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Embryonic Stem Cells As an Alternate Marrow Donor Source: Engraftment without Graft-Versus-Host Disease

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Abstract

A single embryonic stem cell (ESC) line can be repetitively cryopreserved, thawed, expanded, and differentiated into various cellular components serving as a potentially renewable and well-characterized stem cell source. Therefore, we determined whether ESCs could be used to reconstitute marrow and blood in major histocompatibility complex (MHC)-mismatched mice. To induce differentiation toward hematopoietic stem cells (HSCs) in vitro, ESCs were cultured in methylcellulose with stem cell factor, interleukin (IL)-3, and IL-6. ESC-derived, cytokine-induced HSCs (c-kit$^+$/CD45$^+$) were isolated by flow cytometry and injected either intra bone marrow or intravenously into lethally irradiated MHC-mismatched recipient mice. From 2 wk to 6 mo after injection, the peripheral blood demonstrated increasing ESC-derived mononuclear cells that included donor-derived T and B lymphocytes, monocytes, and granulocytes without clinical or histologic evidence of graft-versus-host disease (GVHD). Mixed lymphocyte culture assays demonstrated T cell tolerance to both recipient and donor but intact third party proliferative responses and interferon γ production. ESCs might be used as a renewable alternate marrow donor source that reconstitutes hematopoiesis with intact immune responsiveness without GVHD despite crossing MHC barriers.

Key words: embryonic stem cells • hematopoiesis • in vivo • tolerance • mouse

Introduction

Hematopoietic stem cells (HSCs) obtained from the marrow or peripheral blood are being used worldwide to treat malignancies, inborn errors of metabolism, and autoimmune diseases (1–3). Attempts to maintain HSCs in culture for even relatively short periods of time are unsuccessful due to terminal differentiation. In addition, GVHD is a common morbid and/or lethal complication of allogeneic HSC transplantation (4, 5). HSC graft composition including the number of T cells, dendritic cells, B cells, and CD34$^+$ or other progenitor cells, as well as bacterial contamination, varies depending on patient, source, and harvesting technique. This has resulted in intra-institutional and inter-institutional variation in graft composition. For these reasons, a renewable source of HSCs that is not complicated by GVHD and does not have interpatient, intrapatient, or lot variability would be desirable.

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Embryonic stem cell (ESC) lines are derived from the inner cell mass of the blastocyst and are totipotent and immortal. ESCs can be expanded ex vivo as undifferentiated cells that retain a normal karyotype or, alternatively, can be differentiated ex vivo into cell types of all three germ layers (6, 7). Leukemia inhibitory factor (LIF) is required to maintain the undifferentiated state of mouse ESCs, whereas withdrawal of LIF initiates the formation of embryoid bodies (EB) and cellular differentiation (8, 9). When EB are cultured, cells with hematopoietic progenitor phenotype are routinely observed in vitro (10–14). In the absence of cytokines or stromal cells, multilineage hematopoietic precursors might be detected by colony-forming assays after 4 d of EB culture. C-kit (stem cell factor [SCF] receptor) and CD45 (a hemato-

Abbreviations used in this paper: BFU-E, erythroid burst-forming units; BrdU, bromodeoxyuridine; CFU-GM, granulocyte-macrophage colony-forming units; CFU-Meg, megakaryocyte colony-forming units; CFU-Mix, mixed colony-forming units; EB, embryoid bodies; ESC, embryonic stem cell; ESCT, ESC-derived transplantation; HSC, hematopoietic stem cell; IBM, intra bone marrow; LIF, leukemia inhibitory factor; SCF, stem cell factor; TBI, total body irradiation.

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poietic lineage marker) expression occur simultaneously on day 10 of EB culture (15). One challenge for developing cell therapies from ESCs is whether newly formed tissues can adapt to function effectively in vivo when transplanted into an adult (16). Herein, we demonstrate that murine ESCs induced to differentiate toward c-kit^{+} CD45^{+} HSCs by removal of LIF and addition of SCF, IL-3, and IL-6 reconstitute hematopoiesis in lethally irradiated MHC-mismatched mice. This occurs without clinical or histological evidence of GVHD and with bidirectional host/donor tolerance and intact third party immune responses.

Materials and Methods

ESCs

The 129/SvJX129/SV-CP F1 (MHC H2^{b}) hybrid, 3.5-d mouse blastocyst–derived ESC line R1 was provided by A. Nagy (Mount Sinai Hospital, Toronto, Canada). To maintain ESCs in an undifferentiated state they were cultured on gelatinized tissue culture dishes in high glucose Dulbecco's modified Eagle's medium supplemented with 15% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1× nonessential amino acids, 1× sodium pyruvate, and 1,000 U/ml LIF (Specialty Media and StemCell Technologies Inc.). Mitomycin C–treated primary embryonic fibroblasts (StemCell Technologies Inc.) were used as a feeder layer for a long-term culture of R1 ESCs.

Induction of ESCs toward Hematopoietic Progenitors (HSCs)

To induce differentiation toward HSCs in vitro, the ESCs were cultured on low adherent Petri dishes in Iscove's modified Dulbecco's medium containing ~1% methylcellulose, 15% FBS, 150 μM monothioglycerol, 2 mM L-glutamine, 500 ng/ml murine SCF, 46 ng/ml human IL-3, and 500 ng/ml human IL-6 (StemCell Technologies Inc. and Sigma-Aldrich; cytokines were provided by Kirin Brewery). Cells were cultured at 37°C in 5% CO_2 atmosphere incubator for 7–10 d. The single cell suspension was collected, washed, and suspended in PBS 10^7 cells/0.2 ml for i.v. injection or 0.5×10^7 cells/30 μl in the case of intra bone marrow (IBM) injection.

