Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation.

Short title: hematopoietic differentiation of human ES cells

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In order to realize the therapeutic potential of human embryonic stem cells (hESCs), it will be necessary to regulate their differentiation in a uniform and reproducible manner. We have developed a method in which known numbers of hESCs in serum free medium were aggregated by centrifugation to foster the formation of embryoid bodies (EBs) of uniform size ('spin EBs'). These spin EBs differentiated efficiently and synchronously, as evidenced by the sequential expression of molecular markers representing stem cells, primitive streak and mesoderm. In the presence of hematopoietic growth factors, reproducible differentiation was achieved with blood cells formed in over 90% of EBs. Using chimeric EBs generated from mixtures of GFP+ and GFP- hESCs in a clonogenic assay, the hematopoietic precursor frequency was estimated to be approximately 1:500 input cells. This method of EB formation provides a generally applicable means for modulating and objectively monitoring the directed differentiation of hESCs.

Introduction

Embryoid body differentiation of murine embryonic stem cells (mESCs) recapitulates many aspects of early mouse embryogenesis. Synchronous differentiation is typically achieved by forming mEBs from mESCs seeded at low cell densities. Because hESCs survive poorly as single cells, human EB formation has generally been initiated from enzymatically digested colony pieces of different sizes or from high density suspension cultures. Co-cultivation of hESCs with stromal layers such as OP9 and S17 has successfully induced hematopoietic differentiation, but these systems are compromised by cell-associated and secreted components derived from the feeder layer and by potential interactions between added factors and the stromal layers. The weaknesses inherent in the existing protocols highlight the need for a robust stromal- and serum-free culture system for the differentiation of hESCs.
Study design

Culture and differentiation of hES cells. Human ES cells (hES2, 3, and 4)\textsuperscript{13} and the GFP-expressing hES3 derivative, ENVY\textsuperscript{14}, were cultured essentially as described.\textsuperscript{5} A detailed protocol for hESC differentiation is provided in Supplementary methods. In brief, hESCs were trypsinized into a single cell suspension, washed in phosphate buffered saline and resuspended in serum free medium (SFM).\textsuperscript{15} EB formation was induced by seeding the desired number (300-10,000) of hESCs in 100 µl of SFM supplemented with growth factors in each well of 96-well round bottom, low attachment plates (NUNC, Roskilde) and centrifuging the plates at 1500 rpm (478 g) for 4 mins at 4°C to aggregate the cells. After 10-12d, the EBs were transferred to 96-well flat-bottomed tissue culture plates precoated with gelatin, in SFM supplemented with growth factors and allowed to differentiate further.

Results and discussion

In our initial attempts to direct hematopoietic differentiation of hESCs, hEBs were formed from pieces of undifferentiated colonies in the same SFM that we had employed to induce mesendoderm from mESCs.\textsuperscript{4} However, only the subset of hEBs that developed from larger colony fragments (~500-1000 cells) regularly formed blood cells, suggesting that a minimum number of hESCs was required to generate hEBs that differentiated into mesodermal lineages.

Since it was not possible to reproducibly disaggregate hESC colonies into pieces of exactly the desired size, we attempted to improve the efficiency of hematopoietic differentiation by depositing known numbers of hESCs in round bottomed, low adherence 96-well plates and then aggregating the cells into EBs by centrifugation (Figure 1A). After a period of 8-12d, these 'spin EBs' were transferred to tissue culture treated plates and differentiated further. The wells were scored for the
presence of developing myeloid and hemoglobinised erythroid cells by light microscopy. Visual cell lineages assignments were confirmed by correlating the morphological appearance of cells in the wells with May-Grünwald-Giemsa stained cytocentrifuge preparations, flow cytometry, immunocytochemistry and gene expression analysis in selected cases (see Supplementary Figures 1-3 and Figure 1).

