Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression

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Despite the need for alternative sources of human hematopoietic stem cells (HSCs), the functional capacity of hematopoietic cells generated from human embryonic stem cells (hESCs) has yet to be evaluated and compared with adult sources. Here, we report that somatic and hESC-derived hematopoietic cells have similar phenotype and in vitro clonogenic progenitor activity. However, in contrast with somatic cells, hESC-derived hematopoietic cells failed to reconstitute intravenously transplanted recipient mice because of cellular aggregation causing fatal emboli formation. Direct femoral injection allowed recipient survival and resulted in multilineage hematopoietic repopulation, providing direct evidence of HSC function. However, hESC-derived HSCs had limited proliferative and migratory capacity compared with somatic HSCs that correlated with a distinct gene expression pattern of hESC-derived hematopoietic cells that included homeobox (HOX) A and B gene clusters. Ectopic expression of HOXB4 had no effect on repopulating capacity of hESC-derived cells. We suggest that limitations in the ability of hESC-derived HSCs to activate a molecular program similar to somatic HSCs may contribute to their atypical in vivo behavior. Our study demonstrates that HSCs can be derived from hESCs and provides an in vivo system and molecular foundation to evaluate strategies for the generation of clinically transplantable HSC from hESC lines.

Somatic hematopoietic stem cell (HSC) transplantation is the most common stem cell–based therapy in use today. Currently, somatic stem cells from human bone marrow (BM) and umbilical cord blood (UCB) are the only sources of transplantable cells used clinically for hematopoietic recovery, but they have limited availability, and only one third of patients have a matched related donor. Evaluation of human hematopoietic cells before transplantation into patients is based on both phenotypic properties, such as the number of blood cells (CD45+) expressing putative HSC markers that include CD34, and functional properties using in vitro clonogenic assays detecting CFUs as a measure of multilineage hematopoietic capacity arising from progenitors. Although not used in preclinical assessment, the nonobese diabetic (NOD)/SCID repopulation assay serves as a surrogate in vivo assay to measure repopulation capacity. In the absence of human experimentation, these in vitro and in vivo assays serve as surrogate indices of human HSC (1, 2). Pluripotent human embryonic stem cells (hESCs) can differentiate into primitive hematopoietic cells, but it is unknown whether transplantable HSCs can be generated.

Because somatic HSC transplantation is in wide clinical use and represents the reference standard for HSC, we used surrogate in vitro and in vivo assays both to assess the HSC potential of hESCs and to compare this potential to somatic HSCs. Here, we directly compare the functional hematopoietic potential of the hESC-derived hematopoietic cells (3) with cord blood (CB)–derived somatic CD45+ cells. Direct femoral injection of hESC-derived hematopoietic cells resulted in multilineage human hematopoietic repopulation, providing
direct evidence of HSC function. However, hESC-derived HSCs possessed unique in vivo behavioral properties differing from somatic HSCs that could be associated with a distinct molecular signature of hESC-derived hematopoietic cells. Our study underscores the importance of functional and molecular comparison of hESC-derived progeny with their somatic counterparts for clinical regenerative therapies.

RESULTS
hESC-derived hematopoietic cells have in vitro clonogenic progenitor activity similar to that of somatic cells, but fail to engraft after intravenous delivery and reduce survival of NOD/SCID recipients

Our group has recently shown that CD45<sup>neg</sup>PFV cells isolated from human embryonic bodies (hEBs) are responsible for the hemogenic potential of hESCs (3), giving rise to >98%–pure populations of CD45<sup>+</sup> hematopoietic cells (Fig. 1 A) and to a substantial proportion of primitive hematopoietic cells that express the stem cell marker, CD34<sup>+</sup> (8.5 ± 2.4%; Fig. 1 A). Compared with highly purified (CD45<sup>+</sup>/CD34<sup>+</sup>/CD38<sup>-</sup>/Lin<sup>-</sup>) somatic UCB-derived hematopoietic cells cultured in identical conditions, hESC-derived hematopoietic cells had equivalent progenitor frequency (Fig. 1 B) and myeloid and erythroid lineage representation (Fig. 1 C). Thus, although surrogate in vitro assays demonstrated intrinsic differences in progenitor differentiation, with hESC-derived cells favoring granulocytic potential (Fig. 1 C), these results indicate that primitive somatic and hESC-derived hematopoietic cells possessed similar progenitor content.
Bona fide HSCs can be defined functionally only by sustained multilineage in vivo reconstitution upon transplantation. Experimentally, the NOD/SCID xenotransplant assay has provided a powerful surrogate to define candidate human hematopoietic stem cells functionally, defined as human SCID-repopulating cells (SRCs) (4). To evaluate in vivo repopulating capacity of hESC-derived hematopoietic cells, $5 \times 10^5$–$1.6 \times 10^7$ hESC-derived hematopoietic cells were transplanted by i.v. tail-vein injection into sublethally irradiated NOD/SCID mice. In contrast with 100% survival of recipient mice receiving a similar dose of cultured primitive somatic hematopoietic cells, $<40\%$ of the mice transplanted with hESC-derived hematopoietic cells survived 8 wk after i.v. transplantation (Fig. 1 D). Unlike cultured somatic hematopoietic cells that successfully repopulated the BM of recipients 8 wk after i.v. transplantation, no chimerism was detected in surviving recipients of hESC-derived hematopoietic cells (Fig. 1 E). Therefore, despite equivalent frequency of CD34$^+$ cells and progenitor (CFU) capacity, the in vivo behavior of i.v.–transplanted hESC-derived hematopoietic cells is distinct from that of their somatic counterparts. We surmised that either HSCs were not generated under these conditions or that HSCs were generated but lacked appropriate intrinsic properties to enable their detection in a traditional human HSC in vivo assay system.

hESC-derived hematopoietic cells form pulmonary emboli in vivo via aggregation in response to rodent serum

Because of poor survival of recipients transplanted by i.v. injection with hESC-derived hematopoietic cells, we examined lungs, heart, brain, spleen, kidneys, liver, pancreas, intestines, and muscle of transplanted mice by serial sectioning and staining 24 h after i.v. transplantation of somatic or hESC-derived hematopoietic cells. In contrast with normal lung structures in mice receiving similar doses of somatic hematopoietic cells (Fig. 2 A), lung tissue from mice receiving hESC-derived hematopoietic cells revealed numerous emboli lodged in small pulmonary capillaries that obstructed the vessel lumens (Fig. 2, B and C). The human origin of these emboli was confirmed by i.v. transplantation of GFP-tagged hESC-derived hematopoietic cells (Fig. 2 C, inset). Because human emboli were detected in the lungs of mice transplanted with untransduced and GFP-transduced hESC-derived hematopoietic cells, transduction is probably not contributing to pulmonary obstruction. To exclude potential physical differences, the cellular size of CD45$^+$ hESC-derived hematopoietic cells was compared with somatic hematopoietic cells after 7 d of liquid culture using forward and side light-scatter properties detected by flow cytometry. hESC and somatic hematopoietic cells were indistinguishable in both size and complexity (Fig. 2 D). In addition, the mean cellular diameter was evaluated and quantitated by microscopy, revealing an average of $11.8 \pm 2.0 \, \mu m (n = 200)$ for somatic hematopoietic cells and $12.3 \pm 2.2 \, \mu m (n = 200)$ for hESC-derived hematopoietic cells. These analyses suggest that difference in the physical size of hESC versus somatic cells is not significant and therefore is unlikely to explain emboli formation in vivo.