Flow Cytometric Analysis

Two or three color cell cytometric analysis was performed using standard procedures on an Epics XL (Beckman Coulter). The single cell suspension was aliquoted and stained with either isotype controls or antigen-specific antibodies. Cell surface antigens were labeled with the combinations of the following monoclonal antibodies: FITC-, PE-, or biotin- (followed by CyChrome staining) conjugated H2K^{b}/D^{b}, CD117 (c-kit), CD34, Sca-1, CD45, CD19, CD11b, and CD3 (BD Biosciences). Dead cells were excluded from analysis using propidium iodide staining. Samples were run on an Epics XL flow cytometer and analyzed with CELLQuest™ software (BD Immunocytometry Systems).

In Vitro Hematopoietic Progenitor Assays

The single cell suspension of ESC-derived, cytokine-stimulated cells was washed and stained with the following antibodies: CD45, c-kit, and CD34. The cells were sorted using the gated dot diagrams in an Epics-Elite ESP flow cytometer cell sorter (Beckman Coulter). Four different populations of cells were used for clonal cell culture including CD34^{+} cells (purity 75%), c-kit^{+} cells (purity 63%), CD45^{+} cells (purity 75%), and a heterogeneous population consisting of CD45^{+} c-kit^{-} (12%), CD45^{-} c-kit^{+} (23%), and CD45^{+} c-kit^{+} (49%) subsets. Cells were plated in prepared methylcellulose-based cultures supplemented with a cocktail of growth factors in 35-mm Lux suspension culture dishes (Nunc) as previously described (17–19). In brief, 200 cells per 1 ml were cultured in medium containing 1.2% methylcellulose, 30% FCS (Hyclone), 1% deionized fraction V bovine serum albumin (Sigma-Aldrich), and 5×10^{-5} mol/liter 2-mercaptoethanol (Sigma-Aldrich). The following colony-stimulating factors were used: 20 ng/ml murine SCF, 10 ng/ml human GM-CSF, 20 ng/ml human G-CSF, 10 ng/ml murine IL-3, 30 ng/ml murine IL-6, 3 U/ml human recombinant erythropoietin, and 100 ng/ml human TPO (StemCell Technologies Inc.). After 12 d of culture in an incubator at 37°C in humidified atmosphere with 5% CO_2, all colonies were counted under an inverted microscope. The identification of erythroid burst-forming units (BFU-E), granulocyte-macrophage colonies (CFU-GM/CFU-G/CFU-M/CFU-Eo), megakaryocyte colony-forming units (CFU-Meg), and erythrocyte-containing, mixed colony-forming units (CFU-Mix) colonies was based on the typical morphological features.

Enrichment of ESC-derived Hematopoietic Progenitors

The suspension of single cells differentiated from ESCs was collected, washed, and stained with the following antibodies: CD45 and c-kit. The cells were sorted using an Epics-Elite ESP flow cytometer cell sorter (Beckman Coulter) as a heterogeneous population consisting of CD45^{+} c-kit^{-}, CD45^{-} c-kit^{+}, and CD45^{+} c-kit^{+} subsets. The phenotypic purity of sorted cells determined by postsorting flow cytometry analysis was 86 ± 11% for c-kit and 49 ± 18% for CD45. After sorting, cells were resuspended in PBS and used immediately for IBM (10^5 cells/30 μl) or i.v. injection (10^6 cells/0.2 ml).

Long-Term Repopulation Model

Mice. 6–7-wk-old female BALB/cJ mice (MHC H2^{d}; Jackson ImmunoResearch Laboratories) were used as recipients of both ESCs and cytokine induced ESCs. Mice were irradiated (total body irradiation [TBI] 5.5 or 8.0 Gy) 16 h before injection. The mice were housed in microisolator cages under specific pathogen-free conditions and provided with γ-irradiated food in the animal facilities of Northwestern University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Northwestern University.

Transplantation. Cells prepared as described above were injected either i.v. or IBM. i.v. injection was performed into one of the lateral tail veins. IBM injection was performed according to the procedure previously described (20). In brief, mice were anesthetized and after shaving and disinfection, a 5-mm incision was made on the thigh. The knee was flexed to 90° and the proximal side of the tibia was drawn anteriorly. A 26-gauge needle was inserted into the joint surface of the tibia through the patellar tendon and advanced into the bone marrow cavity. Using a 50-μl microsyringe (Hamilton), the cells were injected through the bone hole and into the bone marrow cavity. The skin was then closed using 6-0 vicryl sutura (Ethicon).

Chimerism. The presence of donor-derived (R1 ESC, H2^{b}) T lymphoid, B lymphoid, monocytic, and granulocytic lineage was determined using flow cytometric analysis of mononuclear cells isolated from peripheral blood of mice 2, 4, 8, 12, and 20 wk after infusion of ESC-derived cells. Cell surface antigens were labeled with the following monoclonal antibodies: FITC-, PE-, or biotin-conjugated H2K^{b}/D^{b}, H2^{d}, CD45, CD45R/B220, CD19,

CD11b, CD14, and CD3 (BD Biosciences). Mononuclear cells isolated from the peripheral blood of an untreated BALB/c mouse were used as a negative control. Mononuclear cells from a 129/Sv mouse served as a positive control (see Fig. 4, a–c).

MLR In Vitro. Immune responses in recipient BALB/cJ mice toward donor histocompatibility antigen of 129/Sv strain, recipient MHC, and third party antigens were evaluated by one way MLR tests. MLR tests were performed in six animals transplanted with ESC-derived cells 6 mo after transplantation. 10^6 splenocytes from chimeric mice were cultured separately in 24-well plates (Falcon; BD Labware) with 10^6 irradiated splenocytes (30 Gy) obtained from 129/Sv, BALB/cJ, and SJL/J ($H2^S$) mice. Cells were cultured in a total volume of 2 ml RPMI 1640 medium (Cellgro; Mediatec) supplemented with 2 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 50 μg/ml gentamicin, and 10% FSC. After 48 and 72 h of culture in a 37°C humidified CO_2 incubator, cells were pulsed with bromodeoxyuridine (BrdU), adding 20 μg per each 2-ml well as previously described (21). 20 h after the second pulse of BrdU, cells were harvested and processed with a BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol. Cells were stained with FITC anti-BrdU and 7-amino-actinomycin. Flow cytometric data were acquired using an Epics XL flow cytometer and analyzed with CELLQuest™ software. Syngeneic and allogeneic splenocytes were used as negative and positive controls, respectively.

