

Controlled, Scalable Embryonic Stem Cell Differentiation Culture

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ABSTRACT

Embryonic stem (ES) cells are of significant interest as a renewable source of therapeutically useful cells. ES cell aggregation is important for both human and mouse embryoid body (EB) formation and the subsequent generation of ES cell derivatives. Aggregation between EBs (agglomeration), however, inhibits cell growth and differentiation in stirred or high-cell-density static cultures. We demonstrate that the agglomeration of two EBs is initiated by E-cadherin-mediated

cell attachment and followed by active cell migration. We report the development of a technology capable of controlling cell-cell interactions in scalable culture by the mass encapsulation of ES cells in size-specified agarose capsules. When placed in stirred-suspension bioreactors, encapsulated ES cells can be used to produce scalable quantities of hematopoietic progenitor cells in a controlled environment. *Stem Cells* 2004;22:275-282

INTRODUCTION

Embryonic stem (ES) cells are pluripotent cells capable of extensive proliferation while maintaining their potential to differentiate into any cell type in the body [1, 2]. ES cells can therefore be considered a renewable source of useful cell types such as cardiomyocytes [3], insulin-secreting cells [4], dopaminergic neurons [5], and hematopoietic progenitors [6, 7]—cells that have proven difficult to expand in vitro. While ES-derived cells have tremendous potential in many experimental and therapeutic applications, their utility, and indeed investigations into the scope of their utility, is dependent on the availability of relevant cell quantities. This can be accomplished by increasing the

scale of cell production and by optimizing culture conditions for the generation of target cells.

In an ideal scenario, differentiation of ES cells could be directed to a pure population of the desired cell type. For example, *Tropepe et al.* (2001) [8] described culture conditions that exclusively permit the formation of neural progenitor cells from mouse ES cells, albeit at very low cell frequency. In most cases, the knowledge to precisely control mouse or human ES cell fate decisions is lacking. Consequently, the most robust method for generating most differentiated cell types is through the embryoid body (EB) system where ES cells spontaneously differentiate as tissue-like spheroids in suspension culture. EB differentiation

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has been shown to recapitulate aspects of early embryogenesis, including the formation of a complex three-dimensional architecture wherein cell-cell and cell-matrix interactions are thought to support the development of the three embryonic germ layers and their derivatives [9, 10].

Presently, all human and most mouse ES cell lines require aggregation of multiple ES cells to efficiently initiate EB formation [10, 11]. Standard methods of EB formation include hanging drop, liquid suspension, and methylcellulose culture. These culture systems maintain a balance between allowing ES cell aggregation necessary for EB formation and preventing EB agglomeration for efficient cell growth and differentiation [11]. However, these culture systems are limited in their production capacity and are not easily amenable to process-control strategies. The ability to culture differentiating ES cells in stirred-suspension bioreactors, either alone or in combination with other technologies [12], would overcome many of the current culture limitations. Stirred-suspension bioreactors are readily scaled in size to match production needs. Stirring also homogenizes bulk media conditions and thus facilitates measurement and control. In addition to its cell-production-related role, the ability to control culture conditions would be useful for experimental investigations of extrinsic factors (such as glucose concentration, cytokine concentration, pH, and oxygen tension) and their effects on ES cell growth and differentiation. Unfortunately, direct addition of ES cells to stirred suspension culture results in significant cell agglomeration and consequently poor cell growth and differentiation [11].

We primarily used mouse ES cells to investigate the process of EB agglomeration and to develop a method for overcoming this cell culture issue. We then verified the relevance and application of these findings for human ES cells. A two-step mechanism for mouse EB agglomeration was elucidated. First, cell-cell adhesion molecule E-cadherin was determined to mediate attachment between neighboring EBs. Following attachment, cells actively migrated and remodeled, assimilating cells into a single spheroid. This mechanism was found to be common to human EBs, and these findings were consistent with the observation that blastocyst-stage human embryos constitutively express E-cadherin [13]. To control cell-cell interactions, mouse and human ES cells were encapsulated in size-controlled agarose hydrogel capsules. Encapsulation permitted the use of high-cell-density culture and enabled EB formation and differentiation to hematopoietic cells in controllable stirred-suspension bioreactors. The importance of this culture system was demonstrated using oxygen tension as an extrinsically controlled inductive signal for hematopoietic development. Hematopoietic progenitor yield was significantly greater in cultures maintained at 4%

versus 20% oxygen tension. Together these results demonstrate our capacity to produce scalable quantities of human hematopoietic progenitor cells from human ES cells in bioreactors.