With the use of established quantitative assays (5), hESC-derived hematopoietic cells were directly cocultured with isolated mouse blood cells, which were unable to induce a rosette reaction or significant human cell aggregation (unpublished data). However, addition of mouse serum to cultured hESC-derived hematopoietic cells caused immediate aggregation in vitro. Quantitative analysis indicated that, after 2 h of exposure, up to 80% of hESC-derived hematopoietic cells aggregated in response to adult mouse and rat serum, 32% aggregated in response to adult human serum, 21% aggregated in response to human CB and human fetal blood serum, and only 12% aggregated in response to fetal bovine serum (Fig. 2, E and F). In contrast, adult mouse serum did not cause aggregation of somatic hematopoietic cells (Fig. 2, E and F). Based on these observations, the inability to detect human engraftment in NOD/SCID mice from hESC-derived hematopoietic cells may be a result of cellular aggregation and pulmonary emboli formation after systemic delivery by i.v. transplantation that prohibits host survival and seeding of hESC-derived hematopoietic cells to the BM of NOD/SCID recipients.

Detection of hESC-derived human hematopoietic repopulating stem cells via intrafemoral injection into NOD/SCID recipients

To bypass the circulatory system and complications associated with systemic delivery, we transplanted hESC-derived hematopoietic cells by means of intra–bone marrow transplantation (IBMT; references 6–8) directly to the femur. Approximately $4\,–\,15 \times 10^5$ hESC-derived hematopoietic cells differentiated from hemogenic precursors were injected into the femur of sublethally irradiated NOD/SCID mice by IBMT. In contrast with i.v. transplanted mice (Fig. 1 D), $>90\%$ of the mice survived IBMT $>8$ wk (Fig. 3 A), and most surviving mice demonstrated human reconstitution, indicative of human SRC function. Detection of human engraftment in the BM of the injected femur by Southern blot analysis for human-specific $\alpha$-satellite sequences is shown for representative independent experiments (Fig. 3 B). Human hematopoietic graft composition from hESC-derived SRCs was similar to that previously shown for somatic SRCs derived from UCB and included lymphoid (CD45$^+$/CD19$^+$), myeloid (CD45$^+$/CD33$^+$) and erythroid (glycophorin Na$^+$ CD45$^+$/human MHC-1$^+$) hematopoietic lineages as shown in a representative example in Fig. 3 C.

Because dissemination of the hematopoietic graft beyond the injected site of delivery serves as an established in vivo functional property of somatic HSCs (9–11), BM cells obtained from noninjected contralateral femurs and remaining long bones were examined for human chimerism. Using human-specific $\alpha$-satellite sequences, human engraftment from hESC-derived hematopoietic cells was detected in contralateral femurs and other bones but at lower levels than in with the injected femur (Fig. 3 D). Using detection of human
Figure 2. In vivo pulmonary emboli and aggregation of hESC-derived hematopoietic cells in response to mouse serum. Hematoxylin and eosin-stained lung cross-sections of NOD/SCID recipient mice receiving (A) somatic hematopoietic cells and (B) hESC-derived hematopoietic cells, 24 h after i.v. transplantation. Normal lung structures and pulmonary capillary (dashed lines) were observed in mice receiving somatic hematopoietic cells, but mice receiving a similar dose of hESC-derived hematopoietic cells had numerous emboli in the serial lung cross sections. Bar, 10 μm. A representative embolus (B, inset) lodged in the pulmonary capillary (dashed line) causes blood vessel obstruction (B and C). The human origin of pulmonary emboli was confirmed by i.v. transplantation of GFP-expressing hESC-derived hematopoietic cells (C, inset; bar, 10 μm; green, GFP-expressing hESC-derived hematopoietic cells; blue, nucleus stained by DAPI [arrows]). (D) Size and complexity of somatic and hESC-derived hematopoietic cells were evaluated by flow cytometric measurement and by direct comparison of physical size by microscopy. No differences in these properties were detected between somatic and hESC-derived hematopoietic cells. Bar, 50 μm. (E) Microscopic examination for cellular aggregation of hESC-derived hematopoietic cells and somatic hematopoietic cells after 1–2 h in vitro treatment with or without serum from different mouse and human sources. Addition of adult mouse serum did not trigger aggregation of somatic hematopoietic cells but did cause rapid aggregation of hESC-derived hematopoietic cells. Bar, 50 μm. (F) Quantitative analysis of cellular aggregation indicated that up to 80% of hESC-derived hematopoietic cells aggregated in response to addition of adult mouse and rat serum (n = 7); 32% aggregated in response to human adult serum (n = 3); 21% aggregated in response to human neonatal CB serum (n = 3); 12% aggregated in response to human fetal blood serum (n = 3); and 10% aggregated in response to FBS (n = 3). All sera tested were used at 20% by volume.
chimerism in the injected femur and the other long bones as the criterion for engraftment, 11 out of 19 mice transplanted with hESC-derived hematopoietic cells by femoral IBMT demonstrated hESC-derived SRC activity, compared with six of six mice injected with purified somatic cells derived from UCB (Fig. 3 E). Although IBMT delivery of hESC-derived hematopoietic cells provides direct evidence for hESC-derived SRCs, levels of human reconstitution and frequency of detection were limited compared with UCB-derived somatic SRC (Fig. 3 D). To compare in vivo behavior of hESC-derived SRC with somatic SRC further, the level of human chimerism established in the injected femur from IBMT SRCs was compared with that in contralateral femur and other bones (Fig. 3 F). In contrast with somatic SRCs, the majority of hESC-derived SRC engraftment was detected in the injected femur, with only low levels observed beyond the delivery site (Fig. 3 F). The low level of sustained human reconstitution and localized detection suggests that SRCs generated from hESCs are distinct from somatic SRCs and possess limited proliferation and migratory ability in vivo.

