamine, non-essential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma), and 4 ng/ml human recombinant basic fibroblast growth factor (bFGF; Invitrogen). Media were change every day. Cell cultures were passaged every 5–6 days. At passaging, cells were treated with 200 u/ml collagenase (Invitrogen) for 5–10 min at 37 °C, and then gently pipetted to break the big clumps into even sizes. Cells were passaged at a split ratio of 1:3 or 1:4.

CELLstart culture

Manufacture's culture conditions were followed for CELLstart (Invitrogen). Briefly, the CELLstart matrix was diluted in PBS containing Ca^{2+} and Mg^{2+} at 1:50 dilution. Tissue culture vessels were coated for 2 h at 37 °C before use. Cell cultures were treated with Accutase (Millipore) for 1–2 min at 37 °C. Accutase was removed and replaced with fresh StemPro medium (Invitrogen) supplemented with 0.1 mM β-mercaptoethanol and 8 ng/ml human recombinant bFGF, and then the big clumps were gently pipetted to break into even sizes. Cells were seeded at ratio of 1:3 or 1:4 into CELLstart-coated vessels.

Human ES cell suspension culture

Prior to suspension adaptation, hESCs were maintained in CELLstart-coated plates with StemPro hESC SFM supplemented with 8 ng/ml bFGF and 0.1 mM β-mercaptoethanol. For the initial suspension adaptation, hESCs are first dissociated into single cells using Accutase for 3–5 min after 1 h of 10 μM Y-27632 (EMD Chemicals) treatment. After wash the single cells were seeded in even suspension at a density of 1.5 to 2.5×10^5 cells/ml in mTeSR, or StemPro supplemented with 8 or 40 ng/ml bFGF and 0.1 mM β-mercaptoethanol. To enhance formation of cell aggregate, 10 μM Y-27632 were added into the media. Single cell suspension was seeded in 6-well plates with orbital shaking at 80 rpm or spinner flasks with magnetic stirrers (Thermo Scientific 50119114) set at 70 RPM. Subsequent medium changes were performed daily via demi-depletion using the same medium without Y-27632. Cells are passaged every 3–4 days post-seeding via dissociation into single cells with Accutase and seeded at 1.5 to 2.5×10^5 cells/ml in the same medium. Cultures were maintained in a 5% CO_2 air environment with 95% relative humidity at 37 °C.

Flow cytometry

Cell surface markers

Dissociated single cells were washed in PBS, resuspended in FACS buffer (0.5% BSA in PBS) and incubated with primary antibodies on ice for 30 min. After incubation, cells were washed in FACS buffer and incubated with secondary antibody for 30 min on ice. Cells were washed, resuspended with FACS buffer and analyzed by Accuri C6 flow cytometry (Accuri). Primary antibodies were mouse IgM anti-Tra-1-60, mouse IgM anti-Tra-1-81, mouse IgM anti-SSEA-1 (Millipore), and mouse IgG_3 anti-SSEA-4 (R&D systems). Secondary antibody was R-PE goat anti-mouse IgM (Southern Biotech).

Intracellular markers

Cells were fixed in 4% paraformaldehyde solution (USB) in PBS for 5 min at room temperature. Subsequently, cells were washed in PBS and permeabilized with buffer containing PBS, 0.1% soponin and 0.1% BSA, for 10 min at room temperature. Cells were resuspended in permeabilization buffer and stained with antibodies as procedures mentioned above using permeabilization buffer. Primary antibodies were mouse IgG_{2b} anti-Oct-4 (Santa Cruz) and mouse IgG_1 anti-Troponin-T (Neomarkers). Secondary antibody was R-PE goat anti-mouse IgG (Southern Biotech).

Alkaline phosphatase (AP) staining

A Millipore's AP detection kit was used to characterize ES cells for AP activity. The staining was performed following the procedure described in the kit.

Quantitative RT-PCR

Total RNA from cells was extracted using RNeasy kit (Qiagen). Complementary DNA was synthesized from 2 μg of RNA per sample using iScript cDNA Synthesis Kit (BioRad). Real time PCR was performed to determine expression level of each gene. cDNA corresponding to 0.1 μg of total RNA was used as a template in the PCR consisting of MasterMix and TaqMan gene expression system (Applied Biosystems), according to the manufacturer's instructions. Quantitative PCR was performed with an Stratagene MX3000P System using the default thermal cycling conditions (Initiate at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s). Relative quantification was performed using the comparative cycle threshold method ($2-\Delta\Delta CT$). Primer sets were purchased from Applied Biosystems (Supplemental Table 1).