IFN-γ Level Analysis. Spleen cells were isolated from surgically removed spleen of mice transplanted with ESC-derived cells and passed over nylon wool columns. 5×10^5 (in 0.2 ml culture medium) chimeric splenocytes were cultured in presence of irradiated (30 Gy) donor, recipient, or mismatched (SJL/J) splenocytes in 96-well plates for 72 h. Culture supernatants were collected and levels of IFN-γ in supernatants were determined by ELISA kit according to the manufacturer's protocol (R&D Systems).

Grading of Histologic Changes of GVHD. All mice were killed 6 mo after ESC-derived transplantation (ESCT). For evaluation of presence and degree of hepatic and intestinal inflammation, tissues were removed from all mice in both groups and kept in 10% formaldehyde. Tissue sections were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by standard procedures. The degree of inflammation of liver and small bowel was graded in a 0–4 scale as previously described (22).

Statistical Analysis

All data are presented as the mean ± standard error of the mean. Two groups of data were analyzed by the Mann-Whitney U test (Student's *t* test for nonparametric distribution). $P < 0.05$ was considered statistically significant.

Results

Injection of Undifferentiated ESCs. Murine ESCs were maintained in the undifferentiated state by coculture on irradiated primary fibroblasts in the presence of LIF. Flow

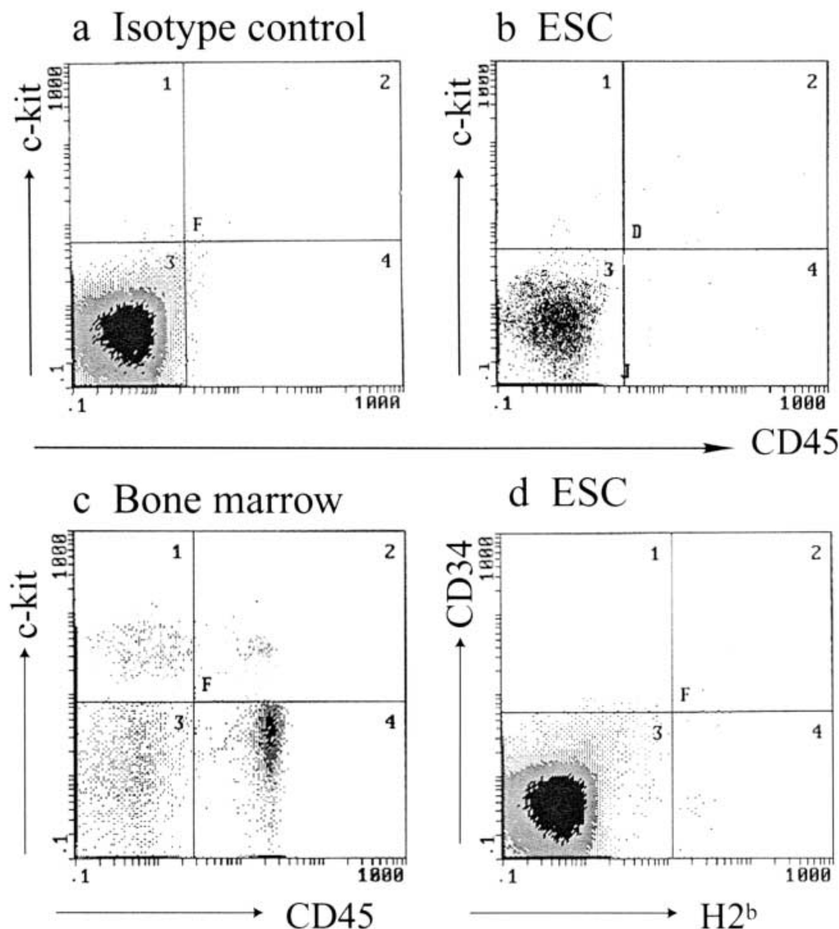

Figure 1. Immunophenotype of undifferentiated ESCs. Expression of c-kit, CD45 (b), and CD34 and $H2^b$ (d) compared with isotype control (a) and normal murine bone marrow (c).