The differentiation process was affected by several parameters. If the hESCs were aliquoted into flat bottomed wells, they failed to form large aggregates even following centrifugation of the cells (Figure 1B) and both survival and differentiation were unpredictable. Conversely, deposition of hESCs into low attachment round bottomed wells followed by brief centrifugation encouraged cellular association into a close pellet (Figure 1C) from which uniform EBs reproducibly formed and differentiated (see examples in Figure 2). Hematopoietic differentiation was also influenced by the number of cells seeded into each well. In the representative experiment shown in Figure 1D, efficient blood formation required in excess of 500 hESCs per well and optimum erythropoiesis was observed when 1000 cells were seeded per well. In 5 experiments in which 3000 hESCs were deposited per well, EBs developed in 1112/1164 (95.5%) wells, with an average of 95.8±1.6% of wells containing viable EBs and 91.8±7.8% of wells containing blood cells per experiment.

Gene expression analysis of hEBs revealed sequential expression of differentiation-stage related genes, similar to the pattern of gene expression observed in cultures of mouse EBs (Figure 1E). During hESC differentiation, a gradual attenuation of the expression of the stem cell gene OCT4 was observed. The primitive streak genes MIXL1 and BRACHYURY were strongly expressed from d4-8 indicating a wave of mesendodermal induction. VEGF receptor 2 (FLK1/KDR) expression increased and remained at high levels throughout the differentiation period consistent with the ventral patterning of mesoderm in these cultures, followed at d10 and d20 by peak expression of the
hematopoietic and endothelial gene surface marker CD34 and the hematopoietic transcription factor RUNX1, respectively.\textsuperscript{17,19}

Most EBs generated by this protocol generated large numbers of hemoglobinised and/or non-hemoglobinised blood cells (Figure 1F, G). May-Grünwald-Giemsa stained cytospins showed neutrophils, macrophages and mast cells in wells containing myeloid cells (Figure 1H) and maturing erythroid cells in wells containing overtly hemoglobinised cells (Figure 1I). Although most erythroid cells were nucleated, consistent with a primitive, yolk-sac lineage, occasional cells undergoing enucleation were observed, suggesting the presence of definitive erythropoiesis (Figure 1I, inset). Flow cytometry of EBs disaggregated at d11 showed a high proportion of CD34\textsuperscript{+} and/or CD38\textsuperscript{+} cells (Figure 1J), confirming the efficiency of differentiation. Analysis at d26 showed that the cultures supported the expansion of hematopoietic mesoderm, because most cells expressed either CD45 or Tie-2, marking hematopoietic or endothelial lineages respectively (Figure 1K).

The reproducibility of the spin EB method was exploited to estimate the frequency of hematopoietic precursors arising from a defined number of hESCs. EBs were formed from 3000 hESCs comprising variable combinations of wild type hES3 cells and a GFP-expressing derivative, denoted ENVY\textsuperscript{14}. Aggregation of GFP\textsuperscript{+} and GFP\textsuperscript{−} hESCs led to the formation of chimeric EBs into which GFP\textsuperscript{+} cells were integrated in proportion to their input number (Figure 2A). Analysis of these data demonstrated an excellent correlation ($R^2 > 0.99$ for the 3 experiments shown in Figure 2B) between the number of GFP\textsuperscript{+} input cells and the percentage of EBs forming GFP\textsuperscript{+} blood cells. The very high correlation coefficient is a testament to the reproducible differentiation observed over many hundreds of wells. Using Poisson statistics, the frequency of hematopoietic precursors was estimated at approximately 1 in 500 input cells.
In preliminary experiments examining the development of methylcellulose colony forming cells (CFCs), we observed CFCs from d6 spin EBs giving rise to primitive erythroid and macrophage colonies at a frequency of 92 per $10^4$ cells and d10 CFC at a frequency of 23 per $10^4$ cells. Taken together, the high frequencies of d11 CD34$^+$ cells and hematopoietic precursors observed in our system compared favourably with the results reported using conventional EB or stromal cocultivation methods for hESC differentiation $6^{-8},10,12$ and were superior to the precursor frequencies reported by others for human EBs differentiated in serum free cultures. $7$ At a molecular level, the spin EBs passed through a transient in vitro gastrulation stage that antedated the expression of mesodermal genes and the emergence of hematopoietic cells, similar to differentiating mESCs. $4,16^{-18}$ The ability to faithfully reproduce early human embryonic differentiation in vitro provides a powerful tool for the molecular and cellular dissection of this previously inaccessible phase.