hESC-derived hematopoietic cells possess a distinct molecular signature from somatic counterparts

Although our results provide direct evidence that hematopoietic cells derived from hESCs possess the developmental potential to generate transplantable HSCs, in contrast with in vitro progenitors, in vivo behavior of hESC-derived HSCs is limited. To explore the molecular basis that may account for these functional differences, purified CD34+/H11001+/CD38- cells enriched for primitive hematopoietic cells were isolated from hESC and somatic UCB. With the use of microarray, global gene expression analysis revealed that broad families of genes associated with cell–cell contact and migration, cell replication, and transcriptional regulation (including HOX clusters A and B) were differentially expressed in purified hESC-derived and somatic cells (Fig. 4 and Table S1, available at http://www.jem.org/cgi/content/full/jem.20041888/DC1.

hESC-derived cells express higher levels of negative hematopoietic regulator CD164 (12) as well as the migratory

Figure 3. Femoral IBMT of hESC-derived hematopoietic cells in NOD/SCID mice. (A) 8-week survival of NOD/SCID recipients receiving somatic or hESC-derived hematopoietic cells by IBMT. (B) Southern blot analysis of recipient mouse BM DNA using the human-specific α-satellite sequences to demonstrate human chimerism from independent experiments as indicated. (C) Representative example of multilineage human hematopoietic (CD45+) reconstitution of BM cells from the injected femur of NOD/SCID recipients receiving hESC-derived hematopoietic cells that include lymphoid, myeloid, and erythroid lineages. (D) PCR analysis of recipient mouse DNA extracted from BM cells using the human-specific α-satellite sequence to demonstrate human chimerism in the injected femur, contralateral femur, and other long bones. (E) Summary of level of human chimerism in individual mice transplanted with somatic or hESC-derived hematopoietic cells from hemogenic CD45-PPV precursors. (F) Comparative analysis of average level of human chimerism in injected femur, contralateral femur, and other long bones.
and/or adhesion proteins CKLF-1 (13), integrin-β3 (14), matrix metalloproteinase 9, macrophage-inhibiting factor, and monocyte chemoattractant protein–1 (15), which may reduce the ability of hESC-derived hematopoietic cells to migrate beyond the injected site and enter the circulation. In addition, higher levels of CD47 (16), CD24 (17), MMRN (18), PF4 (19), PF4V1 (18), GPIIIA (20), and NINJ2 (21, 22) expression in hESC-derived cells provides a mechanistic basis for the observed aggregate formation resulting in pulmonary emboli. Reduction of CD44 by HAS1 expression may negatively regulate normal in vivo migratory properties of HSCs (23). In contrast with hESC-derived cells, somatic cells expressed substantially higher levels of CXCR4, CD44, and l-selectin, which are involved in appropriate homing and engraftment behavior of candidate human HSCs (24, 25), as well as matrix metalloproteinases, ADAM8 (26) and ADAM17 (27) that are involved in establishing HSC residence within the BM niche.

In addition to inappropriate homing, migration, and aggregation behavior of hESC-derived hematopoietic cells, hESC-derived HSC possessed limited proliferative capacity in vivo based on low levels of human chimerism (Fig. 3 E). Overall, hESC-derived cells differentially expressed genes involved in accelerated cell-cycle progression and loss of stem cell self-renewal ability, whereas somatic cells expressed genes required for maintenance and control of the quiescent cell-cycle status, essential for function of transplantable HSCs (Fig. 4 B). For example, up-regulated genes in somatic cells included cyclin G2 (28), p53 binding protein (29), p21 (30), and p57 (31), all involved in regulating quiescence, pool size, and differentiation of mammalian HSCs (30).

Aside from genes directly associated with the cellular behavior and physiology of HSCs in vivo, specific transcription factors involved in the intrinsic developmental program of candidate HSCs were differentially expressed in hESC-derived and somatic hematopoietic cells (Fig. 4 C). hESC-derived cells overexpress Myc–binding protein that acts downstream of homeobox B4 (HOXB4) (Fig. 4 C) to up-regulate cyclin D3 (Fig. 4 B and reference 32). Also up-regulated are Meis2e, FOG–2, and E2F6, that play crucial roles in the onset of embryonic hematopoiesis, as well as CBF-1 that participates in an essential hematopoietic transcription complex containing RUNX1 and CBF (33). In contrast, somatic cells express genes implicated in the self-renewal and repopulating function of HSCs, such as MLL that regulates HOX genes (34), HLF, which was recently shown to regulate LMO2 that is critical to HSC self-renewal (35), Wnt-targeted TCF4 (36, 37), and Myb that is involved in stem cell commitment (20, 38). Most strikingly, HOX A and B gene clusters demonstrate opposing expression in hESC-derived and somatic hematopoietic cells (Fig. 4 D), suggesting that developmental cues are uniquely specified for HOX genes during hESC differentiation. We propose that limitations in the ability of hESC-derived HSCs to activate a genetic program similar to the spectrum of genes expressed by somatic HSCs accounts for their limited proliferative and migratory capacity in vivo.

Our molecular analysis of primitive hematopoietic cells derived from hESC versus somatic sources indicates that multi-

Figure 4. Comparative analysis of the molecular profile of candidate hESC-derived versus somatic hematopoietic stem cells. Hematopoietic cells derived from both hESC-hemogenic precursors and human UCB were isolated based on the expression of CD34 and absence of CD38 (CD34+/CD38–/CD45–). Total RNA from both hESC-derived and somatic stem cell populations was extracted, and amplified RNA was generated to hybridize human HG-U133AB Affymetrix chips. Expression levels of hESC and somatic CD34+/CD38–/CD45– cells were calculated relative to undifferentiated hESC cells. Differentially regulated genes were defined as those being up-regulated more than twofold and being statistically significant (P < 0.01). Genes differentially expressed in primitive hematopoietic cells derived from hESC and UCB were categorized as those involved in (A) cell–cell contact and migration, (B) cell replication, (C) transcriptional regulators, and (D) HOX genes. A complete dataset of the differential microarray profile is provided in Table S1.
ple gene profiles—specifically the HOX A and B clusters—are inappropriately programmed, suggesting that is unlikely that a single gene will promote a molecular signature to reprogram hESC-derived hematopoietic appropriately. Despite the inability to generate HSC from mouse embryonic stem cell (ESC) lines, similar molecular profiling has not been reported for interspecies comparisons. Recently however, Kyba and colleagues demonstrated that hematopoietic progenitors derived from mouse ESC could be endowed the ability for in vivo reconstitution by ectopic expression of HOXB4 (39). Based on this seminal finding and the limited reconstitution levels detected by intrafemoral delivery of hESC-derived hematopoietic cells (Fig. 3), we tested the potential role ectopic expression of HOXB4 into the hESC-derived hematopoietic cells. Using methodology recently created by our group (40), hESC-derived hemogenic precursors were retrovirally transduced using bicistronic retroviral constructs (40) to express HOXB4 ecotopically (Fig. 5, A and B).