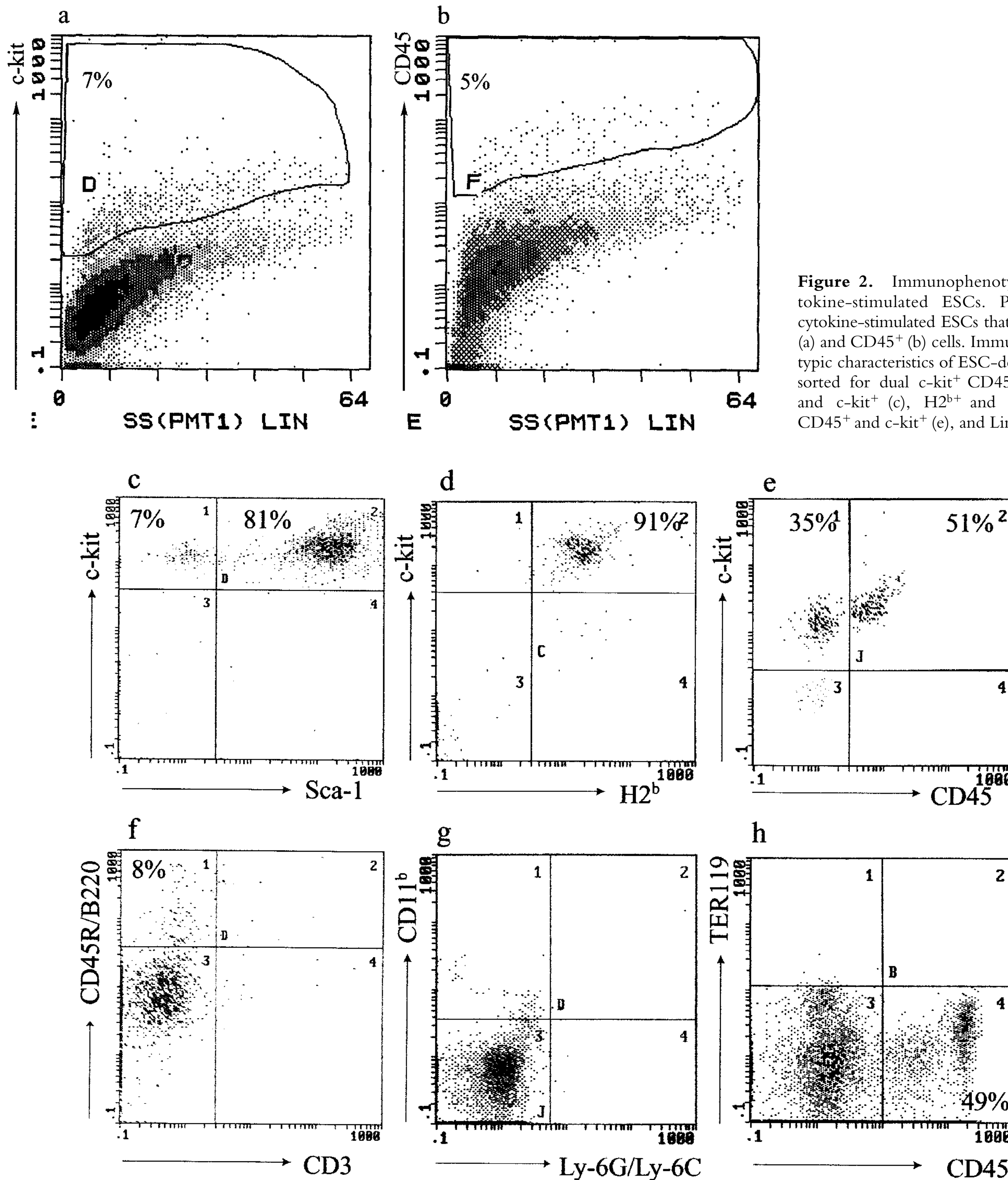

Figure 2. Immunophenotype of cytokine-stimulated ESCs. Percent of cytokine-stimulated ESCs that are c-kit$^+$ (a) and CD45$^+$ (b) cells. Immunophenotypic characteristics of ESC-derived cells sorted for dual c-kit$^+$ CD45$^+$: Sca-1$^+$ and c-kit$^+$ (c), H2^{b+} and c-kit$^+$ (d), CD45$^+$ and c-kit$^+$ (e), and Lin$^-$ (f–h).

cytometric analysis of undifferentiated ESCs showed the absence of CD117 (c-kit), CD45, CD34, or MHC molecules on their surface (Fig. 1, a–d). When undifferentiated ESCs were injected either i.v. or IBM into lethally irradiated mice, marrow/hematopoietic failure resulted in 100% mortality within 8–13 d (not depicted).

Immunophenotype of Ex Vivo Cytokine-stimulated Hematopoietic Differentiation of ESCs. To promote ex vivo hematopoietic differentiation, undifferentiated ESCs were cultured in methylcellulose medium by the withdrawal of LIF and the addition of the hematopoietic cytokines SCF, IL-3, and IL-6 for 7–10 d resulting in formation of EB. Flow cytometric analysis of presorted population revealed that 7% of cultured cells presented the HSC marker c-kit and ~5% presented the panleukocytic marker CD45 (Fig. 2, a and b). The immunophenotype of c-kit$^+$ CD45$^+$ ESC-derived progenitor cells is Sca-1$^+$ (Fig. 2 c), H2^{b+} (Fig. 2 d), and lineage$^-$ for B cell marker B 220 (Fig. 2 f), mono-