We believe that modulating medium composition, extracellular matrix substrate and hESC cell numbers will enable spin EBs to be readily adapted to the generation of many cell types. In addition, the ability to form chimeric EBs with marked cells, such as ENVY, will permit an estimation of clonogenic frequency in cell types for which simple precursor assays are not available.
References

Figure Legends

Figure 1. Differentiation of defined numbers of hESCs as spin EBs. (A) Schematic diagram of the spin EB method for hESC differentiation. (B,C) Immunofluorescent images of 3,000 ENVY hESCs deposited in (B) flat and (C) round bottomed wells immediately following centrifugation. Aggregation of ES cells was only induced in the round bottomed wells. Original magnification x 50. (D) The percentage of wells containing myeloid or erythroid cells graphed as a function of the input number of cells in each well. 72 wells were assayed at each point. (E) Sequential expression of differentiation-stage related genes in EBs harvested after the indicated number of days of differentiation. (F-K) Hematopoietic cells derived from spin EBs. (F,G) Brightfield microscopy of EBs differentiated for 11d in suspension culture and then plated down for a further 14d, revealing large numbers of (F) non-hemoglobinised and/or (G) hemoglobinised blood cells. Original magnification x 100. (H,I) May-Grünwald-Giemsa stained cytocentrifuge preparations after 28d of differentiation showing neutrophils (n), macrophages (m) and mast cells (mc) in wells containing (H) myeloid cells and large numbers of maturing erythroid cells (ery) in wells containing (I) overtly hemoglobinised cells. Some cells with condensed chromatin apparently undergoing enucleation were observed (inset). Original magnification x 400. (J,K) Flow cytometry of EBs dissociated (J) at d11 and stained for expression of CD34 and CD38 and (K) at d26 and stained for expression of Tie-2 and CD45. The percentages of cells within the indicated quadrants or regions are shown.

Figure 2. Formation of hES3/ENVY chimeric EBs enables the frequency of hematopoietic precursors to be estimated. (A) Brightfield (BF) and fluorescence (GFP) images of d7 chimeric EBs formed from a total of 3000 hESCs comprising the indicated ratios of GFP⁻ hES3 and GFP⁺ ENVY cells. Further differentiation of the EBs led to the formation of hematopoietic cells in each well which were all GFP⁻ (left panels), all GFP⁺ (right panels) or included a mixture of GFP⁻ and GFP⁺ cells (middle panels), depending on the proportion of ENVY cells contributing to the EB. (B)
Plotting the frequency of wells with no GFP⁺ cells against the ENVY input cell number/well demonstrated an excellent correlation ($R^2 > 0.99$). Results shown are the mean of 3 experiments with error bars representing the s.d. The clonogenic frequency of hematopoietic precursors (1:523) was estimated by determining the ENVY input cell number that resulted in 37% of the wells containing only GFP⁻ blood cells.
Ng et al Figure 1

**A**
- Dissociate cord blood
- 300-5000 cells per well
- Spin
- Aggregate
- 8-12 d culture
- Transfer to TC plate
- Analysis d15-30

**B**
- Images of green and red fluorescence

**C**
- Images of green fluorescence

**D**
- Graph showing % wells with blood cells vs. input cell number/well

**E**
- Gel electrophoresis showing
  - OCT4
  - MIXL1
  - BRACHY
  - FLK1
  - CD34
  - RUNX1
  - β ACTIN

**F**
- Image of tissue

**G**
- Image of brown tissue

**H**
- Images showing m and n

**I**
- Images showing ery and n

**J**
- Flow cytometry showing CD38 and CD45
  - Control: 8, 20, 29
  - Treatment: 2, 3, 1.3

**K**
- Flow cytometry showing CD38 and CD45
  - Control: 2, 3, 2
  - Treatment: 2, 3, 1.3
Ng et al Figure 2

A

3000 hES3/ENVY cells in 96 well plates

EB formation

proportion of hES3:ENVY

hematopoietic differentiation

BF

GFP

100:0 90:10 80:20 67:33 50:50 30:70 0:100

B

ENVY input cell number/well

y = 100e^{0.0516x}

R^2 = 0.9989

% wells with no GFP+ve blood cells

% cells with GFP+ve blood cells

0 100 200 300 400 500 600 700 800 900 1000 1100 1200
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