Ectopic expression of HOXB4 has been shown previously to confer a profound in vivo growth advantage on human adult somatic CD34+ cells while impairing myeloid differentiation (41, 42). To ensure that our HOXB4-expressing retroviruses transduce and produce biologically functional HOXB4 proteins, we transduced somatic Lin−/CD34+ cells from CB with vector alone compared with HOXB4-expressing constructs shown in Fig. 5 A. Average transduction efficiencies of 35 and 34% were achieved for vector and HOXB4, respectively. Quantitative PCR confirmed that HOXB4-transduced cells overexpressed HOXB4 (Fig. 5 C). Functionally, cultures seeded with CB Lin−/CD34+ cells expressing HOXB4 showed a threefold increase in cell expansion as compared with vector-transduced CB Lin−/CD34+ cells (Fig. 5 D). Transplantation of HOXB4-transduced CB Lin−/CD34+ cells into NOD/SCID mice demonstrated an eightfold increase in reconstitution capacity compared with vector-transduced human HSCs and a marked enhancement in the generation of primitive CD34+ cells (24.7 vs. 16.6% on average; Fig. 5 F). As predicted, HOXB4 overexpression reduced myeloid differentiation from transduced human HSCs, reflected in both myeloid CFU capacity in vitro (Fig. 5 E) and myeloid lineage composition in vivo (Fig. 5 F). These results indicate that our retroviral system for inducing HOXB4 ectopic expression in human stem cells is functional and consistent with the reported effects of HOXB4. Previous findings support the functionality of the HOXB4 protein produced by our retroviral system in human somatic stem cells (41, 42).

hESC-derived hemogenic precursors were transduced with vector or with HOXB4-expressing retrovirus using recently reported approaches (40). Like techniques used to evaluate HOXB4 expression in transduced somatic cells (Fig. 5 C), quantitative PCR demonstrated that HOXB4 expression in hESC-derived hematopoietic cells transduced with HOXB4 was 60-fold greater than HOXB4 expression in vector-transduced cells (n = 3). Analogous to HOXB4-transduced somatic cells, ectopic expression of HOXB4 increased the total cell expansion of cultured hESC-derived hematopoietic cells (>2.5-fold) as compared with vector-transduced cells (Fig. 5 G), whereas the survival of HOXB4- and vector-transduced hematopoietic cells, measured by percentage excluding 7-AAD, was equivalent (79.4 ± 16.1 vs. 78.2 ± 15.1, respectively; n = 4; Fig. 5 G). These observations suggest that ectopic expression of HOXB4 enhances the proliferative capacity of hESC-derived hematopoietic cells that results in overall cell expansion. However, HOXB4 did not affect the developmental capacity of hematopoietic cells. Progenitor frequency and lineage development by CFU (Fig. 5 H) and phenotype (Fig. 5 I) of HOXB4-transduced hESC-derived hematopoietic cells were similar to vector-transduced cells. We were unable to achieve a more robust reconstituting ability by IBMT of HOXB4-transduced hESC-derived hematopoietic progeny into NOD/SCID recipients (Fig. 5, J and K). Our data suggest HOXB4 is unable to induce hematopoietic repopulating capacity from hESCs, underscoring the notion that single genes, such as HOXB4, are unlikely to represent a master gene capable of conferring engraftment potential to hESC-derived hematopoietic cells. Collectively, these results support our original predication from combined functional assays and comparative molecular profiling analysis (Figs. 1–4) that single genes are unlikely to alter in vivo functionality of hESC-derived cells.

**DISCUSSION**

This study establishes that candidate HSC can be derived from hESC and can be functionally examined with a variety of in vitro and in vivo surrogate measures of stem cell and progenitor function. Given the difficulty of obtaining transplantable murine HSCs from mouse ESCs, it is interesting that, with the exception of ectopic HOXB4 expression (39), the repopulating function of hESC-derived hematopoietic cells could be detected here. The IBMT delivery method used seems essential for detecting hematopoietic potential from hESC-derived progeny, similar to that recently shown using mouse ESC-derived hematopoietic cells (43). Although hematopoietic repopulating cells derived from hESC were observed in our study, the proliferative and migratory properties of these cells was significantly different from those of somatic HSCs. Insights into the molecular basis of these differences could be correlated to changes in gene expression of broad families of genes that are involved specific cellular processes (e.g., homing, adhesion, cell cycle). Because these differences between hESC-derived progeny and somatic cells may not be restricted to the hematopoietic lineage alone, our results have a number of important implications for future application of hESC for therapeutic purposes that extend beyond development of HSC from hESC lines. This notion is supported by years of studies in the murine model, in which mouse ESC progeny behave differently than would be expected from adult cells of the same lineage (44, 45).

Our study provides additional insights into hESC biology and strategies. First, comparisons must be made between...
Figure 5. Ectopic expression of HOXB4 induces proliferation but does not confer engraftment potential on hESC-derived hematopoietic cells. (A) Control vector (GFP) and HOXB4 bicistronic retroviral construct. HOXB4 cDNA was subcloned into the control vector backbone upstream of an IRES. Enhanced GFP (eGFP) cDNA downstream of IRES sequence acts as a reporter for selection of stable cell lines and tracking of transduced cells. (B) Western blot analysis of PG13 packaging cell line transduced with vector or HOXB4 retrovirus showing specific HOXB4 expression from HOXB4-containing retrovirus. (C) Quantitative RT-PCR confirmed that HOXB4-transduced cells had up to 300-fold increase in HOXB4 expression as compared with controls shown. (D) HOXB4-overexpressing CB Lin-CD34+ cells show a threefold in vitro proliferative advantage after 4 d culture as compared with their GFP-expressing counterparts (n = 4). (E) Vector and HOXB4-transduced CB Lin-CD34+ were seeded in methylcellulose assays,
hESC-derived stem cells and somatic stem cells. Importantly, the most appropriate surrogate stem cell assay must be used to gain an accurate picture. The in vitro progenitor assays did not predict the significant functional differences between the two sources of HSC. Indeed, even the reference standard repopulation assay based on i.v. transplantation was ineffective because of the unique cellular aggregation properties of the hESC-derived cells that resulted in lethality. Our study demonstrates that IBMT is an absolute requirement for functional in vivo assessment of candidate HSCs derived from hESC and demonstrates the power of this method to assess HSC activity of novel populations (8).