MATERIALS AND METHODS

ES Cell Culture and Hematopoietic Cell Assays

Maintenance of mouse R1 [14] and YC5 [15] ES cells, preparation of cells for flow cytometry, and preparation of hematopoietic colony-forming cell assay were previously described in *Dang et al. (2002)* [11]. Maintenance of human ES cells (H9.2 and I6) and EB formation were also previously described [10].

Agglomeration of Two EBs

Mouse R1 and YC5 ES cell spheroids (also referred to as EBs for simplicity) cultured in ES media containing leukemia inhibitory factor (LIF; Chemicon; Temecula, CA) were formed in hanging drop culture initiated with 100 ES cells/drop for 24 hours and transferred into nontreated conical 96-well plates (Sarstedt; Newton, NC) containing 100 μ l of mouse ES cell medium. One YC5 EB and one R1 EB of the same size were placed together in each well. The plates were gently agitated until the two EBs came in contact with one another. Starting at time 0, photos were taken every 4 hours to record EB interactions.

Test wells were filled with 100 μ l of mouse ES cell medium with 40 μ g/ml α -mouse E-cadherin (U3254; Sigma; St. Louis, MO), 50 μ g/ml Cytochalasin D (Sigma), or 50 μ g/ml mitomycin C (Sigma). EBs were incubated separately for 2 hours in their respective solutions before transferring YC5 EBs into the R1 EB wells.

Human EB agglomeration was similarly studied. We placed two human EBs within the same well containing 100 μ l of human ES media and observed agglomeration of the EBs over time. Human EBs treated with 40 μ g/ml α -human E-cadherin (67A4; Chemicon) were incubated separately for 2 hours before transfer into agglomeration cultures.

Encapsulation Process

Mouse ES cell aggregates were formed by generating a single cell suspension of 3×10^5 cells/ml in ES cell media and allowing cells to aggregate for 1 day. Human ES cell aggregates were formed by partial dissociation by incubating human ES cell maintenance cultures for 20 minutes with 2 mg/ml collagenase B (Sigma). ES cell aggregates were collected and added to molten 2% (weight) low-gelling-temperature agarose (type VII; Sigma) in phosphate buffered saline (PBS; GIBCO-BRL; Rockville, MD) at 2×10^6 cells/ml. The molten agarose mixture was dispensed into 200-centistroke

viscosity dimethylpolysiloxane (DMPS; Sigma) at 37°C and subjected to impeller shearing using the CellSys Microdrop Maker (One Cell Systems; Cambridge, MA) to create agarose hydrogel capsules [16]. Microcapsules were washed twice with Hank's buffered saline solution (HBSS; GIBCO-BRL) and suspended in the appropriate ES cell differentiation media.

Bioreactor Culture

We used the Cellferm-pro system (DasGip; Julich, Germany) for stirred-suspension culture of encapsulated ES cells under controlled conditions. Cellferm-pro consisted of a control, monitoring, dosing, gassing, and cultivation system. We simultaneously operated four pH- and dissolved-oxygen-regulated 400-ml vessels in batch culture mode. Vessels were filled with 200 ml of ES cell media without LIF and inoculated with 5×10^5 ES cells (12,500 ES-cell-containing capsules), achieving a starting cell density of 2.5×10^3 ES cells/ml (60 ES-cell-containing capsules/ml). Cells were cultured for 7 days before harvesting and analyzed by cell counting, flow cytometry, and myeloid-erythroid colony-forming cell assay.

