Embryonic stem cell-derived hematopoietic stem cells

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Despite two decades of studies documenting the in vitro blood-forming potential of murine embryonic stem cells (ESCs), achieving stable long-term blood engraftment of ESC-derived hematopoietic stem cells in irradiated mice has proven difficult. We have exploited the Cdx-Hox pathway, a genetic program important for blood development, to enhance the differentiation of ESCs along the hematopoietic lineage. Using an embryonic stem cell line engineered with tetracycline-inducible Cdx4, we demonstrate that ectopic Cdx4 expression promotes hematopoietic mesoderm specification, increases hematopoietic progenitor formation, and, together with HoxB4, enhances multilineage hematopoietic engraftment of lethally irradiated adult mice. Clonal analysis of retroviral integration sites confirms a common stem cell origin of lymphoid and myeloid populations in engrafted primary and secondary mice. These data document the cardinal stem cell features of self-renewal and multilineage differentiation of ESC-derived hematopoietic stem cells.

Cdx4 | clonal analysis | HoxB4

Transplantation of bone marrow (BM)-derived hematopoietic stem cells (HSCs) is the standard treatment for high-risk leukemia and a range of genetic disorders of the blood. However, a shortage of HLA-matched BM donors and the inability to culture and genetically repair BM-derived HSCs in vitro have limited more widespread therapeutic applications (1). When generated by somatic cell nuclear transfer, pluripotent embryonic stem cells (ESCs) provide a theoretically unlimited source of autologous hematopoietic progenitors and an alternative strategy for treating leukemia and genetic bone marrow disorders (2, 3). Although ESCs can differentiate into all lineages of the blood system in vitro, efficient production of functional HSCs that can reconstitute all hematopoietic lineages in vivo has proven difficult (4).

One approach to obtain definitive HSCs from ESCs is to enforce expression of genes that stimulate hematopoiesis or enhance HSC function. The homeodomain gene HoxB4 has been shown to enhance competitive engraftment of murine BM-HSC and induce proliferation of progenitors from human cord blood without inducing leukemia, thereby making HoxB4 an excellent candidate gene for our studies (5–11). Previously, we successfully engrafted lethally irradiated mice with ESC-derived hematopoietic progenitors engineered to ectopically express HoxB4. When introduced into hematopoietic precursors dissected from the precirculation murine yolk sac, HoxB4 promoted long-term multilineage engraftment, suggesting that this homeodomain gene helped specify definitive hematopoietic fate from primitive hematopoietic progenitors (12). However, the extent and durability of lymphoid engraftment from either ESCs or yolk sac populations was minimal in these engrafted animals, possibly due to the inability to fully pattern definitive HSCs from these embryonic populations.

Our understanding of how Hox genes promote hematopoietic specification has been greatly advanced by insights into the role of Cdx4, which along with Cdx1 and Cdx2 represent a family of caudal-related homeobox-containing transcription factors that specify posterior tissue fates and mediate anterior-posterior patterning through modulation of hox gene expression (13–15). Cdx4 was shown to be necessary for blood formation in the zebrafish and to promote hematopoietic colony formation when ectopically expressed in ESCs (16). Cdx4 null zebrafish have reduced expression of hematopoietic genes, including SCL, Runx1, and GATA1, whereas overexpression of Cdx4 induces ectopic blood formation and alters Hox gene expression patterns, including up-regulation of HoxB4 (16). Cdx1 functions redundantly to promote blood formation in zebrafish (Alan Davidson, personal communication). Cdx2 is a translocation partner of TEL (ETV6) in human acute myeloid leukemia (17), and overexpression of Cdx2 alone results in transplantable acute myeloid leukemia in a mouse model (18). These findings suggest that a genetic pathway involving cdx and hox genes plays an essential role in blood formation and provide a central mechanism for driving hematopoietic specification from ESCs.

