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Multiparameter comparisons of embryoid body differentiation toward human stem cell applications

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Abstract   Applications of differentiated progeny generated from human embryonic stem cells (hESCs) broadly span cell replacement therapies and screening studies (toxicology, disease-drug modeling). These applications require differentiation into lineage-specific cell types from hESCs that are largely dependent on several reported embryoid body (EB) formation methods. However, methodologies for *in vitro* EB differentiation have not been quantitatively evaluated and compared. Using the hematopoietic lineage as a test for differentiation competency, we performed multiparameter comparisons of three prevalent EB methods: (1) suspension (SP), (2) hanging drop (HD), and (3) forced aggregation (FA). Although FA improved the homogeneity between hEBs, the highest hematopoietic induction efficiencies were observed in EBs formed in SP culture independent of the presence or absence of serum. Despite the EB formation method used, EB-based hematopoietic differentiation could be potently influenced by EB size and was augmented by paracrine signaling between cocultured EBs. Our study identifies physical and physiological parameters contributing to the efficiency of hESC differentiation in EB formats and reveals that EB methods are best tailored to specific applications unique to cell replacement vs small molecule screening or early human development.
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Introduction

Human embryonic stem cells (hESCs) are considered a valuable and potentially unlimited resource for cell replacement therapies (Murry & Keller, 2008) and *in vitro* screening for disease-specific drug interactions and toxicity. Efficient identification, isolation, and enrichment of target cells during *in vitro* differentiation are critical if applications of hESC-based derivatives are to be achieved. In the majority of cases,

effective differentiation requires clustering of cells into embryoid bodies (EBs), and is best exemplified in recent studies that have used procedures to enhance hematopoietic differentiation (Vijayaragavan et al., 2009; Ng et al., 2005). Recent reports have also indicated that sheer forces are potent inducers of hematopoietic lineage development (Adamo et al., 2009). This suggests that methods for EB formation are as critical as the continued evaluation of candidate growth factor regulators of lineage control from hESCs.

To date, differentiation from hESCs is induced by three widely used hEB formation methods: (1) suspension (SP) (Denning et al., 2006; Chadwick et al., 2003; Kaufman et al., 2001), (2) hanging drop (HD) (Yoon et al., 2006), and (3) forced aggregation (FA) (Ng et al., 2005; Burridge et al., 2007; Pick et al., 2007; Ungrin et al., 2008). Both SP and HD methods involve aggregates of hESC colonies while FA uses hESC

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cultures dissociated into single cells. The SP method is the most widely used and provides a simple means to generate hEBs. However, the resulting hEBs are highly heterogeneous in both size and morphology (Denning et al., 2006; Kaufman et al., 2001). This creates reproducibility issues between and/or within experiments, thus complicating the use of hEBs for high-throughput screening (Ungrin et al., 2008; Bauwens et al., 2008; Peerani et al., 2007; Itskovitz-Eldor et al., 2000). Several laboratories have developed EB formation methods for specific lineages as well as protocols to reduce size heterogeneity (Ng et al., 2005; Yoon et al., 2006; Burridge et al., 2007; Pick et al., 2007; Ungrin et al., 2008; Bauwens et al., 2008; Peerani et al., 2007; Itskovitz-Eldor et al., 2000). Unfortunately, EB differentiation methods available have not been quantitatively evaluated and compared. This has made it difficult to understand how various EB methods differ at the level of physical and physiological parameters and how they impact candidate growth factor evaluations toward generating lineage-specific cell types for various applications.

Here we evaluated hematopoietic differentiation from EBs generated from hESCs under three different methods (SP, HD, and FA) using multiple parameters including EB size, serum supplementation, and paracrine effects. Our study establishes morphological and functional criteria to predict the best strategies for obtaining hESC derivatives for several applications that include appropriate yields for cell replacement therapies and the consistency required for *in vitro* screening.

Results

Methods used to form human EB formation influence lineage-specific yield

To establish a platform for evaluating parameters regulating hEB differentiation, we first optimized three different hEB formation methods: the SP and HD methods using aggregates and a FA method using defined numbers of single cells from hESC cultures (Fig. 1A). FA methods have been previously reported by several groups (Ng et al., 2005; Burridge et al., 2007; Pick et al., 2007; Ungrin et al., 2008), and differences existing among the protocols used in each study are summarized and compared in Supplementary Table 1. The FA protocol used here deviates from previous reports (Supplementary Table 1); however, the goal of the current study is to compare three independent EB formation methods to predict the best strategies for basic and clinical applications of hESCs.

All hEBs were cultured for 15 days under established conditions for hematopoietic differentiation containing 20% fetal calf serum (FCS) or StemPro34 serum-free medium (Chadwick et al., 2003) (Figs. 1B–D). Regardless of serum supplementation, hEBs formed by SP and HD methods were variable in both morphology and size (Figs. 1B and C). However, FA-EBs were uniform and displayed distinct morphology and growth patterns attributed to culture dissociation followed by reaggregation (Fig. 1D). Most FA-EBs grew in a round shape with consistent cystic formation and even expansion. These results demonstrate that the FA method used here yields hEBs reproducible in both morphology and size.

Previous studies have focused on the generation of EBs homogeneous in both size and morphology (Ng et al., 2005; Burridge et al., 2007; Pick et al., 2007; Ungrin et al., 2008).

Comparative evaluation of spatial distribution and variations between and/or within FA-EBs in parallel with traditional SP- and HD-EBs has not been conducted. Thus, we examined the temporal and spatial expression patterns of pluripotency (Oct4 and Sox2) and differentiation (PECAM-1) markers. Similar frequencies of Oct4 between hEB formation methods and reduction of Oct4 levels during differentiation indicate that hEBs generated by each method efficiently differentiated (Supplemental Figs. S1A and S1B). We conducted whole-mount immunostaining on SP-, HD-, and FA-EBs collected at Days 3 and 5 of hematopoietic differentiation when Oct4 and Sox2 expressions were still detectable (data not shown). Oct4 and Sox2 patterns were irregular in SP- and HD-EBs, whereas expression was evenly distributed and consistent between FA-EBs (Figs. 1E and F). However, the frequency of positively stained hEBs was not different between hEB generation methods (Figs. 1G and H). This pattern was still maintained in hEBs at Day 5 of differentiation, although staining intensity for Oct4 was weaker than Day 3 (Figs. 1I–K). Interestingly, while the number of SP- and HD-EBs with Oct4 and PECAM-1 positive signals at Day 5 of differentiation was decreased, most FA-EBs generated using our methods were positive for Oct4 and PECAM-1 and displayed more consistent staining patterns (Figs. 1L–N). These results suggest that the superior uniformity of pluripotency and differentiation marker expression and spatial organization in FA-EBs may predict higher efficiency and yield of target lineage cells.