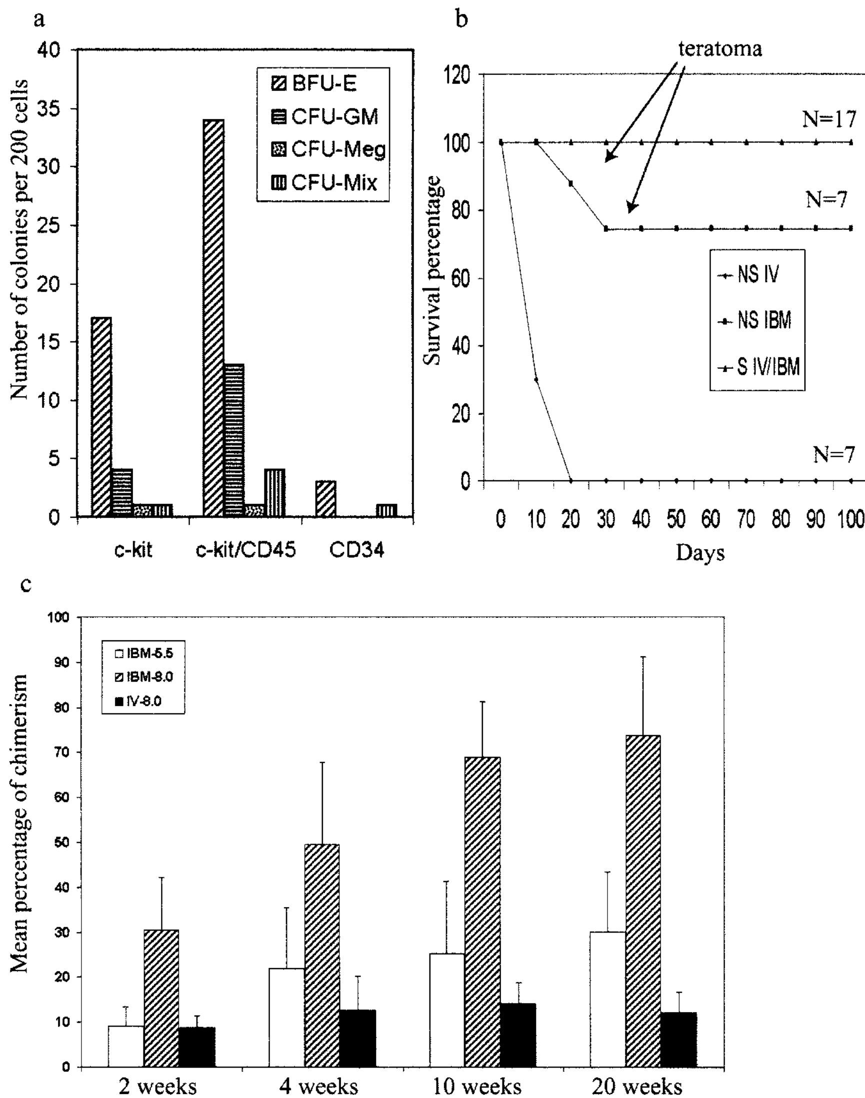

Figure 3. Cytokine-stimulated ESCs ex vivo and in vitro analysis data. (a) Efficiency of hematopoietic colony formation by 200 ESC-derived cells (different population: enriched for c-kit$^+$, c-kit$^+$ CD45$^+$, and CD34$^+$). (b) Survival curve for mice injected i.v. with nonsorted ESC-derived cells, IBM injected with nonsorted ESC-derived cells, and injected i.v. or IBM with sorted c-kit$^+$ CD45$^+$ cells. (c) Mean percentage of donor chimerism in different groups of mice analyzed 2, 4, 10, and 20 wk after ESCT. IBM-5.5, irradiated with 5.5 Gy, injected IBM; IBM-8, irradiated with 8.0 Gy, injected IBM; i.v.-8, irradiated with 8.0 Gy, injected i.v.

cytes/granulocytes marker CD11^b (Fig. 2 g), and red blood cell marker Ter119 (Fig. 2 h).

In Vitro Colony-forming Unit Formation of Cytokine-stimulated ESCs. The in vitro ability of cytokine-stimulated ESCs to form hematopoietic colonies was investigated from sorted ESC-derived hematopoietic progenitor cells expressing either CD34, c-kit, CD45, or both c-kit and CD45. Enriched by flow cytometry, cell subsets were plated in prepared methylcellulose-based cultures supplemented with SCF, IL-3, IL-6, and/or recombinant erythropoietin. Total progenitor frequency of colony-forming units CFU-GM, BFU-E, CFU-Meg, and CFU-Mix, was scored after 12 d of culture (Fig. 3 a). The highest plating efficiency from cytokine-stimulated ESCs was observed with dual positive c-kit$^+$ CD45$^+$ cells that formed the largest number of CFU-GM, BFU-E, CFU-Meg, and CFU-Mix colonies (Fig. 3 a).

In Vivo Injection of Cytokine-stimulated ESCs. i.v. injection of nonsorted cytokine differentiated ESCs into lethally irradiated mice did not result in hematopoietic reconstitution leading to death of all (n = 7) mice between days 8–13 (Fig. 3 b). In the case of IBM injection of nonsorted cytokine-differentiated ESC suspensions, hematopoiesis was reconstituted with a low percentage of donor-mixed chimerism (2–12%; not depicted), however, in two out of seven mice, teratomas that were confirmed histologically arose at the IBM injection site (Fig. 3 b).

As already mentioned, the largest number of ex vivo hematopoietic colonies of myeloid, erythroid, and megakaryocytic lineages arose from cytokine-stimulated ESCs that were enriched for c-kit$^+$ and CD45$^+$ (Fig. 3 a). Based on our in vivo hematopoietic colony-forming assay results and observations by McKinney-Freeman et al. (23) and Cho et al. (24), we chose two immunophenotypic markers to purify hematopoietic progenitors derived from ESCs. Therefore, ESC-derived c-kit$^+$/CD45$^+$ HSCs were isolated by flow cytometry and injected either i.v. (10^6 cells in 0.2 ml) or IBM (0.5×10^5 cells in 15 μl $\times$ 2) into irradiated (TBI 5.5 or 8.0 Gy) 6–7-wk-old BALB/c mice (MHC H2^d; Fig. 3 b). The sorted cell population

Table I. *Mixed Chimerism in Mice Transplanted with ESC-derived c-kit$^+$/CD45$^+$ Cells Analyzed in Different Time Points after Injection*

Mouse	TBI	Injection of ESCs	Chimerism at 2 wk	Chimerism at 4 wk	Chimerism at 10 wk	Chimerism at 20 wk
	Gy					
1	5.5	IBM	9.1	3.1	14.0	27.5
2	5.5	IBM	3.4	28.5	27.6	34.3
3	5.5	IBM	13.2	34.5	47.2	45.3
4	5.5	IBM	11.0	21.3	12.1	13.2
5	8.0	IV	5.7	6.0	10.1	9.5
6	8.0	IV	7.3	12.0	15.1	7.9
7	8.0	IV	11.3	8.7	11.0	12.5
8	8.0	IV	10.5	23.5	20.3	18.6
9	8.0	IBM	22.3	61.5	62.0	87.3
10	8.0	IBM	17.7	46.0	51.5	45.7
11	8.0	IBM	27.6	29.3	68.3	91.0
12	8.0	IBM	33.8	63.0	59.0	57.0
13	8.0	IBM	18.9	73.0	93.0	74.3
14	8.0	IBM	35.6	34.7	75.3	56.0
15	8.0	IBM	54.7	72.1	63.4	85.6
16	8.0	IBM	23.7	25.7	68.1	71.2
17	8.0	IBM	39.0	42.3	79.1	95.5

Data are presented as percent of H2K^{b+}/CD45$^+$ cells.

prepared for injection was analyzed by flow cytometry and immunophenotypically was 86 ± 11% c-kit$^+$, 49 ± 18% CD45$^+$, 80–84% Sca-1$^+$, >90% H2^{b+}, and Lin$^-$ (Fig. 2, c–h).