In this study, we have explored the effect of Cdx4 expression on hematopoiesis in the murine ESC system. Using a murine ESC line with tetracycline-inducible Cdx4, we demonstrate that Cdx4 promotes commitment to hematopoietic mesoderm, stimulates hematopoietic progenitor formation from ESCs, and promotes lymphoid potential of ESC-derived HSCs. Using ESCs engineered to ectopically express both Cdx4 and HoxB4, we demonstrate radioprotection and robust and stable engraftment of hematopoietic lineages in irradiated mice. Moreover, we apply proviral integration analysis in fractionated myeloid and lymphoid lineages of primary and secondary mice to document the clonal derivation of self-renewing, multipotential HSCs from ESCs.

Methods

Cell Culture. ESCs were maintained and differentiated according to published protocols in ref. 12. Doxycycline was added to the culture medium from day 3 to day 4 at 0.1 μg/ml and from day 4 to 6 at 0.5 μg/ml to induce Cdx4 expression. Cells were harvested at day 6 by collagenase treatment. A total of 10⁶ embryoid body (EB) cells were plated onto semiconfluent OP9 cells in six-well dishes and were infected with retroviral supernatants, produced in 293 cells by Fugene (Roche) cotransfection of viral plasmid MSCV-HoxB4-Gires-GFP and packaging-defective helper plasmid, pCL-Eco. Infected EB cells were cultured according to protocols in ref. 12. Blast colony forming/replating assay and hematopoietic colony formation assay were performed as described in refs. 19 and 20.

RT-PCR Analysis and Quantitative Real-Time PCR. Cells were harvested in RNA Stat-60 (Tel-Test), and total RNA was isolated. All RNA samples were treated with DNaseI and purified by RNaseq MinElute kit (Qiagen). cDNAs were prepared according to the manufacturer’s instruction (Invitrogen). Real-time PCR was performed in triplicates with TaqMan reagent kits (Applied Biosystems) on an ABI Prism 7700 Sequence Detector. GFP DNA levels were quantified into arbitrary units by using the comparative Ct method (relative to the TDAG51 gene as an internal normalization control) (21). For Fig. 1 E and F, test gene expression was normalized to

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Abbreviations: BM, bone marrow; CFU-S, colony-forming units of the spleen; EB, embryoid body; HSC, hematopoietic stem cell.

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β-actin and relative expression levels were derived with the comparative C_{\text{t}} method. For Fig. 2, probes labeled with FAM at the 5’ end and TAMRA at the 3’ end were purchased from Integrated DNA Technologies. Multiplex reactions were performed with rodent GAPDH VIC-labeled probe/primer sets as normalization control (Applied Biosystems). Primer/probe sequences and PCR conditions were listed in Tables 2 and 3, which are published as supporting information on the PNAS web site.

**Fig. 1.** Characterization of ESC-derived hemangioblast and hematopoietic progenitors from an inducible Cdx4 cell line. (A) Quantification of blast colony-forming cells (BL-CFCs). A total of 3 × 10^4 EB cells harvested on day 3.2 of differentiation from an inducible Cdx4 cell line were plated in blast-colony forming media in the absence or presence of doxycycline (dox), and colonies were counted 4 days after plating. A photograph of a representative blast colony is shown (Inset). (B) Methylcellulose colony-forming potential of day 6 EB-derived cells plated in methylcellulose containing cytokines (M3434). Colonies were counted from day 5 to 10 after plating. Ery^A/Ery^B, primitive/definitive erythroid; GEMM, granulocyte, erythroid, macrophage, megakaryocyte multilineage; GM, granulocyte macrophage; Mac, macrophage; Mast, mast cell. (C) Flow cytometric analysis of c-kit and CD41 on day 6 EBs. (D) Inducible Cdx4 ESC were treated with doxycycline from days 3 to 6 of EB formation and cultured on OP9 cells in the absence or presence of doxycycline. Fold increase of cell number on day 18 of OP9 culture was calculated relative to the initial cell number. (E) Relative expression levels of fetal (β-H1) and adult hemoglobin (β-major) before and after OP9 expansion by real-time RT-PCR analysis. (F) Relative expression levels of genes specific to different hematopoietic and lymphoid development pathways in Cdx4-induced or HoxB4-induced ESC-derived hematopoietic progenitors 15 days after OP9 expansion.