Immunohistochemistry

Whole mounted staining

Staining-Human ES cell aggregates from suspension of days 2 or 3 were harvested. After washed in PBS, the cells were fixed with 4% PFA for overnight, then permeabilized and blocked with buffer containing 0.3% BM blocking powder (Roche), 5% normal serum, 5% FBS, and 0.1% Triton, for 1 days at 4 °C with gently rocking. Primary antibodies were added at 10 μg/ml for overnight at 4 °C with gently rocking. Primary antibodies used were anti-Oct4, anti-Tra-1-60 (Millipore), anti-CD324 (eBioscience), and Nanog (H-155, Santa Cruz). After wash with PBS containing 0.1% Tween-20 and 0.5% BSA, secondary antibodies (FITC-conjugated IgG and Rhodamine-conjugated IgG) or Streptavidin Cy5 were added at 1:400 dilution for overnight. After additional wash, the aggregates were mounted using Prolong Gold anti-fade reagent with DAPI (Invitrogen).

Cross-sectional staining

Samples were processed by Pathology Core Facility at City of Hope. The slides were blocked in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature. Primary antibodies, anti-sMHC (MF20, Developmental Studies Hybridoma Bank) and anti-βMHC (Millipore), were added at 1:100

dilution for overnight at 4 °C. The slides were then washed and FITC-conjugated IgG antibody were added at 1:400 dilution for 1 h at room temperature and mounted. The whole-mounted and cross-sectional stainings were observed under a LSM 510 Meta Inverted 2 Photon confocal laser scanning microscope.

Cell population doubling time

Cells maintained on MEF or CELLstart adherent or in suspension culture were passaged as mentioned previously. For adherent cultures, cells were seeded in 6-well plates, 3 wells for each time point. For suspension cultures, cells were seeded at 2.5×10^6 cells/ml in 125-ml spinner flasks. After seeding, cells were harvested from wells or aliquoted from spinner flasks everyday from days 1 to 6. Cells were dissociated to single cells with Accutase, and the number of live cells was determined by trypan blue exclusion. Growth curves were generated from these data, and the approximate doubling time was calculated using linear regression. Data points in the linear phase were input into the program, *Doubling Time Software v1.0.10* (http://www.doubling-time.com) (Roth, V. 2006), to calculate population doubling time.

Karyotype analysis

Karyotypes of hESCs were analyzed by Cytogenetics Core Facility at City of Hope. Briefly, cells were harvested according to standard methods and fixed in 3:1 methanol:acetic acid. Slides were GTG banded. Twenty mitotic cells were digitized and karyotyped. Karyograms were reviewed and aberrations were described according to ISCN (2009).

Teratoma assay

hESCs at 2.5×10^6 cells in 0.1 ml serum-free medium were subcutaneously injected into dorsal area of NOD/SCID/IL2R gamma null mice (NSG mice, male, 8–10 weeks old, from Jackson Laboratory, Bar Harbor, Maine) with 23G needle. After the teratoma grew to size about 10 mm in diameter, the mice were euthanized and the teratoma were excised, fixed with 10% formalin, and sent to pathological core lab for paraffin sectioning and H&E staining. The animal procedures were approved by IACUC of Beckman Research Institute of the City of Hope.

EB formation assay

Cell aggregates were harvested, washed with PBS, and resuspended in embryoid body (EB) formation medium (Millipore) supplemented and cultured in suspension. Medium was changed every other day via demi-depletion with EB formation medium for the remainder of the experiment. Alternatively, the EBs can be further differentiated on adherent surface by plating day 1 EBs onto glass-bottom culture dishes (MatTek) coated with Matrigel (BD). Subsequent medium changes were performed every other day with the same EB differentiation medium. Differentiated cell cultures were collected at days 2, 4, 6, 9, 12, and 16 for detection of markers for cell lineages of 3 germ layers.

Cardiomyocyte differentiation

Prior to differentiation in suspension, hESCs were maintained in the suspension culture system as described. For cardiac differentiation, procedures described in previous report (Yang et al., 2008) were used with minor modifications. Briefly, hESC aggregates derived from suspension cultures were directly differentiated in basal medium containing 0.5 ng/ml of BMP-4 (R&D systems) for 1 day. On induction days 1–4, 10 ng/ml BMP-4, 5 ng/ml human bFGF, and 6 ng/ml Activin A (R&D systems) were added. For induction days 4–8 basal medium containing 10 ng/ml VEGF (R&D Systems) and 150 ng/ml Dkk-1 (R&D Systems) was used. From induction day 8 until the end of the experiment, basal medium with 10 ng/ml VEGF and 10 ng/ml human bFGF was used. Cultures were maintained in a spinner flask with stirring rate at 50 rpm.

Cryopreservation

ES cell aggregates from suspension cultures were dissociated to single cells by Accutase and washed as mentioned previously. A freeze medium containing 90% StemPro hESC SFM, 10% DMSO, and 10 μM Y-27632, was compounded to cryopreserve hESCs. Cells were aliquoted into cryovials (Corning) with 4×10^6 cells in 1 ml per vial. The cryovials containing hESCs were placed in Mr Frosty and stored at −80 °C. After 24 h, the cryovials were transferred to liquid nitrogen for long-term storage.

Supplementary data to this article can be found online at doi:10.1016/j.scr.2012.02.001.

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