RESULTS

E-Cadherin-Mediated Cell Aggregation

We studied the process of EB agglomeration by quantitatively tracking the fusion of two EBs or two ES cell spheroids (also referred to as EBs for simplicity). To allow observation of cell mixing, one EB generated from wild type R1 mouse ES cells was agglomerated with one EB generated from YC5-R1 [15] mouse ES cells (that constitutively express the yellow fluorescent protein). We calculated the degree of agglomeration (DOA) as the ratio of the interface diameter to the overall length of the two-EB system (Fig. 1A). We measured DOA at regular time intervals and determined initial EB size (50, 100, 400 ES cells/EB) to have no measurable effect on the kinetics of this process (data not shown). Complete agglomeration (DOA >90%) was achieved after approximately 16 hours and complete cell mixing (homogenous fluorescence intensity) after 48 hours (Fig. 1B).

We investigated the mechanism of mouse ES cell aggregation and EB agglomeration using various loss-of-function treatments (Fig. 1C). Blocking E-cadherin cell-adhesion molecules on the EB surface with α -mouse E-cadherin antibodies significantly inhibited EB agglomeration ($p = 0.021$ at 24 hours). This result supports the observation that homozygous E-cadherin-null ES cells are unable to aggregate [17]. Treatment of EBs with cytochalasin D also impaired agglomeration ($p = 0.002$ at 24 hours). Cytochalasin D inhibits actin-dependent processes including cell migration, which

suggests that EB agglomeration is an active process and not a result of passive cell diffusion [18]. Based on these results, we proposed a two-step mechanism for EB agglomeration: first, neighboring EBs collide and homophilic E-cadherin molecules adhere EBs together. Cells then actively migrate and remodel the structure until all cells are assimilated into a single spheroid. Impairing either E-cadherin-mediated EB attachment or cell migration inhibits EB agglomeration (Fig. 1C).