Formation of SP-EBs allows for highest yield of hematopoietic cells

As cellular transplantation of hESC derivatives is the primary goal of regenerative medicine, the overall yield of lineage-specific cells is critical. However, to date, quantitative analysis of actual cell number has not been conducted, and most studies rely on frequency and not efficiency of cellular output (input of hESCs vs output of mature lineages) (Ng et al., 2005; Yoon et al., 2006; Burridge et al., 2007; Toh et al., 2007). Thus, we further evaluated the impact of hEB methods on output of hematopoietic cell number in the presence (20% FCS) and absence (StemPro34 serum-free medium) of serum. As previously characterized, hematopoietic development from hESCs can be divided into two phases: hemogenic lineage specification phase (Days 0–7) characterized by the appearance of the bipotent hemogenic cells (CD45negPFV) with hematopoietic and endothelial capacity followed by the commitment phase (Days 7–15) characterized as the period in which committed hematopoietic progenitors are detected (Vijayaragavan et al., 2009; Wang et al., 2004). There was no significant difference in the frequency of CD45negPFV cells or hematopoietic (CD45+CD34+ and CD45+) cells between hEB formation methods irrespective of serum supplementation (Figs. 2A–C and Supplemental Figs. S1C–S1E). hEBs cultured with serum displayed an overall higher frequency of CD45+CD34+ and CD45+ cells compared to those in serum-free conditions. Notably, the frequencies of CD45+CD34+ and CD45+ cells were significantly increased in SP-EBs stimulated by serum compared to SP-EBs cultured under serum-free conditions at Day 15 (Figs. 2B and C). However, similar frequencies of hemogenic and hematopoietic cells under both serum-containing and serum-free conditions were observed by Day 20 (Supplemental
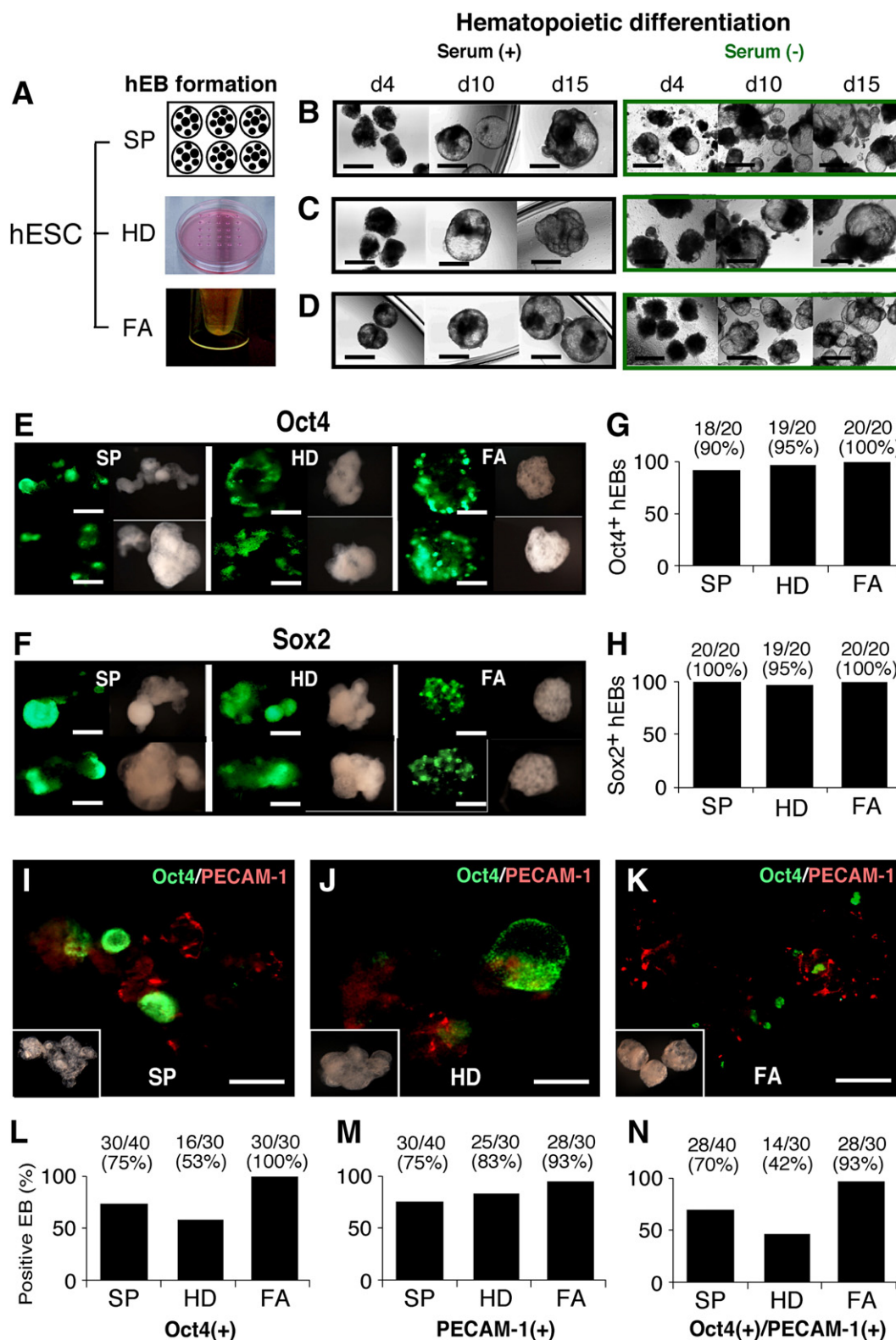
Hematopoietic differentiation

