Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development

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Abstract

To date, hematopoietic development of human embryonic stem cells (hESCs) has been limited to cell lines cultured in the presence of either mouse embryonic fibroblasts (MEF) or MEF-conditioned media (MEF-CM). Anonymous xeno-factors from MEFs or MEF-CM complicate studies of hESC self-renewal, and also raise concerns for the potential clinical applications of generating primitive hematopoietic cells from hESC lines maintained under these ambiguous conditions. Here, we demonstrate that hESCs can be cultured over 30 passages in defined conditions in the absence of MEF or MEF-CM using only serum replacement (SR) media and high concentrations of bFGF (SR-bFGF). Similar to hESCs cultured in MEF-CM, hESCs cultured in SR-bFGF sustained characteristics of undifferentiated hESCs, proliferative potential, normal karyotype, in vitro and in vivo three germ layer specification, and gave rise to hemogenic-endothelial precursors required for subsequent primitive hematopoietic development. Our report demonstrates that anonymous factors produced by feeder cells are not necessary for hESC maintenance and subsequent hematopoietic specification, thereby providing a defined system for studies of hESC self renewal and hESC-derived hematopoiesis.
Introduction

Human ESC lines have been derived from the inner cell mass of human embryos by co-culture on either mouse or human embryonic feeder layers\textsuperscript{1,2}. To better define the conditions to propagate and differentiate hESC lines, Xu et al maintained hESCs in feeder-free conditions by feeding them with mouse embryonic fibroblasts (MEF)-conditioned media (MEF-CM)\textsuperscript{3}. However, significant variations in the production of MEF and harvesting of MEF-CM have hampered reproducibility of these conditions to culture hESC stably\textsuperscript{4}. Moreover, several unknown substances and combinations of factors contained in MEF-CM\textsuperscript{5} further complicate fundamental studies of hESC self-renewal, impact future studies in defining factors that specify lineage maturation, and also become a concern for future clinical applications. Although hESCs have been documented to be capable of hematopoietic specification, maintenance of these hESCs required either MEF or MEF-CM\textsuperscript{6-14}. In this report, we demonstrate that anonymous factors produced by feeder cells are not necessary for hESC maintenance and subsequent hematopoietic development, thereby providing a more defined system for further characterization of hESC self-renewal and lineage maturation.
Materials and Methods

hESC culture and hEB formation. The hESC lines H9 and H11 cultured in MEF-CM were passed into either MEF-CM or serum replacement (SR) media containing high concentration of bFGF (SR-bFGF). MEF-CM was collected as previously described and contained 12ng/mL of basic fibroblast growth factor (bFGF). SR-bFGF media consisted of 80% Knockout Dulbecco’s modified Eagle medium (KO-DMEM, Gibco, Burlington, Canada), 20% serum replacement (SR), 24-36ng/mL bFGF (variants among batches), 1% nonessential amino acids, 1mM L-glutamine (all from Gibco, Burlington, Canada), and 0.1mM β-mercaptoethanol (Sigma, Oakville, Canada). Maintenance of hESC lines was performed as previously described. In brief, hESC colonies were dissociated with collagenase IV (Gibco, Burlington, Canada) for 5 min and passed every 6-7 days. Occasionally, hESC colonies maintained either in MEF-CM or SR-bFGF was physically dissociated during passaging in order to reduce the numbers of fibroblast-like cells. The effect of bFGF on maintenance of hESC was determined by use of bFGF neutralization antibody (Abcam Inc. Cambridge, MA). Formation of human embryoid bodies (hEBs), and preparation of single cells from hESCs and hEBs were performed as previously described.

Immunocytochemical and histochemical staining of Oct4 and alkaline phosphatase (AP). Cultured hESCs were fixed in the culture plates with 10% buffered formalin. Permeabilized hESCs were incubated for 30 min with 3 μg/ml either primary goat anti-Oct4 antibody (Santa Cruz, CA) or normal goat IgG isotype (Sigma, Saint Louis, Missouri) or antibody diluent alone, and visualized with Alexa Fluor 488 conjugated donkey anti-goat IgG secondary antibody (1:400, Molecular Probes, Eugene, OR). AP activity staining was then performed as previously described with “Vector Red” substrate (Vector Laboratories, Burlingame, CA), and followed by DAPI counterstaining. The cells were examined with an Olympus fluorescent microscope.