The earliest reconstitution from ESC-derived HSCs (MHC H2^b) was observed after 2 wk, at which time the percentage of anti-H2K^b/D^{b+}/CD45$^+$ cells was 20.3 ± 14.0% (Table I and Figs. 3 c and 4, a–c). By 4 wk after ESC-derived HSC injection, the population of H-2^{b+}/CD45$^+$ cells increased to 34.4 ± 22.4%. Analysis of chimerism performed 6 mo after transplantation showed a further increase of ESC-derived hematopoiesis to 49.0 ± 31.1% (range: 7.9–95.5%; Table I and Fig. 3 c).

Mice irradiated with 8.0 Gy before IBM injection of ESC-derived c-kit$^+$/CD45$^+$ HSCs had a higher percentage of donor chimerism compared with mice irradiated with a less immune suppressive dose of 5.5 Gy (Table I and Fig. 3 c). When comparing TBI 8.0 to 5.5 Gy, percent donor engraftment was 30.4 ± 11.8 versus 9.2 ± 4.2 ($P < 0.05$) at 2 wk and 73.7 ± 17.6 versus 30.1 ± 13.4 ($P < 0.05$) at 20 wk, respectively (Table I and Fig. 3 c). Mice injected IBM compared with the i.v. route of administration had faster and more effective reconstitution of hematopoiesis from ESC-derived hematopoietic progenitor cells. Between IBM and i.v. routes of administration, the percent donor engraftment was 30.4 ± 11.8 versus 8.7 ± 2.6 at 2 wk ($P < 0.05$) and 73.7 ± 17.6 versus 12.1 ± 4.7 ($P < 0.01$) at 20 wk, respectively (Table I and Fig. 3 c). Flow cytometric analysis of PBMC subpopulations revealed that the population of donor-derived T lymphocytes (H-2^{b+}/CD3$^+$ cells) comprised 18.3 ± 4.7 and 17.3 ± 6.5% of PBMC at 10 and 20 wk, respectively (Fig. 4, f and g). The population of H-2^{b+}/CD14$^+$/CD11b$^+$ (monocytes/granulocytes) was 47.3 ± 16.5% at 10 wk after transplantation and remained stable for a maximum follow-up of 24 wk (Fig. 4, f–h). Reconstitution of chimeric B lymphocytes (H-2^{b+}/CD19$^+$ cells) was 3.1 ± 3.4% at 20 wk (Fig. 4, f and g). No mouse receiving sorted cells developed a teratoma or had evidence of malignant or abnormal growth.

Immunologic Competence of ESC-derived Hematopoiesis. No mouse developed runting (ruffled fur, hunched back, and weight loss) consistent with GVHD despite stable MHC-mismatched engraftment. There was no histologic evidence of GVHD in autopsy specimens of liver or bowel. MLRs were evaluated by means of BrdU incorporation using splenocytes from chimeric, 129/Sv (ESC donor), BALB/c (recipient), and SJL/J (third party) mice. Splenocytes collected from chimeric mice were characterized by low MLR proliferative responses to cells of either the donor or host MHC compared with proliferative responses to SJL/J (third party, MHC-mismatched) splenocytes (9.2 ± 2.1, 5.6 ± 3.4, and 24.3 ± 9.1%, respectively; Fig. 5 a). IFN-γ production from chimeric mice correlated with the MLR results (Fig. 5 b) in that there was an inverse correlation between percentage of donor chimerism and either proliferative response or IFN-γ production against donor genotype MHC splenocytes ($R = -0.89$ and $R = -0.87$, $P < 0.01$, respectively; Fig. 5, c and d). The highest IFN-γ production