Second, although HSCs were generated from hESC, the current methodology was not sufficient to induce a cellular and molecular program reflective of a somatic HSC. This result parallels the inability to reprogram accurately all types of nuclei by nuclear transfer (46). The fact that several genes classes are differentially expressed among hESC-derived and somatic primitive hematopoietic cells reinforces the notions that single gene products do not define the molecular nature of HSCs and that a mosaic of combinatorial factors are required to confer HSC function. This finding suggests that single-gene reconstitution strategies are unlikely to restore HSC function of hESC-derived hematopoietic cells. Recent evidence for the role of HOX gene products (47) and cell cycle regulators (48) in mammalian HSC behavior indicates that modulation of these classes of genes is liable to affect in vivo behavior of HESC-derived HSCs by affecting a broad range of target genes necessary to establish functional HSCs. Our study evaluates this issue directly by expressing HOXB4, the best-known candidate for conferring HSC function on mammalian hematopoietic cells derived from ESC lines. We demonstrate that ectopic expression of HOXB4 induced proliferation but had no affect on HSC function of hESCs, thereby supporting our original supposition.

Third, this report provides the foundation for optimizing and improving differentiation methodology using extrinsic factors that mimic better the in utero orchestration of genetic programs required for generation of HSCs. These factors may include signaling pathways shown to be involved in embryonic specification of the hematopoietic lineage and also involved in somatic HSC self renewal, such as the Notch, Wnt, and hedgehog pathways. A strategy using both in vivo and molecular comparisons of hESC-derived cell types with their somatic counterparts will be instrumental in elucidating the fundamental principles required to generate tissue-specific stem cells from hESCs and to guide future applications of hESC-based regenerative therapies independent of lineage and disease target. We propose that similar preclinical modeling approaches will be required to develop responsibly appropriate and successful methods for using hESC in cell replacement.

MATERIALS AND METHODS
Culture of hESCs, formation of embryoid bodies, and isolation of hemogenic CD45**-PFV precursors. Maintenance of undifferentiated hESC lines H9 and H1 originally derived by Thomson et al. (49) were cultured in feeder-free conditions (50), and formation and dissociation of hEBs were performed as previously described by our group (51). The hemogenic precursor population from d 10 hEBs was purified according to their expression of PECAM-1, coexpression of Flk1+ and VE-cadherin, and lack of CD45 (3). Single cells dissociated from d 10 hEBs were stained with human PECAM-1 or Flk-1-FITC (BD Biosciences) and CD45-APC (Becton Dickinson), followed by 7-AAD (Immunotech) staining to exclude dead cells (3). The hemogenic precursors were separated by FACSVantage SE (Becton Dickinson). Isolation gates, including histogram markers and dot plot quadrants, were set based on respective IgG isotype controls. Purity was determined immediately after sorting and was consistently >95%.

Differentiation of hESC-derived hematopoietic cells. The hemogenic precursor population was seeded on fibronectin-coated plates (10⁵ cells/cm²) and cultured for 7 d in serum-free liquid medium (3) previously shown to sustain human hematopoietic stem cells (52). Serum-free medium consisted of 9500 BIT media (StemCell Technologies, Inc.), 2 mM 1-glutamine (GIBCO BRL), 10⁻⁴ M β-mercaptoethanol, 300 ng/ml rhu-stem cell factor (SCF; Amgen), 50 ng/ml rhu-granulocyte colony-stimulating factor (G-CSF; Amgen), 50 ng/ml rhu-granulocyte colony-stimulating factor (G-CSF; Amgen), 300 ng/ml rhu-Flt-3L, 10 ng/ml rhu-IL-3, and 10 ng/ml rhu-IL-6 (all obtained from R&D Systems). The serum-free medium and growth factors were replaced every 2 d.

Histopathological examination. Mouse tissues were embedded in Tissue-Tek OCT (Sakura Finetechnical Co., Ltd.) and snap-frozen in liquid nitrogen. Serial cryosections (5 μm) were cut and stained with hematoxylin and eosin. To track cell locations after i.v. injection, hESC-derived hematopoietic cells differentiated from GFP-expressing H7 hESCs were used. Serial cryosections from the lungs were directly visualized with an Olympus confocal laser-scanning microscope.
Aggregation assay of hESC-derived hematopoietic cells. hESC-derived hematopoietic cells were resuspended in serum-free medium and seeded in a 96-well plate (2 x 10^4 cells/well) for 2 h at 37°C in the absence or presence of 20% individual adult mouse serum (n = 3), adult human serum (n = 3), human CB serum (n = 3), human fetal blood serum (n = 3), or fetal bovine serum (n = 3). Three microphotographs per well were taken on contiguous fields at 10-fold magnification. Singletons, doublets, and triplets were counted as nonaggregated cells (5). Results were expressed relative to the number of nonaggregated cells obtained in serum-free medium (N0). The percentage of aggregated cells was calculated as [N - N0/N] x 100%, where N represents the number of nonaggregated cells in medium containing n serum (~3), human CB serum (~3), human fetal blood serum (~3) or presence of 20% individual adult mouse serum (n = 3). Adult human serum (n = 3) was used in the absence of individual adult mouse serum, and adult human serum was used in the presence of individual adult mouse serum.

CFU assays. Cells indicated in the text were plated into methylcellulose H4230 (StemCell Technologies, Inc.) supplemented with human growth factors, including 50 ng/ml SCF, 3 U/ml erythropoietin (Epo; Amgen), 10 ng/ml H4230 (StemCell Technologies, Inc.) supplemented with human growth factor and 20% serum. Specimens were coded during analysis for blinded assessment.

Intravenous and IBMT of NOD/SCID mice. hESC-derived hematopoietic cells were transplanted into sublethally irradiated 8–10 wk-old NOD/SCID (3.5 Gy) or NOD/SCID/H11024m−/− mice (3.25 Gy) using either an i.v. (54) or IBMT technique (6–8). For IBMT, the leg of the anesthetized mouse was flexed; a 27-gauge needle was inserted into the femur at the knee joint and then replaced with a 28-gauge insulin syringe containing 25 µl of cell suspension. Cell doses ranged from 5 x 10^4–1.6 x 10^5 for i.v. and 4–15 x 10^4 for IBMT. All experiments using mice received approval from our local authority, the Animal Care and Veterinary Services of the University of Toronto.