Assessment of undifferentiated markers by flow cytometry. Expression of Oct4, Tra-1-60, Tra-1-81 and SSEA-4 was considered as undifferentiated indicators of hESCs and analyzed by flow cytometry. For intracellular Oct4 assay, 100μl of dissociated hESCs (1x10^5 cells) were fixed for 15 min by addition of 100μl fixation solution (Caltag Laboratories, CA). After washing with 1mL of PBS and centrifugation at 1,800g for 3 min, the cells were treated with permeabilization solution for 5 min, and then stained with 1μl of primary mouse anti-Oct4 antibody or normal mouse IgG isotype (BD, San Jose, CA) for 20 min. The cells were washed once prior staining with FITC-conjugated goat anti-mouse IgG (Sigma, Oakville, Canada) for 20 min, and washed twice prior to flow cytometry.
acquisition. For detection of Tra-1-60 and Tra-1-81, primary Tra-1-60 mAb (3.3 µL), or Tra-1-81 mAb (2.6 µL) (both from Chemicon, Temecula, CA) was pre-incubated for 30 min at 4°C with 2.5 µL of FITC-conjugated goat anti–mouse IgG antibody (Immunotech, Marseille, France) in 100 µL of PBS containing 3% FBS. Dissociated hESCs were suspended in PBS containing 3% FBS at a concentration of 2-5X10^4 cells/100 µL and added to the pre-incubated Tra-1-60 or Tra-1-81. After 30 min at 4°C, the cells were washed twice and re-suspended in PBS containing 3% FBS, and finally stained with 7-aminoactinomycin D (7-AAD) viability dye (Immunotech) at 4 µL/200 µL for 15 min at room temperature. Live cells identified by 7-AAD exclusion were analyzed for surface marker expression using FACSCalibur (BDIS) and Cell Quest software (BDIS). Cell surface expression of SSEA-4 was determined as previously described.3,7.

Cell proliferation and karyotype of hESCs. Cell proliferation was determined as previously described3,7. London Health Sciences Centre, Cytogenetics Laboratory, London, Ontario, Canada performed karyotype analysis.

Characterization of hESC-derived hemogenic precursors and hematopoietic cells. CD45negPFV (CD45 negative, PECAM-1+, Flk1+ and VE-Cadherin+) hemogenic precursors and CD45+ hematopoietic cells derived from hESCs were characterized as previously described 9.

Colony-forming unit (CFU) assays and Wright-Giemsa staining. Human clonogenic progenitor assays were performed by plating hESC derivatives (1x10^4 cells) into methylcellulose H4230 (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with the following human recombinant growth factors: 50 ng/mL stem cell factor (Amgen Inc., Thousand Oaks, CA), 3 U/mL erythropoietin (Amgen Inc.), 10 ng/mL granulocyte monocyte colony-stimulating factor (Novartis, Dorval, QC, Canada), and 10 ng/mL IL-3 (R&D, Minneapolis, MN). After incubation for 10 to 14 days at 37°C and 5% CO2 in a humidified atmosphere, colony counts were performed based on standard morphological characteristics. Isolation of the cells from individual colonies, cytospin preparation and Wright-Giemsa staining were performed as previously described 7.

Quantitative Real Time PCR (Q-PCR). Total RNA was extracted and reverse-transcribed as described previously 8. An initial regular PCR was performed for each gene of interest (GOI) to optimize the conditions and check the amplicon size. The conditions for Q-PCR reactions were reported elsewhere 16. Forward and reverse primer sequences were: 5’-gtggctccaggatgttagga-3’ and 5’-gcctgagttcatgttgctga-3’ for HNF3α; 5’-ccggcagaagattgtagagc-3’ and 5’-cgttggacacgtttgattg-3’ for PAX6; 5’-tgccccactgtgctagc-3’ and 5’-ggcatggactgtgcatgag-3’ for glyceraldehyde-3-phosphate
dehydrogenase (GAPDH). The amount of GOI was normalized to the endogenous housekeeping gene GAPDH.

**Teratoma formation and histological analysis.** The University Western Ontario Council On Animal Care approved animal protocols. Male nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (5 to 7-week old) were anaesthetized by intraperitoneal injection of 2.5% Avertin (0.14-0.18 ml/g of body weight). Small (1 cm) sagittal skin and body wall incisions were made slightly above the penis on the side of the abdominal midline with fine scissors after removing hairs. The testis was positioned fully outside the body on sterile gauze. Fifteen to twenty clumps prepared from SR-bFGF-maintained hESC colonies (each clump containing approximately 200-500 cells) were suspended in 20µl of KO-DMEM, and injected into one testis capsule of each mouse using 0.5ml syringe with a 30G short needle. Incisions were closed with 1-2 stitches. All operations were performed in sterile conditions. After surgical operations, each mouse was subcutaneously administered with 0.5-1.0 ml of Buprenorphine (0.05-0.1 mg/Kg of body weight) and 1 ml of 0.9% saline. Testicular lesions were palpable from about four weeks after inoculation. Mice were sacrificed at week 6-12. Tumors were embedded in frozen tissue embedding gel (Fisher), snap-frozen in liquid nitrogen and stored at −80°C. Cryostat sections (5µm) were examined histologically after hematoxylin and eosin staining.