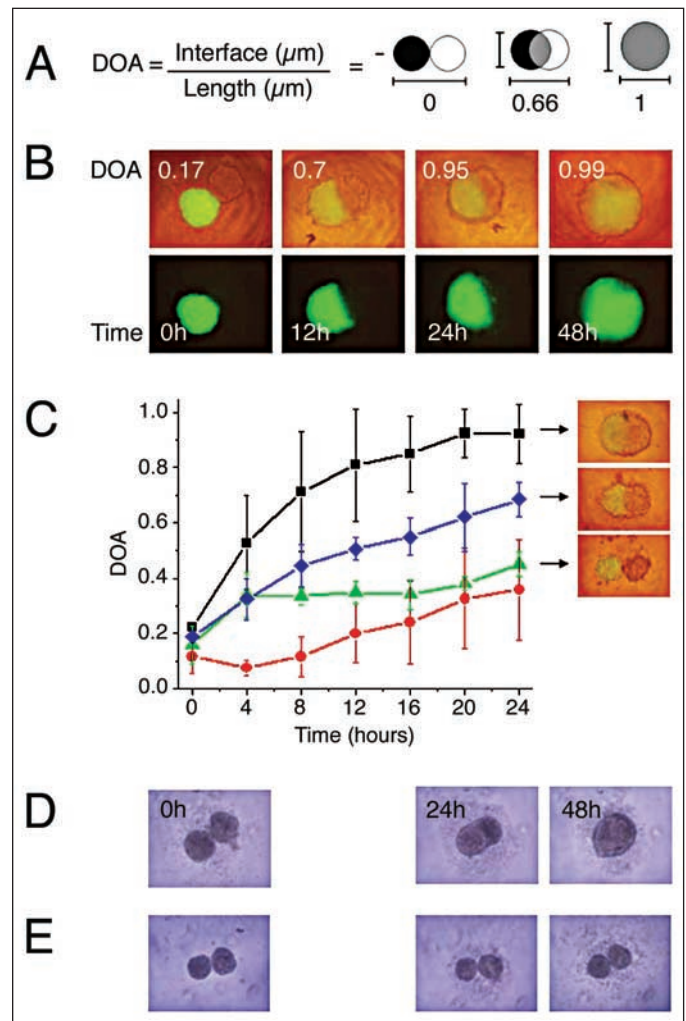


Figure 1. EB agglomeration is initiated by E-cadherin-mediated cell attachment. A) DOA was calculated by dividing the interface diameter by the overall length of the two-EB system. B) Untreated mouse EBs agglomerating over time with calculated DOA at each time interval. Photos taken at 200 \times magnification. C) Linear graph of DOA (y-axis) versus time (x-axis), $n = 5$. Compared with the control (\blacksquare), mouse EB agglomeration is impaired by treatment of EBs with α -mouse E-cadherin-blocking antibody (\blacklozenge) and with cytochalasin D (\blacktriangle). Day 6 EBs with downregulated E-cadherin expression (\blacklozenge) did not agglomerate. D) Untreated human EBs agglomerating over time. Photos taken at 100 \times magnification. E) Human EB agglomeration is inhibited by treatment with α -human E-cadherin-blocking antibody. Photos taken at 100 \times magnification.

Figure 2. A) Percent E-cadherin-expressing cells (y-axis) versus time (x-axis). Mouse EBs differentiated for 3 or fewer days express E-cadherin (>80% cells), correlating with EB agglomeration, $n = 3$. B) Flow cytometric histogram plots showing percentage of differentiating human ES cells (huESC and HuEB) expressing E-cadherin over time.

To determine whether this mechanism of EB agglomeration was relevant to the human EB system, we performed similar agglomeration studies with human EBs.

Untreated human EBs agglomerated more slowly than mouse EBs, requiring 48 hours for complete agglomeration (Fig. 1D). Similar to mouse EBs, agglomeration of human EBs was inhibited by treatment with α -human E-cadherin antibodies (Fig. 1E).

E-cadherin expression by ES cells is downregulated as the cells differentiate [19]. Using flow cytometry, E-cadherin expression was tracked in differentiating EBs over time. Expression of E-cadherin by mouse ES cells remained high (>80%) over the first 3 days of differentiation before being downregulated to approximately 25% by day 5 of differentiation (Fig. 2A). Human ES cells (huESC) also expressed E-cadherin that was downregulated as cells differentiated over time (Fig. 2B). As expected, E-cadherin expression correlated with the rate of EB agglomeration. Mouse EBs differentiated for three or fewer days agglomerated at the same rate as the control whereas day 6 mouse EBs did not agglomerate (Fig. 1C). These results were consistent with the observation that mouse ES cells placed directly into stirred culture aggregated into large cell clumps, whereas mouse EBs grown in static culture for a minimum of 4 days could be transferred to stirred culture with little agglomeration [11].

Control of Cell Aggregation by Encapsulation

Mouse ES cell aggregates (20 to 50 ES cells per aggregate) were individually encapsulated in agarose hydrogel capsules that permitted EB formation while physically preventing EB agglomeration. Capsule diameters between 100-150 μm were designed to encapsulate cells for the first 4 days of differentiation culture when E-cadherin expression remained high (Fig. 3A). EBs emerged from the capsules at an efficiency of $85\% \pm 10\%$. The encapsulation procedure was also readily adapted for human ES cell application. To ensure efficient human EB formation, larger ES cell aggregates (1,000-5,000 ES cells per aggregate) were required. These were encapsulated in 200-300 μm diameter capsules