Figure 1 Morphological and phenotypic observations in hEBs generated by SP, HD, and FA methods. (A) Schematic of hEB formation methods using aggregates of hESC colonies and single cell suspensions. (B–D) Representative images of SP- (B), HD- (C), and FA-EBs (D). hEBs were cultured under hematopoietic conditions for 15 days in the presence (black box) or absence (green box) of serum and analyzed. (E and F) Whole-mount staining of Oct4 (E) and Sox2 (F) in hEBs formed by SP, HD, and FA methods at Day 3 of hematopoietic differentiation. (G and H) Percentage of Oct4 (G) and Sox2 (H) positive hEBs at Day 3 of hematopoietic differentiation. (I–K) Costaining of Oct4 and PECAM-1 was performed in SP-EB (I), HD-EB (J), and FA-EB (K) at Day 5 of hematopoietic differentiation. (L–N) Percentage of Oct4, PECAM-1, and Oct4/PECAM-1 positive EBs shown in at Day 5 of hematopoietic differentiation. Scale bars, 500 μm.
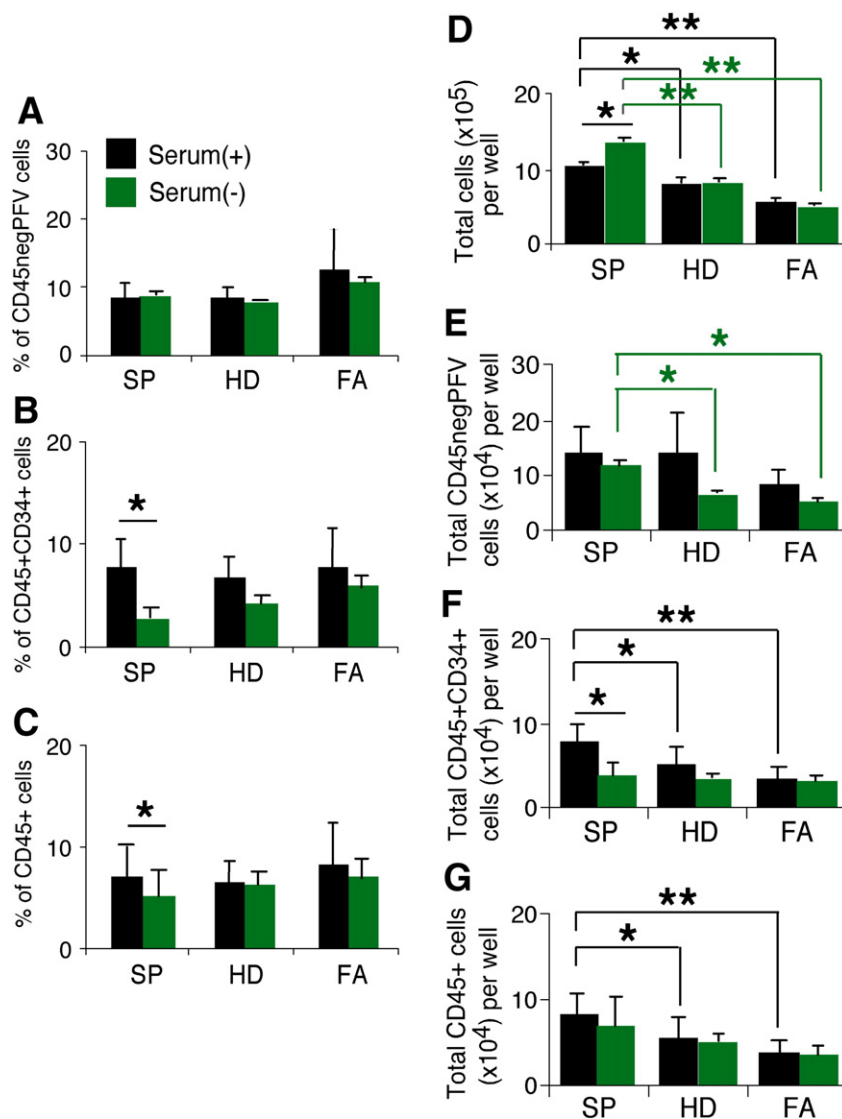
Figure 2 Comparisons of hematopoietic potential in SP-, HD-, and FA-EBs cultured in the presence or absence of serum. (A–C) Percentage of CD45negPFV (A), CD45+CD34+ (B), and CD45+ (C) cells from dissociated SP-, HD-, and FA-EBs. (D) Total cell number of differentiated hESCs. (E–G) Total number of CD45negPFV (E), CD45+CD34+ (F), and CD45+ (G) cells. hEBs were cultured in the presence (black bars) or absence of serum (green bars). All bars indicate mean±SD from H1 and H9 cell lines. * $P<0.05$; ** $P<0.01$.

Fig. S2), indicating a slight temporal delay of hematopoietic differentiation on serum withdrawal. This suggests that lack of serum delays but does not impair differentiation. Thus, serum-free conditions make it possible to define the precise role of growth factors during hEB differentiation.

Overall yield of lineage-specific cells from pluripotent stem cells is mainly a product of total number of cells generated during a given differentiation protocol. The total number of cells following 15 days of hEB differentiation was significantly increased in SP-EBs compared to both HD- and FA-EBs independent of serum supplementation (Fig. 2D). Assessment of hematopoietic output revealed that significantly more CD45negPFV cells were generated in SP-EBs compared to HD- and FA-EBs in the absence of serum (Fig. 2E). In contrast, the total number of CD45+CD34+ and CD45+ cells was higher in SP-EBs compared to all other hEB methods tested in the presence of serum (Figs. 2F and G). These results demonstrate that the highest yield of both precursor and committed

hematopoietic cells was obtained using the SP-EB methodology. In addition, the presence of serum increased hematopoietic cell numbers but inhibited total cell expansion when using the SP-EB method. While our FA method produces more consistent hEB morphology and size, the SP method is superior in generating hematopoietic cells in terms of total yield, and may therefore be better suited for differentiation assays prior to clinical transplantation. Additionally, these results show that homogeneity in hEB morphology and phenotype is not an accurate predictor of functional output or yield of tissue-specific cell types, using the hematopoietic lineage as an example.