**Fig. 2.** Hox gene expression profile in hematopoietic populations isolated from EBs by flow cytometry, determined by quantitative real-time RT-PCR analysis. (Upper) Flk1^+^ cells from day 4 EBs with (+ dox) or without (− dox) Cdx4 induction from days 2 to 4 of EB differentiation. (Lower) CD41^+^ cells from day 6 EBs with (+ dox) or without (− dox) Cdx4 induction from days 3 to 6 of EB differentiation.

**Cell Transplantation.** Six-week- to 3-month-old Rag2^{−/−}/γc^{−/−} female mice were given two doses of 400 cGy γ-irradiation, separated by 4 h and were injected via lateral tail vein with 2 × 10^6 cells in 400 μl of IMDM/2% IFS. Transplanted mice were maintained under sterile conditions. Experiments were carried out with Institutional Animal Care and Use Committee approval.

**Spleen Colony Forming Assay.** Six- to 10-week-old Rag2^{−/−}/γc^{−/−} female mice were irradiated with a single dose of 900 cGy γ-irradiation and 10^5 whole BM or 10^6 ESC-derived hematopoietic progenitor cells were administered retroorbitally in 200 μl of PBS. An equal number of mice were irradiated and injected with PBS to control for host-derived spleen colonies. Mice were killed on different time points, and their spleens were fixed in Bouin’s buffer and scored for the colony-forming units of the spleen (CFU-S).

**FACS Analysis.** Peripheral blood leukocytes, splenocytes, and bone marrow cells were treated with red cell lysis buffer (Sigma). Antibodies were purchased from Pharmingen BD Biosciences. Propidium iodide was added to exclude dead cells. Gr1^+^, B220^+^, or...
CD3+ cells were isolated either by FACS sorting or by positive selection with magnetic streptavidin-conjugated Dynabeads M280 (Dynal Biotech). The purity of sorted cells was verified by post-sorting FACS analysis.

**Genomic DNA Isolation and Southern Hybridization.** GFP and HoxB4 probes were obtained separately by purification of an NcoI/ClaI digested fragment from MSCV-ires-GFP and an EcoRI/XhoI fragment from MSCV-HoxB4-ires-GFP with a MinEli gel purification kit (Qiagen). Probes were labeled and Southern hybridization was performed according to standard protocols. Band intensity was measured by IMAGEQUANT.

**Results**

**Cdx4 Expression Enhances Hemangioblast Formation.** The first hematopoietic cell to be detected in EBs is the hemangioblast, a bipotential precursor of hematopoietic and endothelial lineages (19, 22). Hemangioblasts are detected on days 3 and 4 of *in vitro* differentiation of ESCs into EBs, the time interval when Cdx4 expression is highest (16). Therefore, we examined whether enforced expression of Cdx4 could promote hemangioblast formation. To achieve conditional gene induction of Cdx4, a mouse Cdx4 cDNA was cloned into the Ainv15 ESC line such that its expression is controlled by a tetracycline responsive promoter element (Fig. 6A, which is published as supporting information on the PNAS web site; 12). RT-PCR confirmed the induction of Cdx4 after incubation of the cells for 24 h with tetracycline analogue doxycycline (Fig. 6B). Doxycycline was added to the EB differentiation media from day 2 to 3.2 (to allow maximal gene induction at 24 h, ref. 23) before blast colony assay in methylcellulose. Additionally, doxycycline was added to some methylcellulose cultures. Induction of Cdx4 expression during EB development stimulated the formation of blast colonies, and the yield was further increased when Cdx4 was continuously induced during methylcellulose culture (Fig. 1A). Individual blast colonies were picked, replated, and shown to generate both endothelial and hematopoietic progeny (Table 1 and Fig. 7, which are published as supporting information on the PNAS web site). Cdx4 induction not only enhances formation of the hemangioblast, but appears to favor its differentiation toward hematopoietic fates.