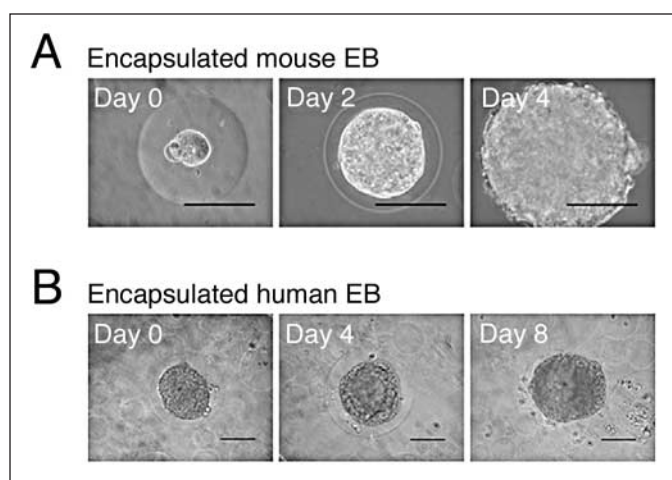
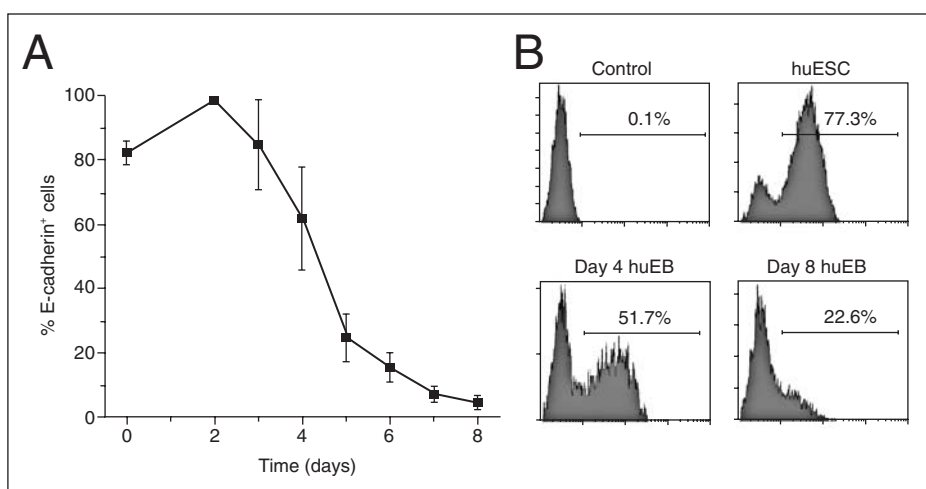


Figure 3. ES cell aggregates were encapsulated in agarose hydrogel microcapsules designed to contain developing EBs while E-cadherin expression remained high. A) Mouse EBs emerge from capsules after 4 days. B) Human EBs emerge from capsules after 8 days. Scale bars = 100 μm .

designed to retain EBs for the first 8 days of differentiation culture (Fig. 3B). Human EBs emerged from these capsules at an efficiency of $42\% \pm 15\%$.

We first tested the efficacy of the encapsulation approach for controlling EB agglomeration in high-cell-density (input 10^5 mouse ES cells/ml) static cultures. We compared changes in the number of cell aggregates over time between encapsulated and nonencapsulated cells. Non-encapsulated mouse ES cells began culture as individual cells that agglomerated, reducing the number of cell aggregates over time and resulting in varying aggregate sizes (Fig. 4A). In contrast, encapsulation maintained a consistent number of cell aggregates of more uniform size by preventing mouse EB agglomeration (Fig. 4B). After 4 days, encapsulated cultures contained 10 times the number of cell aggregates as non-encapsulated cultures. Having downregulated E-cadherin at