SP-EBs produce the greatest number of hematopoietic clonogenic progenitors

The colony forming unit (CFU) assay is commonly used to evaluate multilineage hematopoietic progenitors with

immense clonogenic proliferative capacity (Chadwick et al., 2003; Wang et al., 2004). In parallel with the phenotypic analysis above, we also conducted functional CFU assays to compare multilineage hematopoietic progenitor potential derived from SP-, HD-, and FA-EBs. Equal cell numbers of dissociated EBs were plated and the estimated number and phenotype of hematopoietic progenitors were examined after 15 days. We observed CFU colonies including CFU-G, CFU-M, CFU-GM, and CFU-E in each group (Supplemental Fig. S3A). We also characterized the phenotypes and hierarchical relationship of the hematopoietic compartments derived from hESCs. In order to compare clonogenic potentials between cell populations within hEBs, we isolated specific subpopulations based on the expression of hematopoietic lineage markers such as CD34, CD45, CD43, and CD38. We found that CD45+CD34+ cells produce the most CFU colonies compared to other phenotypes (Supplemental Fig. S3B). Therefore, we calculated the total number of hematopoietic progenitors based on the frequency of CD45+CD34+ cells. Similar frequencies of CFU colonies were seen from defined cell numbers (15 000 cells) of SP-, HD-, and FA-EBs

independent of serum supplementation (Fig. 3A). The SP method resulted in significantly higher total numbers of hematopoietic progenitors compared to HD and FA regardless of serum presence (Fig. 3B). While no differences in colony distribution were seen with each hEB generation method, the production of CFU-E was significantly promoted from EBs cultured under serum-free conditions (Fig. 3C). These demonstrate that hEBs generated by SP, HD, and FA methods possess similar functional potential; however, the SP-EB method results in production of the greatest number of total hematopoietic progenitors. Accordingly, our analysis reveals that the SP-EB generation method is the most appropriate for applications requiring high yields of hematopoietic cells, such as *in vivo* transplantation studies using hematopoietic cells.

Hematopoietic capacity is dependent on the size of EBs

Previous studies have reported on differential EB size and morphology generated by SP and HD methods and suggested a relationship between EB size and differentiation capacity to a
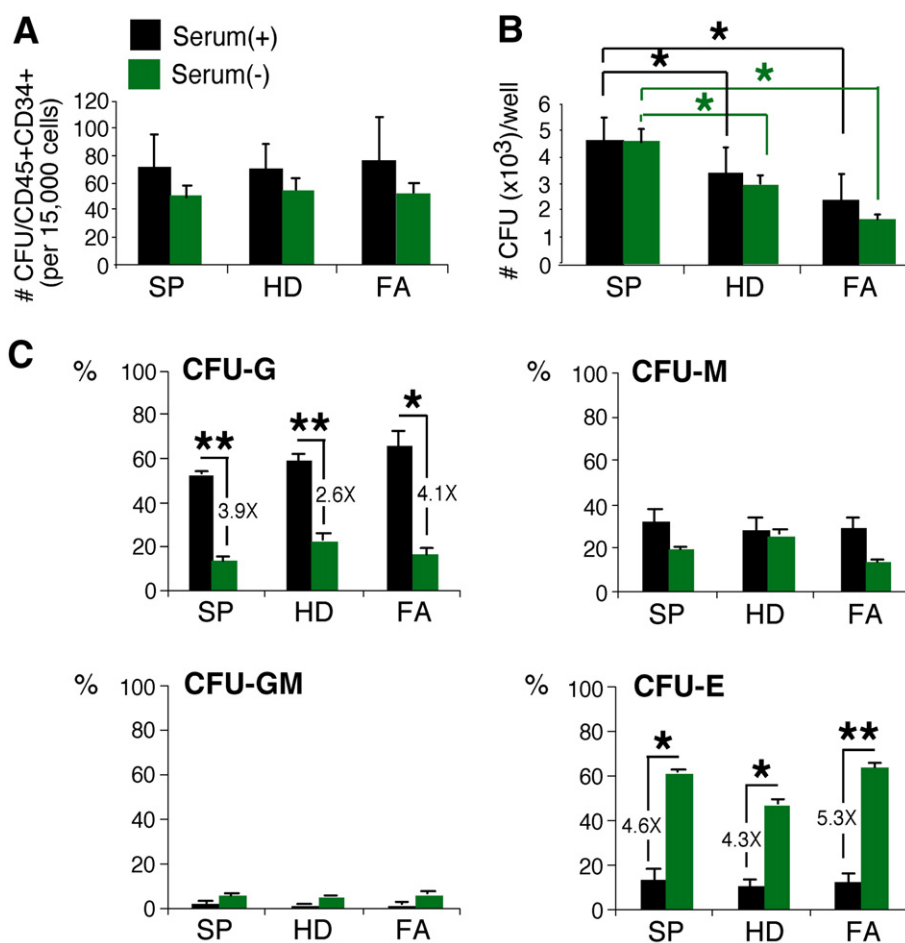

Figure 3 Comparison of clonogenic capacity in SP-, HD-, and FA-EBs cultured in the presence or absence of serum. (A) Hematopoietic progenitor capacity detected by plating 1.5×10^4 cells from dissociated SP-, HD-, and FA-EBs cultured for 15 days. (B) Total number of CFUs from dissociated SP-, HD-, and FA-EBs. (C) Distribution of CFU subtypes including macrophage (CFU-M), granulocyte (CFU-G), erythroid (CFU-E), and bipotent colonies (CFU-GM). hEBs cultured in the presence (black bars) or absence of serum (green bars) were seeded into methylcellulose. All bars indicate mean±SD from H1 and H9 cell lines. * $P < 0.05$; ** $P < 0.01$.