**Statistical analysis.** Results were expressed as means ± SEM. Statistical significance was determined using an unpaired Student t test. Results were considered significant when P<0.05.
Results

SR-bFGF is capable of maintaining undifferentiated hESCs

To determine whether established hESCs could be grown in the absence of factors produced by feeders, we passed the hESC line H9 previously cultured in MEF-CM for 35 passages into either MEF-CM or defined SR-bFGF media conditions that was never directly or indirectly in contact with MEFs. In side-by-side comparisons with the same passage of hESCs cultured in MEF-CM, hESC cultured in SR-bFGF possessed similar properties. Using hESCs maintained in MEF-CM as our standard, undifferentiated colonies cultured in SR-bFGF had similar morphology, activity of alkaline phosphatase (AP) (Fig 1a) and expression of Oct4 (Fig 1b). Flow cytometry analysis showed that SR-bFGF-maintained hESCs had comparable levels of Oct4, SSEA4, Tra-1-60 and Tra-1-81 expression (Fig. 1c). The hESCs cultured in SR-bFGF also showed identical proliferation capacity to those cultured in MEF-CM (Fig. 1d) and normal karyotype (Fig. 1e). To assure the support of hESC growth using defined SR-FGF conditions was not restricted to one cell line alone, another hESC line, H1, was also maintained in an undifferentiated state in SR-bFGF over 8 passages (Fig. 2a and c).

To examine which components of SR-bFGF conditions were crucial to hESC maintenance in an undifferentiated state, we cultured hESCs in: a) media without SR but supplemented with bovine serum albumin, insulin, transferrin and 36ng/mL bFGF, b) media supplemented with SR in the absence of bFGF or c) lower concentrations of bFGF (<12ng/mL), and finally in d) SR-bFGF media without pre-coating of the culture plates with matrigel or laminin (the major component in matrigel). None of the conditions (a-d) supported hESCs with undifferentiated properties past day 21 (3 passages) of culture (data not shown). Under conditions a) to c), hESCs gradually lost their typical colony morphology and diminished expression of undifferentiated markers. Under condition d), hESC colonies were unable to attach to the plate, resulting in cell death after one passage (7 days). The role of bFGF in hESC maintenance was further examined by use of a neutralizing antibody specific to bFGF. Addition of the bFGF-neutralizing antibody to the MEF-CM media induced both H1 and H9 hESC differentiation and loss of hESC colony maintenance (Fig. 2e and f), suggesting that bFGF is an essential factor released by MEFs to support hESC lines. Collectively, these experiments indicate that the combination of SR media, high concentrations of bFGF, and matrigel or laminin are crucial for the maintenance of hESCs. In addition, these comparative analyses underscore the importance of long-term culture to define robust conditions for hESCs maintenance since hESCs seemingly possess autonomous survival responsiveness over limited (4-7 days) short-term culture.
Human ESCs cultured in SR-bFGF are capable of characteristic hematopoietic development

The ability to form embryoid bodies (EBs), and subsequently undergo differentiation, is an important measure of the overall quality of hESC lines\(^7,8\). To determine whether SR-bFGF-maintained hESCs are capable of EB development and differentiation as previously documented for hESCs cultured in MEF-CM \(^7,8\), hESCs were allowed to aggregate into EBs \(^7\). Compared with EBs formed from MEF-CM-cultured hESCs, SR-bFGF-cultured hESCs produced similar number and size of EBs (Fig. 3a).