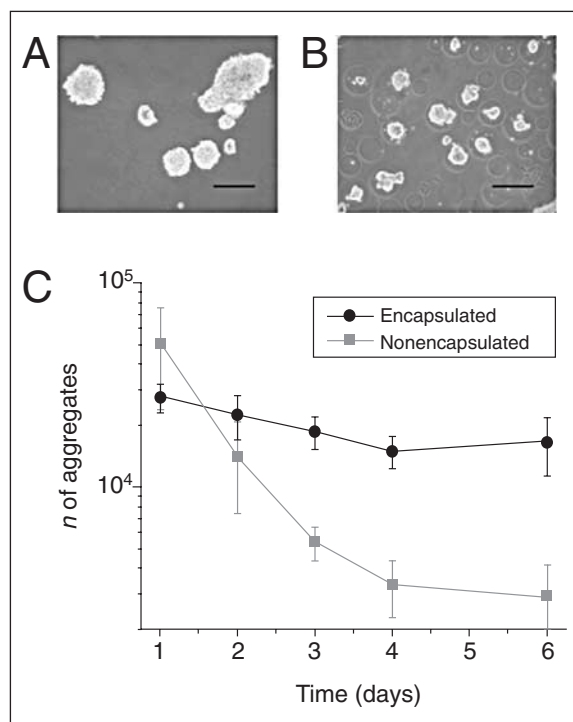


Figure 4. Encapsulation prevents EB agglomeration. A) Comparison of total cell aggregate number over time between encapsulated and nonencapsulated mouse ES cells in static culture initiated with 10^5 ES cells/ml, $n = 3$. B) Sample photographs of nonencapsulated and C) encapsulated mouse cell aggregates after 2 days of culture. Scale bars = 100 μ m.

this point, mouse EBs emerged from their capsules and did not aggregate (Fig. 4C). We had previously reported that cell agglomeration, beyond that required for mouse EB formation, negatively affects cell yield [11]. Consistent with this observation, cell expansion after four days of encapsulated culture was significantly higher (23 ± 3 -fold over input) than non-encapsulated culture (12 ± 3 -fold over input), $p = 0.007$.

We next evaluated cell growth and differentiation potential of encapsulated EBs in stirred culture. EB formation efficiency and cell growth of encapsulated mouse ES cells in stirred culture were similar to standard (non-encapsulated, static) liquid suspension cultures initiated at a typical cell density of 10^4 ES cells/ml (Table 1). This indicated that encapsulation and stirring-related shear stress (at 50 revolutions per minute

Table 1. Cell fold expansion in different culture systems

	Static control	Encapsulated stirred	Non-encapsulated stirred
Fold expansion	61 ± 11	61 ± 10	< 1

Cultures were initiated with 10^4 R1 ES cells/ml, and cell fold expansion was measured after 7 days, $n = 5$.

[rpm] using a glass ball stirrer) did not affect cell yield. Importantly, the differentiation capacity of EBs was also unaffected. The focus of this report involves the generation of hematopoietic progenitors, although other cell types such as cardiac and neural cells were similarly unaffected by encapsulated stirred culture (data not shown). Hematopoietic progenitor frequency was measured by flow cytometric analysis of CD34 and CD45 expression (Fig. 5) and by myeloid-erythroid colony-forming cell (CFC) assays. These phenotypic and functional assays demonstrated that neither the encapsulation process nor encapsulation combined with stirred culture significantly affected hematopoietic progenitor generation when compared with standard liquid suspension culture (Table 2).

Controlled ES Cell Differentiation Culture

Process control is most easily employed in stirred-suspension culture because point measurements accurately reflect bulk media conditions, and changes in culture conditions can be rapidly implemented. We used these capabilities to maintain differentiating mouse ES cells at different oxygen

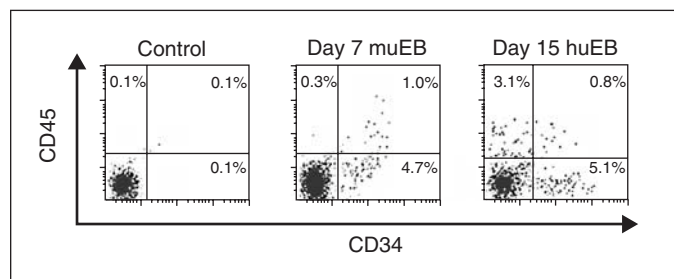


Figure 5. Representative flow cytometric analysis plots of cell surface marker CD34 (x-axis) and CD45 (y-axis) expression after 7 days of mouse ES cell (MuEB) differentiation culture and 15 days of human ES cell (HuEB) differentiation culture.

Table 2. Comparison of mouse hematopoietic progenitor frequency in various encapsulated (E) culture systems with standard liquid suspension culture (LSC). Flow cytometric analysis of CD34 and CD45 coexpression and myeloid-erythroid colony-forming cell (CFC) assay results are tabulated. Hypoxic and normoxic populations were compared by Student's t test, and the calculated p value is shown, $n = 5$.