specified lineage (Burridge et al., 2007; Bauwens et al., 2008; Peerani et al., 2007; Bader et al., 2001). To address this issue during hematopoiesis, we examined the effect of EB size using all three hEB formation methods available to the field. Fig. 4A depicts hEB formation by SP and HD methods and grouping depending on hEB size. hEB size measurements were taken by averaging the longest and shortest diameter of selected hEBs that were round shape, cystic, and nonfused. From these measurements, SP-EBs and HD-EBs were divided into three groups at Day 15 as follows: large (SP-L and HD-L, 1790±40 μm, n=28), medium (SP-M and HD-M, 1100±30 μm, n=28), and small size hEB (SP-S and HD-S, 570±30 μm, n=26). Individually isolated and dissociated hEBs were analyzed by flow cytometry

to determine frequency of hematopoietic cells including CD45negPFV, CD45+CD34+, and CD45+ cells at Day 15 of differentiation (Figs. 4B–G and Supplemental Fig. S4). There were no significant differences in the frequency of hemogenic or hematopoietic cells generated between medium and large size SP- or HD-EBs cultured with hGFs (Figs. 4B–G). However, small SP- and HD-EBs generated significantly less CD45negPFV (Figs. 4B and E), CD45+CD34+ (Figs. 4C and F), and CD45+ cells (Figs. 4D and G) compared with medium and large hEBs, with virtually no hematopoietic cells being generated in the small hEBs (Table 1). In order to confirm the accuracy of individual hEB analysis, in particular the small hEBs, we pooled large, medium, and small hEBs, respectively, and tested
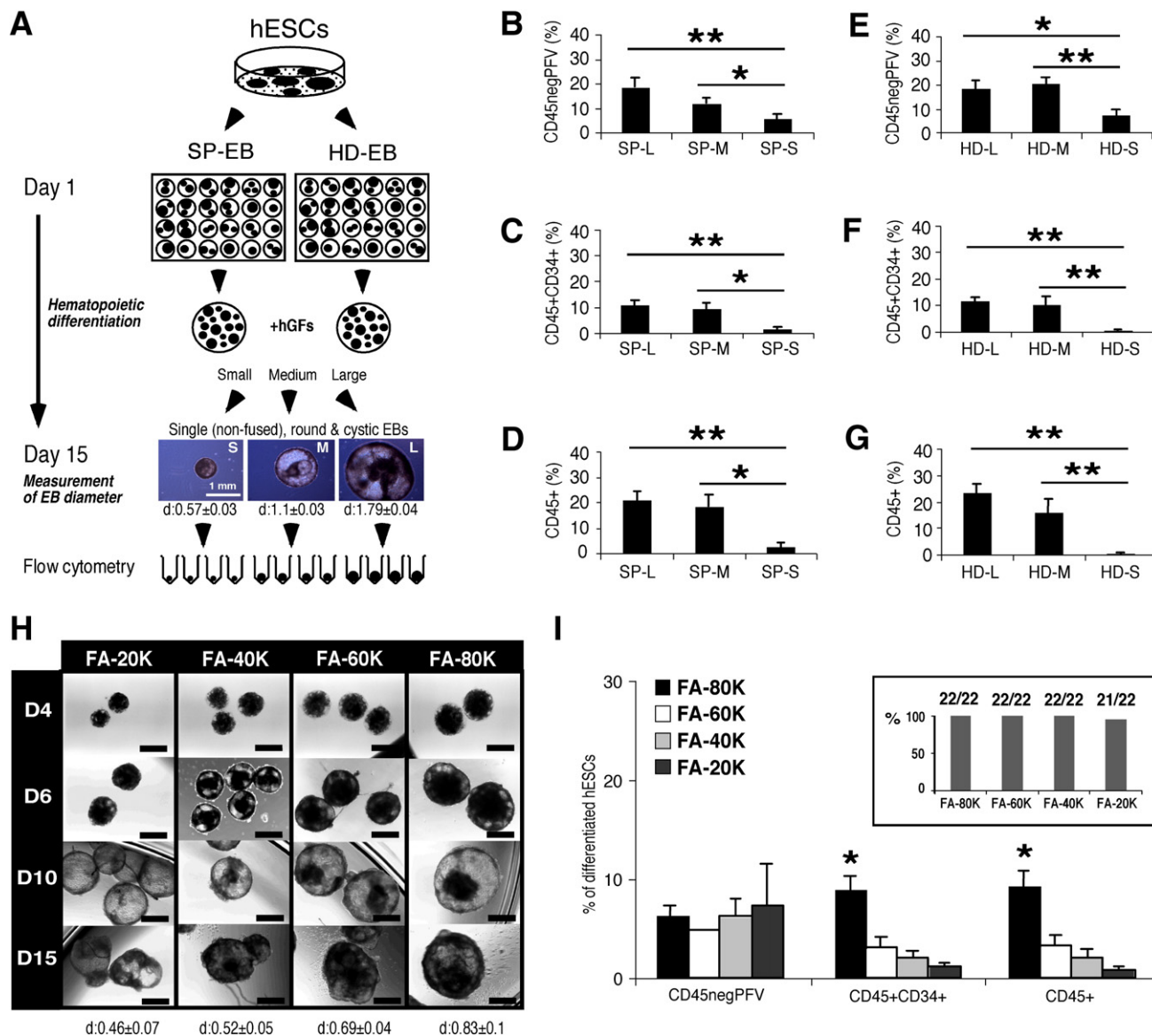

Figure 4   Relationship between hEB size and hematopoietic differentiation. (A) Schematic of hEB formation by SP and HD methods, measurement of hEB diameter, and flow cytometry at Day 15 of hematopoietic differentiation. (B–G) Percentage of hemogenic precursors (CD45negPFV; B and E), primitive hematopoietic cells (CD45+CD34+; C and F), and committed blood cells (CD45+; D and G) from dissociated EBs cultured with hGFs. (H) Representative images and diameter of FA-EBs formed using defined numbers of single cells (20, 40, 60, and 80 K cells). Scale bars, 500 μm. (I) Percentage of CD45negPFV, CD45+CD34+, and CD45+ cells in FA-EBs generated from 20, 40, 60, and 80 K cells. Efficiency of FA-EB formation (inset). L, large EB; M, medium EB; S, small EB. * $P<0.05$; ** $P<0.01$. All bars indicate mean±SD from H1 and H9 cell lines.