Retroviral vectors, packaging cell lines, and transduction of CB Lin−/CD34− cells and hESC-derived hematopoietic cells. A 1.15-kb EcoRI-Xhol fragment encoding for the full length human HOXB4 gene (41, 42, 55–57) from pBluescript II KS-HOXB4 (a gift from G. Sauvageau, Institute of Research in Immunology and Cancer, Montreal, Quebec, Canada) was subcloned into EcoRI-Sall sites of the MIEV retroviral vector (58) (a gift from R. Hawley, Holland Laboratory, American Red Cross, Rockville, MD), upstream of an internal ribosomal entry site (IRES) and an enhanced GFP reporter gene, as previously described (59).

Construction of GalV-pseudotyped PG13 stable packaging cell line releasing control vector (GFP) or HOXB4 retroviral particles as well as production and collection of the retroviral particles was done as previously described in detail (59). Purification and retroviral transduction of both CB Lin−/CD34− cells and hESC-derived CD45+94FV precursors was done as recently reported (40, 59). The PG13 packaging cell line was transduced with control vector (GFP) or HOXB4 retrovirus, sorted for GFP+ expression and analyzed by Western blotting for detection of HOXB4 protein.

Cell lysates were prepared from 10^6 cells by using lysis buffer containing 10% Triton X-100, 1M Tris-HCl, 0.5 EDTA, and protease inhibitors leupentin and aprotonin at 10 mg/ml. Proteins were separated using 10% SDS-PAGE and transferred to polyvinyl difluoride membrane. Equal amounts of proteins were loaded per lane as determined by Ponceau staining (0.1% wt/vol in 5% acetic acid). Membranes were blocked with 5% skim milk and blotted with rat anti-HOXB4 antibody 112 at 1 µg/ml (purchased from Developmental Studies Hybridoma Bank at the University of Iowa). Membranes were washed and stained with rabbit anti-rat horseradish peroxidase antibody at 200 ng/ml (Santa Cruz Biotechnology, Inc.). Signal was detected by means of enhanced chemiluminescence system (ECL; Amsham Biosciences) and quantified using an imaging detection and analysis station (Alpha Innomtech Corporation).

Quantitative RT-PCR. mRNA was extracted from FACS-isolated populations and reverse transcribed into cDNA using mRNA extraction and first-strand cDNA synthesis kits (Amsham Biosciences) according to the manufacturer’s instructions. Expression of HOXB4 (forward primer: 5′-AGCACGGTAACAACCCTATTAC-3′ and reverse primer: 5′-CGTGTT CAGGTAGCGGTGTAG-3′) was quantified by quantitative RT-PCR (Mx4000, Stratagene) using SYBR green (Invitrogen) DNA-binding dye. The PCR conditions were 2 mM MgCl2, 0.4 mM dNTP, 8% glycerol, 3% DMSO, 150 nM of each primer, 0.75 µl of 1/1,000 dilution of reference dye, and 2.5 µl of 1/2,000 dilution of SYBRGreen. Quantitative PCR reaction conditions were primary denaturation at 95°C for 1 min and 40 cycles of PCR consisting of 95°C for 10 s, 60°C for 1 min, and 72°C for 30 s, followed by analyzing the amplified products using the dissociation curve. The signal intensities were normalized against GAPDH (forward primer: 5′-TGACACCCACAAGCTTGACC-3′ and reverse primer: 5′-GGCATGT GAATGGTTCATGAC-3′), and the 2^ΔΔCt equation (60) was used to calculate the relative expression of HOXB4.

Analysis of NOD/SCID mouse engraftment. To prepare mouse BM cells for flow cytometric analysis, red cells were lysed with 0.8% ammonium chloride solution, and the remaining cells were washed in PBS containing 5% FBS. The presence of human cells in the transplanted NOD/SCID and NOD/SCID/H11024m−/− mice was determined by flow cytometry using FITC-conjugated antibody against either human CD45 or glycoporphin-A and phycoerythrin-conjugated antibody against human CD19, CD3, CD34, or CD36 (all conjugated antibodies from Becton Dickinson). In parallel, Southern blot and PCR analyses were performed to detect human DNA in mouse bone marrow.

High-molecular-weight DNA was isolated using phenol/chloroform extraction or DNAzol reagent (GIBCO BRL) according to the manufacturer's instructions. 1 µg DNA was digested with EcoRI restriction enzyme at 37°C overnight, separated on an agarose gel, transferred to Hybond-N+ nylon membrane (Amsham Biosciences) and hybridized with a 3P-labeled human chromosome 17–specific α-satellite probe as previously described (54). The level of human cell engraftment was quantified using a phosphomorphager and ImageQuant software (Amsham Biosciences) by comparing the characteristic 2.7-kb band with human-mouse DNA mixture control (limit of detection, <0.1% human DNA). In cases where the level of human DNA was <0.1%, PCR for the human chromosome 17–specific α-satellite was performed as previously described (7). Forward primer 5′-ACACTCCTTTTTGGAGCATCTA-3′ and backward primer 5′-AGCAATGTGAAACTCTGGGA-3′ were used to amplify a 1171-bp sequence (40 cycles, 94°C for 30 s, 60°C for 30 s, 72°C for 15 s, followed by a final extension of 10 min at 72°C). The criterion for mouse engraftment was the presence of human DNA in both the injected femur and nontransplanted bone marrow.

Molecular profiling. Total RNA was extracted using the QIAGEN RNAeasy kit and was amplified using Message Amp aRNA kit (Ambion). 15 µg of fragmented antisense RNA was used for hybridizing human HG-U133AB arrays (Affymetrix, Inc.) on the London Regional Genomic Center, Ontario, Canada. GeneSpring 6.0 was used for data analysis. Genes that were flag-passed in at least one of the populations and significantly (P < 0.01, different by twofold) differentially expressed between hESC- and UCB-derived CD34+/CD45− cells were identified.

Online supplemental materials. Table S1, available at http://www.jem.org/cgi/content/full/jem.20041888/DC1, provides a complete list of differentially expressed genes.
REFERENCES

1. Siena, S., M. Bregni, B. Brando, N. Belli, F. Ravagnani, L. Gandola, A.C. Stern, P.M. Lansdorf, G. Bonadonna, and A.M. Gianni. 1991. Flow cytometry for clinical estimation of circulating hematopoietic progenitors for autologous transplantation in cancer patients. Blood. 77: 400–409.

2. Heimfeld, S. 2003. HLA-identical stem cell transplantation: is there an optimal CD34 cell dose? Bone Marrow Transplant. 31:839–845.

3. Wang, L., L. Li, F. Shojai, P. Menendez, T. Martin, A. Rouleau, and M. Bhatta. 2004. Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangio blasts properties. Immunity. 21:31–41.