Culture system	CD34 ⁺ CD45 ⁺	p value	CFCs	
			(per 10 ⁵ cells)	p value
LSC static normoxic	$1.1\% \pm 0.4\%$		14 ± 6	
E static normoxic	$1.0\% \pm 0.5\%$	0.85	10 ± 3	0.22
E stirred normoxic	$0.8\% \pm 0.2\%$	0.13	8 ± 2	0.09
E stirred hypoxic	$3.6\% \pm 2\%$	0.008	54 ± 21	0.0006

Neither the encapsulation process nor stirring significantly affected hematopoietic progenitor development (significance level $p = 0.05$). However, the ability to culture cells in controlled hypoxic conditions greatly improved hematopoietic progenitor frequency.

tensions. Dissolved oxygen and pH were measured online and maintained at normoxic 20% or hypoxic 4% oxygen tension (Fig. 6A) and pH 7.4.

Hematopoietic progenitor generation was compared between normoxic and hypoxic cultures after 7 days of differentiation. Hematopoietic progenitor frequency, measured by flow cytometric analysis of CD34 and CD45 expression and by myeloid-erythroid CFC assay, was significantly higher in hypoxic versus normoxic culture (Table 2). In terms of numerical CFC yield, $17,000 \pm 4,700$ CFCs were generated from an input of 5×10^5 ES cells per hypoxic bioreactor compared with $2,000 \pm 600$ CFCs per normoxic bioreactor (Fig. 6B). Increase in CFC frequency and yield was due to approximately equal proportional increases in lineage-restricted hematopoietic progenitors, although numerically, erythroid progenitors were responsible for most of the CFC increase under hypoxic conditions.

DISCUSSION

The ability to generate large numbers of ES-derived cells in controlled in vitro conditions is an important step toward their clinical application. Stirred-suspension bioreactors are ideal for this purpose because they can be used to generate scalable quantities of cells, facilitate process control strategies, and simplify the cell production process.

Controlling cell aggregation was necessary for EB formation and growth in stirred-suspension bioreactors. Strategies to prevent global cell aggregation, including addition of dextran sulfate, polyvinyl sulfate, and the use of high impeller rpm [20, 21] were unsuccessful because they prevented initial ES cell aggregation required to induce EB formation and/or disrupted EB architecture, thus impacting cell proliferation, viability, and differentiation (data not shown). Our investigation showed that blocking the function of E-cadherin could impair EB agglomeration. E-cadherin has been implicated in tissue organization and regulation of gene expression—both of which affect cell differentiation [17]. Use of blocking antibodies in culture would not only be economically impractical, but also may adversely affect cell differentiation.

We demonstrated that mass encapsulation of ES cells under defined conditions (i.e., cells per EB and capsule sizes) was a practical method for controlling cell aggregation in vitro. Our system has advantages over other EB formation systems [11, 22] because it allows for EB development in controlled stirred-suspension bioreactors. Mouse ES cells were used to study the process of EB agglomeration and to optimize our encapsulation system, which we then validated for the human ES cell system. Contrary to differences in the reported conditions for the