Table 1   Comparison of hematopoietic differentiation between different size of EBs generated by SP and HD method

Hematopoietic differentiation (Day 15)

	CD45negPFV		CD45+CD34+		CD45+	
	%	No. of EB	%	No. of EB	%	No. of EB
SP-L	18.8±3.6	(14)	10.6±1.9	(14)	20.8±3.2	(14)
SP-M	12.1±2.1	(14)	9.1±2.4	(14)	18.4±4.3	(14)
SP-S	5.6±1.7	(13)	1.2±0.7	(13)	2.6±1.6	(13)
HD-L	18.2±3.4	(14)	11.5±1.5	(14)	23.3±3.3	(14)
HD-M	20.5±2.9	(14)	10.3±3.4	(14)	15.8±5.1	(14)
HD-S	7.1±2.4	(13)	0.2±0.1	(13)	0.5±0.2	(13)

Analysis was performed in hEBs measured by averaging the longest and shortest diameter at Day 15 of hematopoietic differentiation. SP, suspension; HD, hanging drop; L, large EB; M, medium EB; S, small EB.

hematopoietic differentiation (Supplemental Fig. S5). We obtained similar results as seen for individual hEB analysis. These results indicate that hESC-derived hematopoietic differentiation can be affected by smaller hEB size.

To evaluate the effect of EB size in a more reproducible and homogeneous system, we then generated FA-EBs using a defined number of single cells (20, 40, 60, and 80 K). These hEBs were homogeneous in size and morphology (20 K, 460± 71 μm; 40 K, 520±55 μm; 60 K, 690±48 μm; 80 K, 830±108 μm) (Fig. 4H). In contrast to the influence of size on SP- and HD-EBs, input cell number had no effect on the emergence of CD45negPFV cells in the FA-EBs (Fig. 4I). However, like SP-EBs and HD-EBs, 80 K FA-EBs, which were equivalent in size to "medium" SP- and HD-EBs, produced dramatically more CD45+ CD34+ and CD45+ cells compared to those from 60, 40, and 20 K cells (approximately equivalent to "small" SP- and HD-EBs) (Fig. 4I). Importantly, similar frequencies of hematopoiesis were achieved using all EB generation methods (comparing SP-M and HD-M to FA-80 K), demonstrating that dissociation and reaggregation do not negatively impact hEB developmental potential. Together, these findings provide clear evidence that hEB size, independent of hEB formation methods, profoundly influences hematopoietic output resulting from hESC differentiation and suggest that paracrine or autocrine mechanisms may be augmented with increased cell number.

Paracrine effects between EBs promote hESC-derived hematopoiesis

During hEB differentiation, hEBs are generally cultured in groups and not individually. However, it has not been determined whether cooperative paracrine interactions enhance the production of hESC-derived cells. In our preliminary work, we observed reduced hematopoiesis in single hEB culture compared with group hEB culture (data not shown). This observation, combined with the fact that larger EBs exhibit increased hematopoiesis, prompted the hypothesis that factors diluted in culture medium may be responsible for effects on hematopoietic output. Accordingly, we examined whether paracrine signaling between developing hEBs influences differentiation. To do this, we cultured SP-M and SP-L hEBs for 15 days individually or in groups in StemPro34 serum-free medium containing hGFs, or individually in the presence of hEB conditioned media (CM) (Fig. 5A). All culture methods produced morphologically similar hEBs (Fig. 5B) that contained

hemogenic and hematopoietic cells. There was no difference in the frequency of CD45negPFV cells generated by group or single culture (Fig. 5C). Surprisingly, grouping hEBs in culture produced a significantly higher frequency of both CD45+CD34+ and CD45+ cells compared to single hEB culture (Figs. 5D and E). Culture of single hEBs in the presence of CM resulted in a partial rescue in the frequency of CD45+CD34+ and CD45+ cells (Figs. 5D and E). These findings indicate that reduced hematopoietic development in single hEB culture could be compensated by secreted soluble factors in CM from concentrated hEBs. Thus, paracrine or autocrine effects in hEB culture system promote the production of hESC-derived differentiated cells. Pragmatically, these results demonstrate that caution should be exercised when defining optimal growth factors cocktails for lineage control.

Discussion

Efficient generation of target cells or cell populations from developing hEBs will be beneficial for clinical transplantation, high-throughput screening (new drugs and toxic materials), and identifying molecular pathways important for lineage specification. Our analysis defines physical and physiological criteria that predict functional output of differentiated cells. While all hEB generation methods exhibit similar hematopoietic potentials, differences in morphology and cell numbers demonstrate that differentiation protocols should be chosen based on the desired application. For example, safety assessment of newly developed pharmaceutical drugs should be achieved prior to the direct use in cell replacement therapies. FA methods allow for reproducible generation of homogeneous hEBs in terms of morphology, size and growth making them more amenable to high-throughput screening and drug discovery. In contrast, the HD method, due to limited space of the hanging droplet, may be more useful for the quantification of the auto- or paracrine effects between specific cell populations rather than to induce early lineage specification (Yoon et al., 2006; Gutierrez et al., 2005). For transplantation studies, the SP method is preferable for differentiating hESCs because of the consistently higher yield of total number of cells for a given lineage.

The SP method is the most common and easiest way to generate hEBs and is achieved by scraping undifferentiated hESC colonies after enzyme treatment. However, SP-EBs are
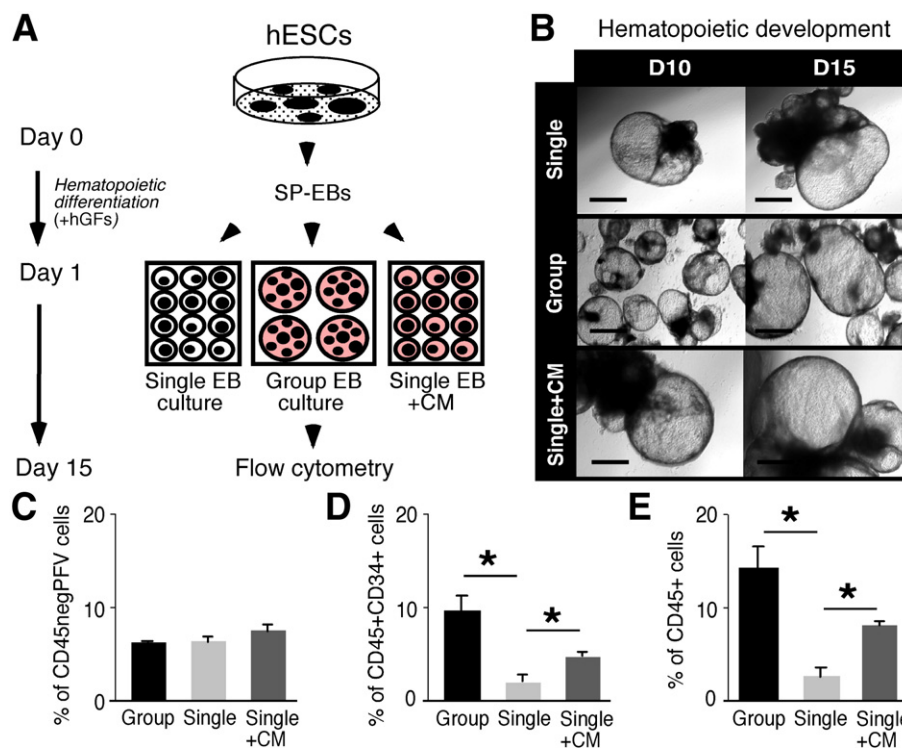
Figure 5 Paracrine interaction between hEBs augments the output of hematopoietic lineage cells. (A) Schematic of hEBs cultured individually, in groups, and individually with conditioned medium (CM). CM was produced by collection of medium used to feed hEBs cultured in groups. hEBs were cultured for 17 days supplemented with hGFs in StemPro34 serum-free medium and the output of CD45negPFV, CD45+CD34+, and CD45+ cells was analyzed. (B) Representative images of developing hEBs. Scale bars, 500 µm. (C–E) Percentage of CD45negPFV (C), CD45+CD34+ (D), and CD45+ (E) cells from hEBs. All bars indicate mean ± SD from H1 and H9 cell lines. *, $P < 0.05$.

