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.

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 hematopoietic stem cell [ESC]-derived transplantation; HSC, hematopoietic stem cell; IBM, intra bone marrow; LIF, leukemia inhibitory factor; SCF, stem cell factor; TBI, total body irradiation.

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.
造血细胞系的表达发生于EB培养的第10天（15）。一个发展挑战在于开发基于ESC的细胞治疗方案，特别是那些对治疗已经形成的新组织可以有效适应的治疗方案。在这里，我们证实，经过LIF和SCF、IL-3、IL-6的去除，使用MHC-错配的胚胎成纤维细胞的供体血液可以促进供体血液的重置并恢复第三方免疫反应。这发生在没有临床或组织学证据的GVHD和方向交叉的宿主/供体耐受和未受累第三方免疫反应的情况下。

材料与方法

ESCs

219/SvJX129/SV-CP F1（MHC H2b）杂交。3.5-d的小鼠空胚胎着床后ESC线R1由A. Nagy（Mount Sinai Hospital, Toronto, Canada）提供。为了维持ESC在未分化状态，培养在去乙醇化后高血糖Dulbecco氏改良的Eagle氏培养液中，15% FBS, 2 mM L-谷氨酸，0.1 mM β-甲基细胞素，1×非必需氨基酸，1×硫胺素，1,000 U/ml LIF（Specialty Media和StemCell Technologies Inc.）。Mitomycin C处理的胚胎成纤维细胞（StemCell Technologies Inc.）被用作饲养层。

诱导ESC向造血前体细胞（HSCs）分化

为了诱导分化，将ESC置于含有15% FBS, 0.1 mM β-甲基细胞素，1×非必需氨基酸，1×硫胺素的培养液中，持续7-10 d。单细胞悬液被收集并进行流式细胞术分析。通过细胞悬液的移植可以检测到血小板前体细胞（CFU-Meg）、粒细胞-单核细胞前体细胞（CFU-GM/CFU-G/CFU-M）和幼红细胞前体细胞（BFU-E）。

流式细胞术分析

使用三色细胞流式细胞术分析在Epics XL（Beckman Coulter）细胞分选器中进行。通过添加CD45、CD19、CD11b、CD3（BD Biosciences）死细胞染色剂对细胞进行标记。死细胞是通过将单个细胞悬液 aliquoted and stained with either iso- staining) conjugated H2K^b/D^b, H24, CD45, CD45R/B220, CD19, 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% denized fraction V bovine serum albumin (Sigma-Aldrich), and 5 × 10^-7 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% CO2, all colonies were counted under an inverted microscope. The identification of erythroid burst-forming units (BFU-E), granulocyte-macrophage colonies (CFU-GM/CFU-GM/CFU-M/CFU-Eo), megakaryocyte colony-forming units (CFU-Meg), and erythroid-containing, mixed colony-forming units (CFU-Mix) colonies was based on the typical morphological features.

富养ESC衍生的造血前体细胞

单细胞悬液通过Epics-Elite ESP流式细胞术细胞分选器（Beckman Coulter）进行分析。四类细胞被用于不同的目的：用IBM（10^7 cells/30 μl）或在PBS中用IBM进行注射（10^6 cells/0.2 ml）。

长期重置模型

小鼠。6-7-wk-old female BALB/c mice (MHC H2b; 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.

移植。细胞被移植在体内或体外。IBM注射是在拔出后立即进行的。IBM在所有情况下被注射到骨髓内。使用50-μl微量注射器（Hamilton），细胞被注射到骨髓内。皮肤被关闭并进行缝合。

移植物。供者-衍生（R1 ESC, H2b）T淋巴系，B淋巴系，单核细胞系，和粒细胞-单核细胞系的 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 bio- tin-conjugated H2K^b/D^b, H24, CD45, CD45R/B220, CD19, CD4, CD8, 4, 12, and 20 wk after infusion of ESC-derived cells.
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. 10⁶ splenocytes from chimeric mice were cultured separately in 24-well plates (Falcon; BD Labware) with 10⁶ irradiated splenocytes (30 Gy) obtained from 129/Sv, BALB/cJ, and SJL/J (H2b) 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% FCS. After 48 and 72 h of culture in a 37°C humidified CO₂ 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 × 10⁵ (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
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), H₂b⁺ (Fig. 2 d), and lineage⁻ for B cell marker B 220 (Fig. 2 f), mono-
cytes/granulocytes marker CD11b (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 × 2) into irradiated (TBI 5.5 or 8.0 Gy) 6–7-wk-old BALB/c mice (MHC H2d, Fig. 3 b). The sorted cell population
preparation for injection was analyzed by flow cytometry and immunophenotypically was 86 ± 11% c-kit+, 49 ± 18% CD45+, 80–84% Sca-1+, >90% H2b+, and Lin− (Fig. 2, c–h).

The earliest reconstitution from ESC-derived HSCs (MHC H2b) was observed after 2 wk, at which time the percentage of anti-H2Kb/Db/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-2b+/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 ± 16.7 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, 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-2b+/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-2b+/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).

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

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Data are presented as percent of H2Kb+/CD45+ cells.

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 (R² = 0.89 and R² = 0.87, P < 0.01, respectively; Fig. 5, c and d). The highest IFN-γ production
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...
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

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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.
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.

References