To further examine whether SR-bFGF-cultured hESCs are capable of hematopoietic lineage specification, EBs were treated with a combination of hematopoietic cytokines (SCF, Flt3 Ligand, IL-3, IL-6, G-CSF) and the ventral mesoderm inducer bone morphogenetic protein-4 (BMP-4) to specify hematopoietic lineage differentiation\(^7,8\). SR-bFGF-cultured hESCs followed a similar spatial and temporal hematopoietic developmental pattern to MEF-CM cultured hESCs. Similar numbers of CD45\(^{-}\)PFV hemogenic precursors \(^9\) were derived after 10 days of EB development from either MEF-CM or SR-bFGF-cultured hESCs in the presence of hematopoietic cytokines and BMP-4 (Fig 3b). Development of hEBs for 22 days from either MEF-CM or SR-bFGF-cultured hESCs gave rise up to 74% of hematopoietic cells expressing pan-leukocyte marker CD45\(^+\) (Fig. 3c), with similar primitive CD34\(^+\) hematopoietic cell composition (49732\(\pm\)15354 vs. 37648\(\pm\)26625, Fig. 3c). Consistent with our previous report\(^7\), hematopoietic cells generated from SR-bFGF-cultured hESCs contained similar hematopoietic progenitor capacity determined by the hematopoietic colony forming unit (CFU) assay\(^18\) (Fig. 3d). Hematopoietic colony types included erythroid, macrophage, granulocyte and granulocyte-macrophage progenitors (Fig.4a-d). Wright Giemsa staining of cytospin preparations of cell isolated from individual erythroid colonies showed mature enucleated erythrocytes and immature erythrocytes (Fig. 4e), whereas cells from monocyte progenitors showed monocytic morphology (Fig. 4f), and mature neutrophils and eosinophils from granulocytic progenitors (Fig. 4g). Further analysis by flow cytometry of cells from individual erythroid colonies demonstrated expression of glycophorin A, and absence of CD45, whereas myelomonocytic markers CD33 and CD13 were absent (Fig. 4h,i). In contrast, the cells comprising granulocyte colonies were stained negative for glycophorin A, but positive for CD45, myeloid markers CD33 and CD13 (Fig. 4j,k).
**SR-bFGF-cultured hESCs maintain potential to generate derivatives of three germ layers**

In addition to hematopoietic specification (representing mesoderm development), SR-bFGF culture conditions sustained hESC capacity to undergo ectoderm and endoderm lineage differentiation. Using quantitative real-time PCR, expression of germ layer specific genes were detected from differentiated EBs generated from hESCs cultured in either MEF-CM or SR-bFGF. Human EBs generated from either MEF-CM or SR-bFGF cultured hESC lines were capable of differentiating into ectoderm and endoderm, as demonstrated by similar levels of Pax-6 (ectoderm) and HNF3α (endoderm) expression (Fig. 5a, b).

To further confirm the potential to form derivatives of all three embryonic germ layers and pluripotent differentiation potential of hESCs cultured in SR-bFGF in vivo, we injected SR-bFGF-maintained hESCs into the testis capsules of NOD/SCID mice. All mice receiving hESCs cultured in SR-bFGF produced teratomas (n=5, independent experiments). Testicular growths were palpable four weeks post injection. There was no gross evidence of metastases to other organs or other sites within the peritoneal cavity (not shown). The cut surfaces of the tumors displayed mucinous or serous cysts and solid components. Microscopically, all tumors contained tissues representing all three embryonic germ layers, including ectoderm (cystic epithelium, neural rosettes, and squamous epithelium), mesoderm (bone, muscle, and adipocytes (not shown)), and endoderm (glandular epithelium) (Fig. 5c-h). These results demonstrate that SR-bFGF-cultured hESCs can be applied not only for studies of hematopoietic specification, but also towards fundamental studies of hESC pluripotency in vivo.

**Discussion**

We report that high concentrations of bFGF in serum free media are sufficient in sustaining the replicative and differentiative potential of hESCs in the absence of MEFs or MEF-CM. Based on our observations, we suggest that bFGF most likely represents a signaling pathway similar to LIF for murine ESCs. It will be critical to evaluate the specific downstream targets of bFGF in hESCs cultured in these defined conditions, and compare them to potential common targets represented by murine ESCs cultured in the media with serum and LIF alone or in serum-free media with LIF and BMP-4.

Currently, hESCs have been found to be capable of incorporating the non-human sialic acid \(N\)-glycolylneuraminic acid (Neu5Gc). Animal sources of Neu5Gc might cause potential
immunogenic reaction with human complement\textsuperscript{20}. Although Neu5Gc can be eliminated by replacement with human orthologs or neutralized by pooled human sera, culture of hESCs on MEF feeder layers prevented complete elimination of Neu5Gc\textsuperscript{20}. These results further emphasize the importance of using less complex culture conditions with defined chemical components. Thus, SR-bFGF provides a foundation for further characterizing self-renewal of the established hESC lines and deriving new hESCs under these defined conditions that are free of potential xeno- or human allogenic containments by replacing animal sources of growth factors or reagents with human orthologs or recombinant proteins.
References:


Figure Legends

Figure 1. SR-bFGF maintain hESCs in an undifferentiated state. Human ESCs (H9 cell line) were cultured over 14 passages in either MEFCM (left column), or in SR-bFGF (right column). (a and b) Double alkaline phosphatase (AP) and Oct4 immunostaining. In the undifferentiated area of the colonies, nuclei were co-stained for Oct4 (green) and DAPI (blue), resulting in a merged color (aqua). The AP (red) was detected in the cytoplasm regions. Bar = 200 µm in a, Bar = 20 µm in b. (c) Expression of intracellular Oct4, and cell surface markers SSEA-4, Tra-1-60 and Tra-1-81 was analyzed by flow cytometry. (d) Proliferation capacity. (e) Karyotype (21 passage in SR-bFGF culture).