highly heterogeneous in both size and morphology, therefore making it difficult to obtain reproducible results between and even within laboratories. Most studies have therefore focused on the generation of homogeneous EBs in both size and morphology (Ng et al., 2005; Burridge et al., 2007; Pick et al., 2007). For example, Ungrin et al. (Ungrin et al., 2008) reported that using single cells, spatially and temporally synchronized hEBs could be generated that were suitable for studying early human developmental processes. However, other EB formation methods were not compared in this study.

To compare the yield of hematopoietic cells between EB formation methods, we started with over 80% confluent hESC cultures consisting of densely packed colonies and an adequate ratio of undifferentiated to fibroblast-like cells (Bendall et al., 2007). This starting criteria is critical for obtaining an adequate total cell and hematopoietic cell yield (Vijayaragavan et al., 2009; Chadwick et al., 2003; Wang et al., 2004). However, the yield of hematopoietic cells was significantly lower in our FA-EBs than SP- and HD-EBs. This was attributed to the loss of single cells during FA-EB formation and cellular damage during initial enzymatic dissociation (Costa et al., 2007). To increase cell–cell adhesion and cellular viability, FBS, polyvinyl alcohol, BSA, or ROCK inhibitor may be added (Burridge et al., 2007; Watanabe et al., 2007); however, exogenous reagents must be evaluated for their potential adverse effects on lineage-specific differentiation.

Investigation into the influence of hEB size on differentiation revealed that larger hEBs resulted in increased frequencies of hematopoietic cells. This was clearly demonstrated using FA-EBs, in which hEBs with <80 K cells exhibited severely diminished hematopoietic potential. While larger (1790 ± 40 µm) hEBs were assessed for hematopoietic development, a point where EB size negatively impacted differentiation could not be reached. Previous studies have reported that there was an upper limit of EB diameter during ESC differentiation, which could reduce the overall yield of differentiated cells. Bader et al. (Bader et al., 2001) monitored EBs with beating cardiomyocytes in EBs made of different numbers of mouse ESC by the HD method. They found that EBs generated from 565 single cells could give rise to a maximum rate of beating cells. Consistently, Burridge et al. (Burridge et al., 2007) showed that the highest efficiency of cardiomyocyte differentiation occurred when they transferred hEBs of 250–350 µm into DMEM with 20% FBS. Recent investigation into micropatterning of hESC culture revealed that differentiation trajectories of hESCs could be controlled by engineering hESC composition and colony size (Bauwens et al., 2008; Peerani et al., 2007; Lee et al., 2009). These findings were attributed to the diffusion of soluble factors, both exogenous and endogenous, into the EB for supply of nutrients and the status of the input hESC composition. Therefore, an optimal number of cells must be present to promote differentiation possibly

through generation of a microenvironment that induces dynamic signaling patterns.

To date, there are no reports evaluating the autocrine/paracrine effects between hEBs during differentiation. We observed that there are paracrine effects that improve hematopoietic differentiation when there are multiple, as opposed to single, hEBs in culture. This effect could also be recapitulated using conditioned medium from concentrated hEB culture. Although we have not identified the secreted factors that mediate these paracrine effects, our study is the first to report the differences in differentiation potential between grouped and single EB culture. The use of serum-free conditions during hEB differentiation would simplify analysis of the conditioned media to identify potential soluble factors involved in hematopoietic development.

In conclusion, our comparative multiparameter analysis of the defining features contributing to hematopoietic differentiation from hESCs reveals that: (1) the EB generation method, (2) serum supplementation, (3) EB size, and (4) cooperative signals between hEBs govern functional output. These parameters are likely not limited to the hematopoietic lineage of hESC derivatives. Therefore, our analysis provides practical guidelines for selecting hEB formation protocols tailored to specific goals within regenerative medicine and stem cell-based applications.