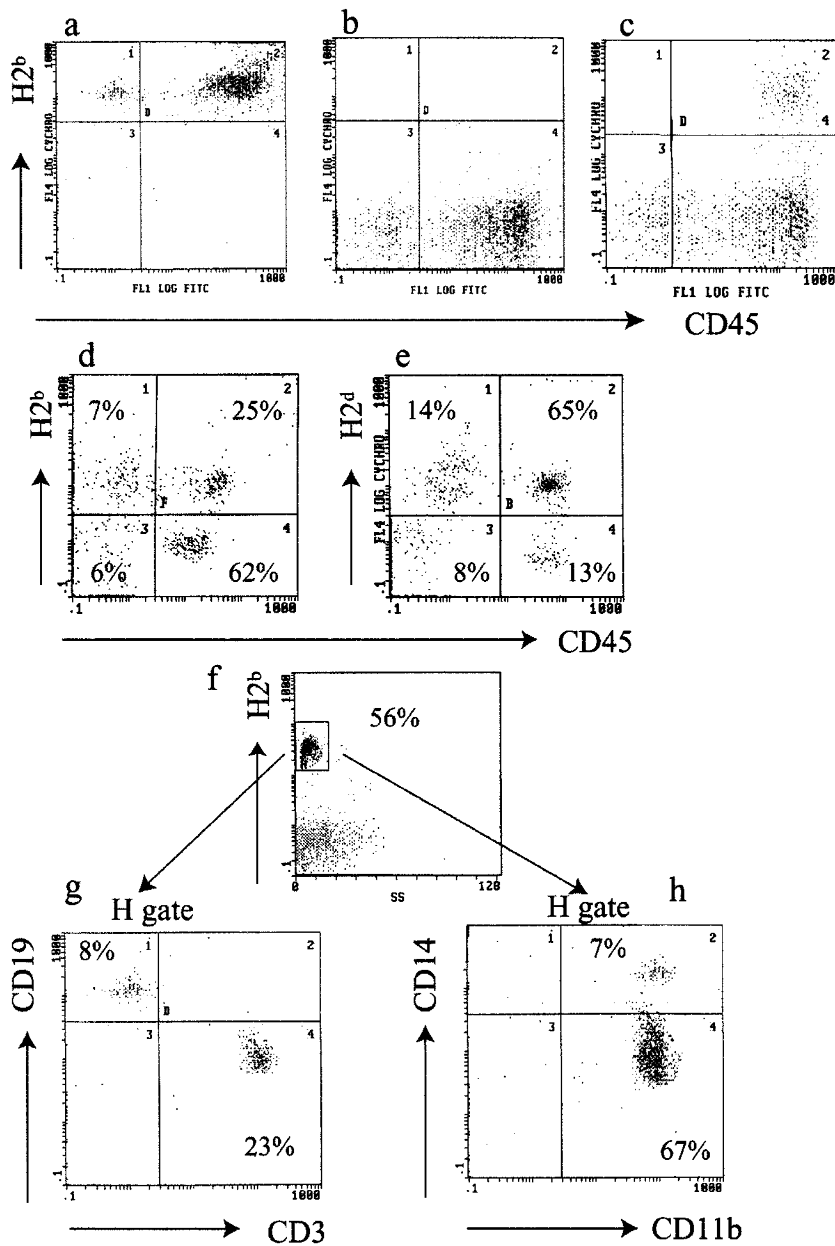

Figure 4. Immunophenotype of peripheral blood after ESCT. Comparison of percentage of $H2^{b+}$ leukocytes in peripheral blood of the C57BL/6J mouse (a), BALB/c mouse (b), and chimeric BALB/c mouse 2 wk after ESC-transplantation (TBI 8.0 Gy/TBI; c). Example of analysis of chimerism based on immunophenotyping of PBMCs in two channels: $H2^b$ (donor-derived) and $H2^d$ (host-derived) and $CD45^+$ (10 wk after ESCT; TBI 8.0 Gy/TBI; d and e). Analysis of $H2^{b+}$ mononuclear cells in peripheral blood from chimeric mouse (20 wk after ESCT; TBI 8.0 Gy/IBM): gating (H gate) positive population $H2^{b+}$ (f) and analysis of percent of T lymphocytes ($CD3^+$), B lymphocytes ($CD19^+$; g), and granulocytes/monocytes ($CD11b^+/CD14^+$; h).

against irradiated third party (SJL/J) splenocytes achieved levels of positive controls (mismatched splenocytes; 1,674.9 ± 534.7 and 2,024.3 ± 234.5, respectively; Fig. 5 b), indicating an intact immune response to foreign antigens.

Discussion

Most of the data concerning ESC-derived differentiation is based on in vitro studies (11, 12, 16, 24–27). The question of whether hematopoietic progenitors derived in vitro from mouse ESCs can support in vivo long-term multilineage engraftment remains unanswered (15, 28). Previous reports suggest that ESCs or cells derived from ESCs have a limited capacity to engraft and reconstitute hematopoiesis in vivo (15). It has been shown that ESCs transduced with a retrovirus containing the BCR/ABL oncogene differentiated in vivo into multiple myeloid cell types as well as T and B lymphocytes (29). Some components of hematopoiesis have also been reconstituted in immune-deficient mice, e.g., SCID or RAG-1–deficient mice (11, 30, 31). However, it has not been previously demonstrated that genetically normal (i.e., nontransduced) ESCs or cells derived from ESCs are capable of reconstituting an intact and functional immune system in normal mice. Our data confirmed failure of hematopoietic engraftment from undifferentiated ESCs. Either i.v. or IBM injection of undifferentiated ESCs into lethally irradiated mice results in 100% mortality from marrow failure. Our findings also demonstrated either no or marginal hematopoietic engraftment and/or teratoma formation after injection of a nonpurified heterogeneous