4. Wang, J.C., C. Dorrell, C.Y. Ito, T. Inamatsu, G. Guenechea, O.I. Gan, and J.E. Dick. 2001. Normal and leukemic human stem cells assayed in immune-deficient mice. In Hematopoesis: A Developmental Approach. L.I. Zone, editor. Oxford University Press, New York. 99–118.

5. Perrault, C., N. Azenberg, P. Legendre, G. Rastegar Lari, D. Meyer, J.A. Lopez, and D. Baruch. 1999. Modulation by heparin of the interaction of the A1 domain of von Willebrand factor with glycoprotein Ib. Blood. 94:4186–4194.

6. Wang, J., T. Kimura, R. Asada, S. Harada, S. Yokota, Y. Kawamoto, Y. Fujimura, T. Tsuji, S. Ichara, and Y. Sonoda. 2003. SCID-repopulating cell activity of human cord blood-derived CD34+ cells assured by intra-bone marrow injection. Blood. 101:2924–2931.

7. Yahata, T., K. Ando, T. Sato, H. Miyatake, Y. Nakamura, Y. Muguruma, S. Kato, and T. Hotta. 2003. A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood. 101:2905–2913.

8. Mauzurier, F., M. Doedens, O.I. Gan, and J.E. Dick. 2003. Rapid myeloid-erythroid repopulation after intrafemoral transplantation of NOD/SCID mice reveals a new class of human stem cells. Nat. Med. 9:559–963.

9. Lapidot, T. 2001. Mechanism of human stem cell migration and repopulation of NOD/SCID and B2mnull NOD/SCID mice. The role of SDF-1/CXCR4 interactions. Ann. N. Y. Acad. Sci. 938:83–95.

10. Plett, P.A., S.M. Frankovitz, and C.M. Orschell. 2003. Distribution of marrow repopulating cells between bone marrow and spleen early after transplantation. Blood. 102:2285–2291.

11. Jetmore, A., P.A. Plett, X. Tong, F.M. Wolber, R. Breese, R. Babic, I., A. Schallhorn, F.P. Lindberg, and F.R. Jirk. 2000. SHPS-1 induces aggregation of Ba/F3 pro-B cells via an interaction with CD47. J. Immunol. 164:3652–3658.

12. Kuppert, M., S. Augnier, M. Hubbe, H. Yagita, and P. Altevogt. 1995. The L1 adhesion molecule is a cellular ligand for VLA-5. J. Cell Biol. 131:1881–1891.

13. Fayard, C.P., G.E. Rivard, W.H. Kane, J. Drouin, S. Zheng, J.C. Moore, and J.G. Kelton. 1996. An autosomal dominant, qualitative platelet disorder associated with multimerin deficiency, abnormalities in platelet factor V, thromboplasmin, von Willebrand factor, and fibronogen and an epinephrine aggregation defect. Blood. 87:4967–4978.

14. Gerotziafas, G.T., I. Eldamy, C. Lecompte, J. Lebras, M. Mirmahfi, F. Potervin, T. Lecompte, and M.M. Sanmana. 2001. The role of platelet factor 4 in platelet aggregation induced by the antibodies implicated in heparin-induced thrombocytopenia. Blood Coagul. Fibrinolysis. 12:511–520.

15. Friedman, A.D. 2002. Runx1, c-Myb, and C/EBPalpha couple differentiation to proliferation or growth arrest during hematopoiesis. J. Cell. Biochem. 86:624–629.

16. Araki, T., and J. Milbrandt. 2000. Ninjurin2, a novel homophilic adhesion molecule, is expressed in mature sensory and enteric neurons and promotes neurite outgrowth. J. Neurosci. 20:187–195.

17. Araki, T., D.B. Zatrchne, N.C. Popescu, and J. Milbrandt. 1997. Mechanism of homophilic binding mediated by ninjurin, a novel widely expressed adhesion molecule. J. Biol. Chem. 272:21373–21380.

18. Brincik, J., and P. Heldin. 1999. Expression of recombiant hyaluronan synthase (HAS) isoforms in CHO cells reduces cell migration and cell surface CD44. Exp. Cell Res. 252:342–351.

19. Kollet, O., I. Pett, J. Kahn, S. Samara, A. Dar, A. Peled, V. Deutsch, M. Gunetti, W. Piacibello, A. Nagler, and T. Lapidot. 2002. Human CD34+CD38(-)CXCR4(-) sorted cells harbor intracellular CXCR4, which can be functionally expressed and provide NOD/SCID repopulation. Blood. 100:2778–2786.

20. Peled, A., I. Pett, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, A. Nagler, H. Ben-Hur, A. Many, L. Shultz, et al. 1999. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science. 283:845–848.

21. Higuchi, Y., A. Yasui, K. Matsuura, and S. Yamamoto. 2002. CD156 transgenic mice. Different responses between inflammatory types. Pathobiology. 70:47–54.

22. Bousso, P., T.R. Lenhard, T. Kirkegaard, J.J. Peschon, R.A. Black, M.J. Delaissance, and M. del Carmen Ovejero. 2003. An assessment of ADAMs in bone cells: absence of TACE activity prevents osteoclast recruitment and the formation of the marrow cavity in developing long bones. FEBS Lett. 553:257–261.

23. Bennin, D.A., A.S. Don, T. Brake, J.L. McKenzie, H. Rosenbaum, L. Ortiz, A.A. DePaoli-Roach, and M.C. Horne. 2002. Cyclin G2 association with p53-mediated transcriptional activation by the p53-binding domain of Mdm2. J. Biol. Chem. 277:27440–27447.

24. Iwabuchi, K., H. Li, H.F. Massa, J. Brisk, T. Date, and S. Fields. 1998. Stimulation of p53-mediated transcriptional activation by the p53-binding proteins, 53BP1 and 53BP2. J. Biol. Chem. 273:26061–26068.

25. Cheng, T., N. Rodrigues, H. Shen, Y. Yang, D. Dombkowski, M. Sykes, and D.T. Scadden. 2000. Hematopoetic stem cell quiescence maintained by p21cip1/waf1. Science. 287:1804–1808.

26. Hayama, I., H. Ohashi, Y. Fukushima, Y. Kaneko, M. Inoue, Y. Komoto, A. Okada, S. Ohashi, A. Nabetani, H. Morosaki, et al. 1996. An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. Nat. Genet. 14:171–173.

27. Satoh, Y., I. Matsumura, H. Tanaka, S. Etoe, H. Sugahara, M. Mizuki, H. Shiyabana, E. Ishiko, J. Ishiko, K. Nakajima, and Y. Kanakura. 2004. Roles for c-Myc in self-renewal of hematopoietic stem cells. Nat. Genet. 36:437–443.