Figure 2. Maintenance of hESC lines H1 and H9 in SR-bFGF conditions and differentiation in response to neutralization of bFGF. H1 (a, c, e) and H9 (b, d, f) hESC lines were cultured in MEF-CM (a and b), SR-bFGF media (c and d), or in the presence of bFGF neutralizing antibody (e and f). H1 hESC line was maintained in SR-bFGF media for more than 6 passages to date (c) and H9 hESC lines for more than 31 passages to date (d). Addition of bFGF neutralization antibody to hESC lines cultured in MEF-CM induced differentiation and loss of colony integrity. Scale Bars=200µm.

Figure 3. SR-bFGF-maintained hESCs undertake hematopoietic development. In comparison to MEF-CM-cultured hESCs (left column), SR-bFGF-cultured hESCs (right column) displayed the similar following properties after differentiation: (a) formation of hEBs (bar = 100 µm), (b) generation of CD45negPFV hemogenic precursors at day 10 of hEB development⁹, (c) expression of the pan-leukocyte hematopoietic marker CD45 and primitive hematopoietic marker CD34 at day 22 of hEB development, (d) generation of hematopoietic progenitors determined by clonogenic colony forming unit assay at day 22 of hEB development.

Figure 4. Hematopoietic cells derived from SR-bFGF-maintained hESCs have characteristic hematopoietic progenitor properties. Hematopoietic cells derived from SR-bFGF-maintained hESCs were identical to those derived from MEF-CM-maintained hESCs, and produced characteristic hematopoietic CFU subtypes, including erythroid (a), macrophage (b), granulocyte (c), and granulocyte-macrophage colonies (d), scale bars = 100 µm. The cells prepared from an erythroid colony showed mature enucleated erythrocytes (arrow) and immature erythrocytes (e), from a macrophage colony showed macrophage morphology (f), and from a granulocyte colony showed mature neutrophils and eosinophils (g), Wright-Giemsa staining, scale bars = 10µm. The cells comprising erythroid colonies were stained positive for erythroid marker glycophorin A, and
negative for pan-leukocyte marker CD45 and myelomonocytic markers CD33 and CD13 (h, i). In contrast, the cells comprising granulocyte colonies were stained negative for glycophorin A, and positive for CD45, CD33, and CD13 (j, k).

**Figure 5. SR-bFGF-cultured hESCs maintain potential to generate derivatives of three germ layers.** (a & b) *In vitro*, in addition to generation of hematopoietic cells (part of mesoderm development, Fig. 3 & 4), SR-bFGF-cultured hESCs also contained capacity to differentiate into endoderm (HNF3α) and ectoderm (Pax6) derivatives according to gene expression determined by a quantitative real-time PCR. (c-h) *In vivo*, 5 out of 5 NOD/SCID mice produced teratomas 6 weeks post injection of SR-bFGF-maintained hESCs (passage 26) into the testis capsule. Microscopic examination from a representative teratoma revealed that the tumor was comprised of tissues representing all three embryonic germ layers, including ectoderm (c-d, c. cystic epithelium, d. neural rosettes, e. squamous epithelium), mesoderm (f-g, f. muscle, g. osteoid island showing bony differentiation), and endoderm (h. glandular epithelium). Hematoxylin and eosin staining. Bars = 50 µm.
Bhatia Figure 1

**hESCs in MEF-CM**

**hESCs in SR-bFGF**

a. Morphology, and AP/Oct4 expression and composition

b. Marker expression associated with undifferentiated state

c. Proliferative capacity

d. Karyotype
Bhatia Figure 3

**hESCs in MEF-CM**

Embryoid body formation

**hESCs in SR-bFGF**

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**b**

CD45negPFV hemogenic precursors

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**c**

Hematopoietic lineage specification

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**d**

Differentiation into hematopoietic progenitors
Bhatia Figure 5

a  Differentiation into endoderm in vitro (HNF3α)

b  Differentiation into ectoderm in vitro (Pax6)

Teratoma formation of hESCs cultured in SR-bFGF
Human embryonic stem cells maintained in the absence of mouse embryonic fibroblasts or conditioned media are capable of hematopoietic development

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