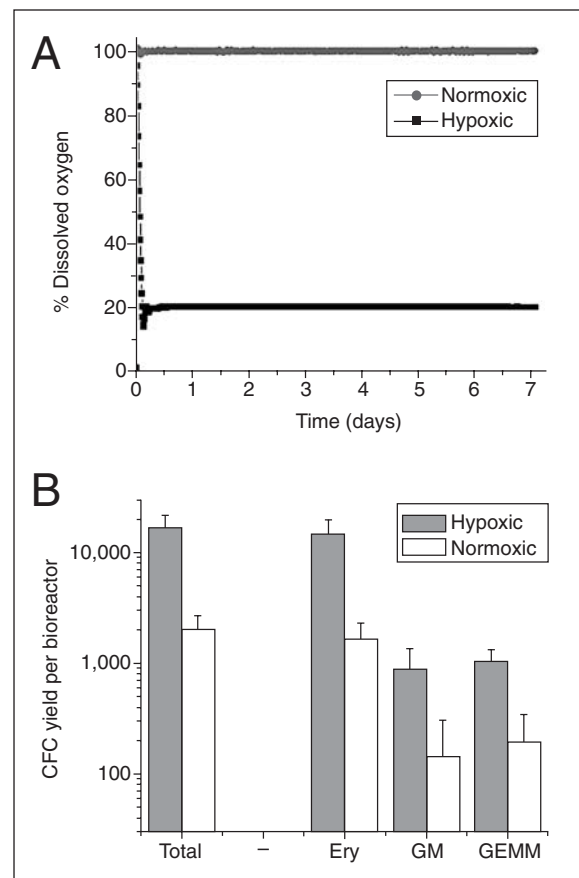


Figure 6. Generation of mouse hematopoietic progenitors in hypoxic and normoxic stirred-suspension cultures. A) Sample dissolved oxygen profiles in controlled stirred-suspension bioreactors. B) Yield of colony forming cells (CFCs) per bioreactor (initiated with 5×10^5 ES cells). Total CFC yield per bioreactor as well as lineage-specific yield of CFC erythrocyte (Ery), granulocyte-macrophage (GM), and granulocyte-erythrocyte-megakaryocyte-macrophage (GEMM) are shown, $n = 5$.

maintenance of undifferentiated ES cells, our results suggest that similar mechanisms may govern ES cell differentiation in mouse and human systems.

We determined that encapsulation of ES cells was necessary for efficient EB formation, cell proliferation, and differentiation in stirred-suspension bioreactors. This enabled, for the first time, the investigation of the controlled manipulation of exogenous factor influences on the differentiation of ES cells. Low-oxygen conditions have been reported to induce expression of various genes in differentiating ES cells, including vascular endothelial growth factor (VEGF) [23, 24] and glycolytic enzymes such as aldolase A [23] via a hypoxia inducible factor-1 (HIF-1)-mediated response [24]. Increased hematopoietic progenitor cell frequency was also reported [24]. We used online oxygen tension measurement and automated gas mix control to maintain cultures at either 4% or 20% oxygen tension. Unlike in

previous investigations, we were able to isolate the effects of oxygen tension (independent of other medium parameters including pH) by controlling pH at a set point value of 7.4, thereby preventing glycolytic acidification of the media. We confirmed that low-oxygen conditions significantly improved hematopoietic progenitor cell frequency and verified that numerical yield of these cells was also improved. The ability to accurately measure and control culture conditions in stirred-suspension bioreactors will be a valuable tool for understanding and optimizing delivery profiles of exogenous factors that affect ES cell differentiation. For example, we have determined that providing hypoxic conditions between days 3 and 7 of mouse ES cell differentiation is critical for the increase in hematopoietic progenitor yield (data not shown). Additionally, the ability to culture encapsulated ES cells at high density may facilitate identification and quantification of secreted factors (in addition to VEGF) involved in hypoxic expansion of hematopoietic progenitors.

We demonstrated the utility of our cell production approach by generating clinically relevant numbers of hematopoietic progenitors that may, at the very least, be useful for rapid short-term engraftment of host animals [25]. This production process is readily compatible with the cell selection strategy described by *Klug et al.* (1996) [12] that confers cell-lineage-specific antibiotic resistance. We are currently developing an integrated production and purification process to generate pure populations of cardiac cells using ES cells transfected with a selection plasmid pgk-hygro-myosin heavy chain-neo [26].

Together, these examples and the results presented herein highlight the importance of stirred-suspension culture for optimizing and controlling physicochemical factors that influence cell growth and differentiation. Our results also demonstrate production of scalable quantities of therapeutically useful cell types. In addition, cell encapsulation may prove useful by providing a scaffold onto which cytokines or extracellular matrix proteins can be attached. Delivery of bioactive molecules in this highly localized manner may allow for the provision of developmentally relevant gradients of molecules in scalable culture and will certainly provide a cost-effective alternative to maintaining the entire bulk media at a specific concentration. Ultimately, encapsulated stirred culture may provide the leverage to control most exogenous factors that affect ES cell growth and differentiation including cell-cell interactions, physicochemical factors, and cytokine delivery.

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