Materials and methods

hESC culture

Undifferentiated hESC lines H1 and H9 were maintained in feeder-free culture as previously described (Chadwick et al., 2003). Briefly, hESCs were cultured on Matrigel (BD Biosciences, Bedford, MA, USA)-coated six-well plate with mouse embryonic fibroblast-conditioned medium (MEF-CM) supplemented with 8 ng/ml human recombinant basic fibroblast growth factor (hbFGF; Invitrogen, Burlington, ON, Canada). hESCs were fed daily with fresh MEF-CM and were passaged at a 1:2 ratio every 6 or 7 days by enzymatic dissociation with 200 U/ml collagenase IV (Invitrogen).

hEB formation

To generate hematopoietic cells from hESCs, we generated hEBs by SP and HD culture methods as previously described (Cerdan et al., 2007). Briefly, for SP culture, confluent undifferentiated hESCs were treated with 200 U/ml collagenase IV for 5−10 min and then transferred to 6- or 12-well ultralow attachment plates (Corning). Clumps of variable size were incubated overnight to allow hEB formation in hEB differentiation medium consisting of KO-DMEM supplemented with 20% nonheat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT), 1% nonessential amino acids, 1 mM L-glutamine, 0.1 mM β-mercaptoethanol. For HD culture, the clumps were scraped after treatment of collagenase IV and transferred to 6-well low attachment plates containing the same hEB differentiation medium overnight and then evenly distributed on the lids of a 60-mm polystyrene petri dish (BD Labware, NJ, USA). For hEB

generation using single hESCs by the FA method, we adapted the "conical tube" method performed by Kurosawa et al. (Kurosawa et al., 2003) to obtain aggregates of single hESCs in a polypropylene 1.5-ml conical tube (Sarstedt Inc., Montreal, QC, Canada). The confluent undifferentiated hESCs were dissociated into single cell suspension by TrypLE (Invitrogen), rinsed with phosphate-buffered saline (PBS), and resuspended in hEB differentiation medium. The single cell suspension was filtered with a 70-μm cell strainer (BD Biosciences) and counted. hEB formation was carried out by placing the defined number ($2−8 \times 10^4$ cells) of single cells reconstituted with 1 ml hEB differentiation medium in a polypropylene 1.5-ml conical tube with a screw cap. After distribution, the tubes were centrifuged at 450 g for 5 min and incubated overnight to allow hEB formation in loosely closed caps for gas supply.

Hematopoietic differentiation in the presence and absence of serum

In order to evaluate hematopoietic differentiation of SP-, HD-, and FA-EBs in the presence and absence of serum, hEBs were cultured in hEB differentiation medium containing 20% FCS and StemPro-34 serum-free medium (Invitrogen) supplemented with hematopoietic growth factors (hGFs) as previously described (Chadwick et al., 2003). Briefly, medium was changed with the hEB differentiation and StemPro-34 serum-free medium supplemented with hGFs as follows: 50 ng/ml granulocyte colony stimulating factor (G-CSF; Amgen, Inc., Thousand Oaks, CA, USA), 300 ng/ml stem cell factor (SCF; Amgen), 10 ng/ml interleukin-3 (IL-3; R&D systems, Minneapolis, MN, USA), 10 ng/ml interleukin-6 (IL-6; R&D systems), 25 ng/ml BMP-4 (R&D systems), and 300 ng/ml Flt-3 ligand (Flt-3 L: R&D systems). The hEBs were cultured for 15 days where the medium including the hGFs was changed at 3-day intervals. To examine paracrine potential of hEBs during hematopoietic development, hEBs cultured in groups (10 hEBs/well) were compared with hEBs cultured alone (one hEB/well) or in conditioned medium (CM). CM was freshly obtained by collection of medium used to feed hEBs cultured in groups. The collected CM was filtered prior to replacement. To rule out undesirable effect of serum, StemPro34 serum-free medium was used.

Flow cytometry analysis

hEBs were dissociated with 0.4 U/ml collagenase B (Roche Diagnostics, Laval, QC, Canada) for 2 h in 37 °C incubator, followed by treatment with cell dissociation buffer (Invitrogen) for 10 min in a 37 °C waterbath and then passed through a 40-μm cell strainer (BD Biosciences). Single cell suspensions were resuspended at approximately 1 to 2×10^5 cells/ml with PBS/3% FBS and stained for 1 h at 4 °C with fluorochrome-conjugated monoclonal antibodies as follows: CD31-phycoerythrin (PE) (BD Pharmingen, San Diego, CA), CD34-fluorescein isothiocyanate (FITC) (Miltenyi Biotech, Bergisch Gladbach, Germany), and CD45-allophycocyanin (APC) (Miltenyi Biotech) or their corresponding isotype controls. Stained cells were washed twice in PBS/3% FBS, resuspended in PBS/3% FBS, and then stained with 7-aminoactinomycin D (7-AAD) (Immunotech, Marseille, France) viability dye for

10 min at RT. Live cells were analyzed for surface marker expression using a FACSCalibur cell analyzer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and Cell Quest software (BDIS).

Sorting of hEBs

To isolate CD45+CD34+, CD45+CD34-, and CD45-CD34+ cells, hEBs were dissociated with collagenase B and stained with CD31-PE, CD34-FITC, CD45-APC, and 7AAD. Each cellular subset was sorted on a FACSAria (BD Pharmingen) as previously described (Wang et al., 2004).

Clonogenic progenitor cell assay

Clonogenic progenitor assays were performed by plating single cell suspensions of dissociated hEB into methylcellulose H4230 (Stem Cell Technologies, Vancouver, BC, Canada) as previously described (Chadwick et al., 2003). Briefly, hEBs were dissociated with collagenase B and cell dissociation buffer and filtered through a 40-μm cell strainer. Dissociated hEBs were counted and 15 000 cells were plated into methylcellulose H4230 supplemented with recombinant human growth factors as follows: 50 ng/ml SCF, 3 units/ml erythropoietin (EPO; Amgen), 10 ng/ml granulocyte monocyte-colony stimulating growth factor (GM-CSF; Novartis, Dorval, QC, Canada), and 10 ng/ml IL-3. After 14 days, differential colony counts were performed based on morphology.

Whole-mount immunological staining

hEBs cultured in hematopoietic differentiation media for 3 days were fixed using fresh methanol:DMSO (4:1) and stored at 4 °C overnight. hEBs were then rehydrated in methanol, washed in PBT (PBS containing 0.1% Tween 20), and incubated with blocking solution (TBST containing 2% nonfat milk carnation and 0.5% Tween) for 1 h. hEBs were stained at 4 °C overnight with primary antibodies as follows: goat polyclonal POU5F1 (1:50 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and goat polyclonal Sox2 (1:40 dilution, R&D systems). After washing in TBST for 5 h, hEBs were incubated with their corresponding secondary antibodies for 5 h and then washed twice in TBS. Stained hEBs were observed under a fluorescence microscope (Nikon, SMZ1000, Japan).

Statistical analysis

All results were expressed as means±SD and generated from at least three independent experiments. Statistical significance was determined using the Student t test and differences were considered significant when $P<0.05$.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2010.04.007.

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