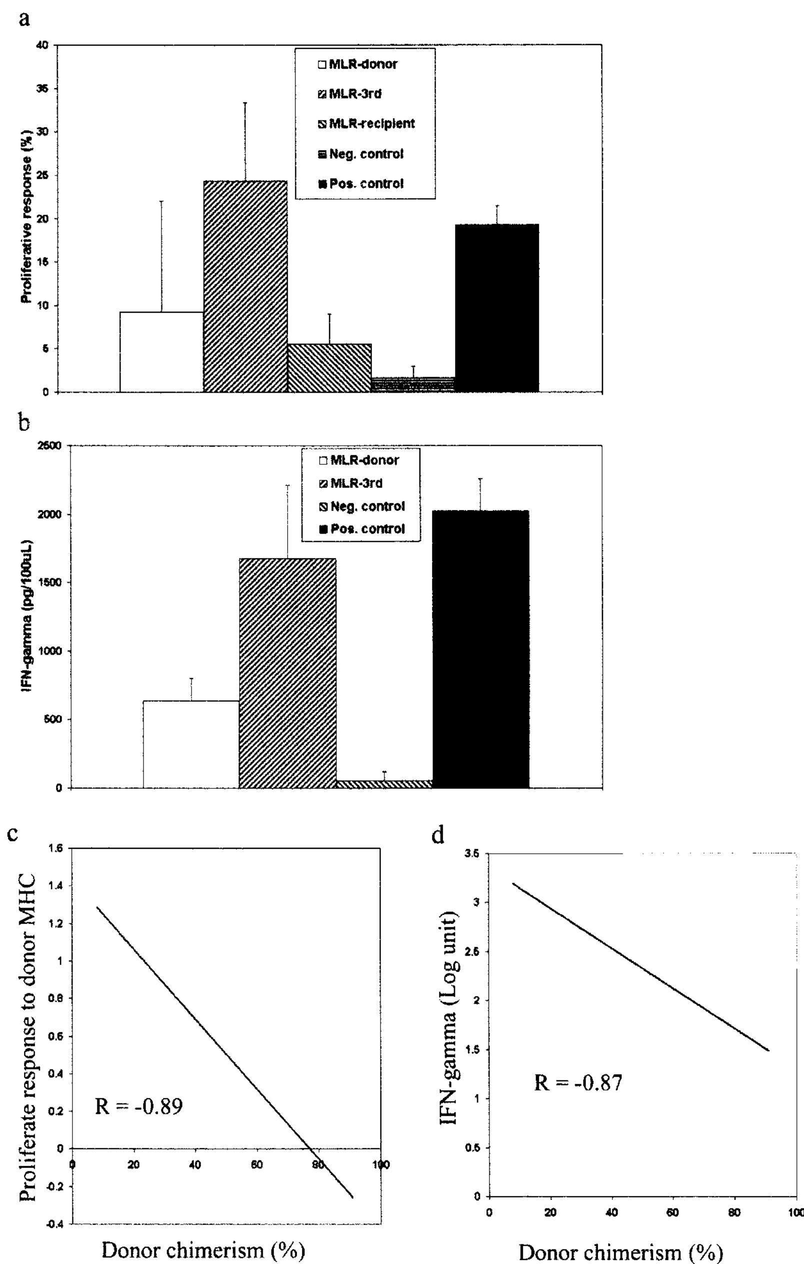

Figure 5. Immunologic competence of ESC-derived hematopoiesis. (a) Proliferative response of splenocytes from chimeric mice to donor and recipient MHC and third party antigen (data are presented as a percent of BrdU incorporated cells). (b) Production of IFN-γ during MLR analyzed by ELISA (mean values). (c) Correlations between donor chimerism analyzed at 20 wk after transplantation and proliferative response to donor MHC (analyzed by MLR) and (d) IFN-γ level assessed by ELISA in MLR (donor and chimeric splenocytes) supernatant. Proliferative response as well as IFN-γ data are presented in log scale, whereas chimerism is shown as the percentage of donor (ESC-derived) cells in peripheral blood in linear scale.

population of cells derived from cytokine-stimulated ESCs. i.v. injection of a cytokine-differentiated, nonsorted ESC suspension resulted in 100% mortality, whereas IBM injection resulted in only low level chimerism (<12%) and in some mice, formation of teratomas at the IBM site. However, we observed that when ESCs, induced to differentiate ex vivo into hematopoietic precursors and sorted for c-kit$^+$ and CD45$^+$ cells, are injected, rapid hematopoietic and immune reconstitution occurs from the ESC donor without development of teratomas.

In this report, ESCs generated EB ex vivo containing hematopoietic precursors in methylcellulose cultures, consistent with previous findings (12, 32). Previous data suggests that multipotent, long-term, repopulating hematopoietic progenitors might be formed within EB around day 4 after LIF withdrawal (29) and that SCF and CD45 receptors arise around day 8 of EB culture (15). Miyagi et al. (30) reported that ESCs express low levels of the c-kit receptor on their surface, whereas Hole et al. (15) reported that expression of c-kit is absent in ESCs until day 8 of EB culture. In agreement with Hole et al., we found no expression of c-kit on undifferentiated ESCs. C-kit expression appeared between days 6 and 8 of ESC culture in methylcellulose medium (unpublished data). To avoid undifferentiated cells that may generate teratomas as well as unwanted excessive differentiation of ESCs into more mature stages, we harvested cells that had been cultured for 7–10 d. As there is no consensus regarding the immunophenotypic features of murine ESC-derived HSCs, we chose the phenotypic markers c-kit/CD45 for the purification based on

in vitro colony-forming assay that found inclusion of the CD45$^+$ cell population along with c-kit$^+$ cells results in more efficient in vitro functionality. Other reports have suggested that sorting for CD41 and c-kit expression may result in better enrichment of definitive hematopoietic progenitors (33, 34).

Analysis of cell population sorted for CD45/c-kit cells (enriched, but not clonal) revealed enrichment for cells expressing the murine HSC marker Sca-1 as well as lacking lineage-specific markers. To this date, the identification and true clonal phenotype of human HSCs remains elusive. For this reason, clinical human stem cell transplants that result in long-term engraftment use a nonclonal but enriched population of marrow or blood cells selected for progenitor markers such as CD34 or CD133. Our data demonstrate that ESC-derived hematopoietic progenitor cells (enriched, but nonclonal) also result in stable, long-term hematopoietic engraftment.

ESCs are allogeneic cells that are immunologically and genetically distinct from the recipient. However, hematopoietic reconstitution of ESC-derived T lymphocytes, B lymphocytes, and monocytes occurred across MHC barriers without evidence of rejection. The percentage of ESC-derived hematopoiesis was greater after IBM injection compared with i.v. injection despite 1 log fewer cells being injected IBM compared with i.v. Our results with ESC-derived hematopoietic progenitor cells are similar to the findings of Kushida et al. (20), Ikehara (35), and Wang et al. (36), who first demonstrated superior engraftment with IBM injection compared with i.v. injection from adult HSCs. These findings suggest that homing of stem cells to the marrow might be inefficient with the i.v. route of administration.

It has been observed that preimplantation-derived, embryonic-like stem cells when injected into liver via the portal vein are able to induce tolerance (37). Several explanations of this phenomenon were proposed including low MHC class I expression, incomplete hematopoietic chimerism with restriction of T and NK cell populations, and successful posttransplant thymic reeducation of T cells (37). In our animal model, after injection of ESC-derived hematopoietic progenitors into either the systemic circulation or IBM, we observed multilineage hematopoietic engraftment. The ESC-derived T cells were bidirectionally tolerant to recipient and host because MLC proliferative responses to recipient and host lymphocytes were diminished. This is consistent with both engraftment and absence of clinical or histologic evidence of GVHD in autopsied tissues. Importantly, immune competence was maintained, as demonstrated by healthy mice without infections and normal third party MLC proliferative responses and IFN-γ production.

Although some groups have previously shown transplantation of ESC-derived blood cells, engraftment was brief and/or deficient in several lineages. Our data demonstrate that ESC-derived cells enriched for a population of c-kit$^+$ CD45$^+$ hematopoietic progenitors may reconstitute long-term multilineage hematopoiesis with a functional immune system and without GVHD. Future studies will focus on clonal analysis of ESC-derived progenitors responsible for long-term hematopoietic engraftment.

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