28. Warren, A.J., W.H. Collode, M.B. Carlton, M.J. Evans, A.J. Smith, and T.H. Rabbitts. 1994. The oncogenic cysteine-rich LIM domain

The authors have no conflicting financial interests.

Submitted: 13 September 2004
Accepted: 6 April 2005
36. Douglas, K.R., M.L. Brinkmeier, J.A. Kennell, P. Eswara, T.A. Harrison, A.I. Patmanakov, B.S. Sprecher, M.A. Potok, R.H. Lyons Jr., O.A. MacDougald, and S.A. Camper. 2001. Identification of members of the Wnt signaling pathway in the embryonic pituitary gland. *Mamm. Genome.* 12:843–851.

37. Murdoch, B., K. Chadwick, M. Martin, F. Shojaei, K.V. Shah, L. Gallacher, R.T. Moon, and M. Bhatia. 2003. Wnt-5A augments repopulating capacity and primitive hematopoietic development of human blood stem cells in vivo. *Proc. Natl. Acad. Sci. USA.* 100:3422–3427.

38. Oelgeschlager, M., R. Janknecht, J. Krieg, S. Schreek, and B. Luscher. 1996. Interaction of the co-activator CBP with Myb proteins: effects on Myb-specific transactivation and on the cooperativity with NF-M. *EMBO J.* 15:2771–2780.

39. Kyba, M., R.C. Perlingeiro, and G.Q. Daley. 2002. HOXB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell.* 109:33–47.

40. Menendez P, W.L. Chadwick, L. Li, and M. Bhatia. 2004. Retroviral transduction of hematopoietic cells differentiated from human embryonic stem cell derived CD45negPFV hemogenic precursors. *Mol. Ther.* 11:1109–1120.

41. Mil İzmir, M.D., R. Duxbury, D. Gagen, R.K. Humphries, M. Schmidt, C. von-Kalle, and L.J. Fairbarn. 2005. Overexpression of HOXB4 confers a myeloerythroid differentiation delay in vitro. *Leukemia.* 19:148–153.

42. Schiedammer, B., H. Klump, E. Will, G. Arman-Kalcek, Z. Li, Z. Wang, A. Kimek, J. Friel, C. Baum, and W. Ostertag. 2003. High-level ectopic HOXB4 expression confers a profound in vivo competitive growth advantage on human cord blood CD34+ cells, but impairs lymphomyeloid differentiation. *Blood.* 101:1759–1768.

43. Butt, R.K., L. Verda, D.A. Kim, Y. Oyama, K. Luo, and C. Link. 2004. Embryonic stem cells as an alternate marrow donor source: engraftment without graft-versus-host disease. *J. Exp. Med.* 199:895–904.

44. Kim, J.H., J.M. Auerbach, J.A. Rodriguez-Gomez, I. Velasco, D. Gavin, N. Lummelsky, S.H. Lee, J. Nguyen, R. Sanchez-Pernaute, K. Bankiewicz, and R. McKay. 2002. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson’s disease. *Nature.* 418:50–56.

45. Lummelsky, N., O. Blondel, P. Laeng, I. Velasco, R. Ravin, and R. McKay. 2001. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. *Science.* 292:1389–1394.

46. Rideout, W.M. III, K. Eggan, and R. Jaenisch. 2001. Nuclear cloning of the Wnt signaling pathway in the embryonic pituitary gland. *Mamm. Genome.* 12:843–851.

47. Davidson, A.J., P. Ernst, Y. Wang, M.P. Dekens, P.D. Kingley, J. Pals, S.J. Korsmeyer, G.Q. Daley, and L.I. Zon. 2003. cdx4 Mutants fail to specify blood progenitors and can be rescued by multiple HOX genes. *Nature.* 423:300–306.

48. Yuan, Y., H. Shen, D.S. Franklin, D.T. Scadden, and T. Cheng. 2004. In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. *Nat. Cell Biol.* 6:436–442.

49. Thomson, J.A., J. Ishkovitz-Skelator, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, and J.M. Jones. 1998. Embryonic stem cell lines derived from human blastocysts. *Science.* 282:1145–1147.

50. Xu, C., M.S. Inokuma, J. Denham, K. Golds, P. Kundu, J.D. Gold, and M.K. Carpenter. 2001. Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* 19:971–974.

51. Chadwick, K., L. Wang, L. Li, P. Menendez, B. Murdoch, A. Rouleau, and M. Bhatia. 2003. Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. *Blood.* 102:906–915.

52. Bhatia, M., D. Bonnet, U. Kapp, J.C. Wang, B. Murdoch, and J.E. Dick. 1997. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. *J. Exp. Med.* 186:619–624.

53. Eaves, C., C. Miller, E. Connelly, J. Audet, R. Oostendorp, J. Cashman, P. Zandstra, S. Rose-John, J. Piret, and A. Eaves. 1999. Introduction to stem cell biology in vitro. *Threshold to the future. Am. NY Acad. Sci.* 872:1–8.

54. Bhatia, M., D. Bonnet, B. Murdoch, O.I. Gan, and J.E. Dick. 1998. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat. Med.* 4:1038–1045.

55. Antonchuk, J., G. Sauvageau, and R.K. Humphries. 2002. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell.* 109:39–49.

56. Brun, A.C., X. Fan, J.M. Bjornsson, R.K. Humphries, and S. Karlsson. 2003. Enforced adenoviral vector-mediated expression of HOXB4 in human umbilical cord blood CD34+ cells promotes myeloid differentiation but not proliferation. *Mol. Ther.* 8:618–628.

57. Giannola, D.M., W.D. Shlomchik, M. Jegathesan, D. Liebowitz, C.S. Abrams, T. Kadesch, A. Dancis, and S.G. Emerson. 2000. Hematopoietic expression of HOXB4 is regulated in normal and leukemic stem cells through transcriptional activation of the HOXB4 promoter by upstream stimulating factor (USF)-1 and USF-2. *J. Exp. Med.* 192:1479–1490.

58. Wiznerowicz, M., A.Z. Fong, R.G. Hawley, and A. Mackiewicz. 1998. Development of a double-copy bicistronic retroviral vector for human gene therapy. *Adv. Exp. Med. Biol.* 451:441–447.

59. Chadwick, K.S.F., L. Gallagher, M. Bhatia. 2005. Smad7 alters cell fate decisions of human hematopoietic repopulating cells. *Blood.* 105:1905–1915.

60. Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–ΔΔC(T)) Method. *Methods.* 25:402–408.