**Cdx4 Promotes both Primitive and Definitive Hematopoiesis in Vitro.** To determine whether induction of Cdx4 over a prolonged time interval might promote hematopoietic progenitor development in EBs, we incubated EBs with doxycycline from days 3 to 6 of differentiation and observed increased numbers of primitive erythroid and multipotent hematopoietic colonies (Fig. 1B). Cells expressing Cdx4 and c-kit, markers on early hematopoietic progenitors in both embryos and EBs (24–26), were also increased in day 6 EBs exposed to doxycycline (Fig. 1C), suggesting that Cdx4 promotes hematopoietic colony formation by enhancing the specification, proliferation, or survival of clonogenic hematopoietic progenitors. Consistent with this finding, we demonstrated by using real-time RT-PCR that the expression level of hematopoietic-specific genes was elevated 2- to 3-fold in whole EBs after Cdx4 activation between days 3 and 6 (Fig. 8, which is published as supporting information on the PNAS web site), suggesting that the enhanced gene expression by Cdx4 reflects an increased percentage of hematopoietic cells in whole EBs. Of the genes we assayed, β-H1, Ter119, LMO2, Scl, and GATA1 reflect both early hematopoietic development and definitive lineage differentiation, whereas β-major, c-myb, and AML1 are markers of definitive hematopoiesis (reviewed in ref. 27). Elevated expression of these genes suggests that Cdx4 activation promotes both primitive and definitive hematopoietic progenitor formation from differentiated ESCs.

OP9 is a stromal cell line derived from M-CSF deficient mice that supports the growth of hematopoietic progenitors (28). When EBs are dissociated and plated onto OP9, we typically observe only scant outgrowth of hematopoietic populations. However, induction of Cdx4 enabled EB-derived hematopoietic progenitors to expand and undergo multilineage differentiation on OP9, as reflected by total cell counts (Fig. 1D) and flow cytometry with hematopoietic markers (Table 1). Compared to the gene expression profile of hematopoietic populations from day 6 EBs, expression of β-H1 embryonic globin was significantly reduced, whereas the expression of β-major, the adult-type globin, was markedly elevated after coculture on OP9 stroma, indicating that Cdx4 promotes maturation of definitive erythroid lineages (Fig. 1E).

**Cdx4 Induction Modulates Hox Gene Expression in Hematopoietic Cells.** Genetic and molecular biological studies in Drosophila, zebrafish, and mouse have established that the Caudal-related family of homeodomain transcription factors regulates *Hox* gene expression patterns (reviewed in ref. 13). To explore the *Hox* gene expression profiles that result from Cdx4 induction during hematopoietic commitment in EBs, we performed real-time RT-PCR

<table>
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Table 1. Surface antigen expression of ESC-derived cells growing on OP9 for 23 days
analysis on the expression of Hox A, B, and C cluster genes in Flk1+ day 4 EB cells and CD41+ day 6 EB cells. Cdx4 induction resulted in enhanced expression of posterior Hox genes (A6, A7, A9, A10, B9, and C6) in these hematopoietic populations (Fig. 2). Comparable induction was not observed in the nonhematopoietic Flk1− population (Fig. 2). These data suggest that Cdx4 promotes blood formation by influencing Hox gene patterning during hematopoietic mesoderm commitment.

Cdx4 Enables Engraftment of ES-Derived Hematopoietic Progenitors.

We next explored whether Cdx4 enables engraftment of ES-derived hematopoietic progenitors in lethally irradiated mice (schema in Fig. 9A, which is published as supporting information on the PNAS website). Contrary to our expectations, hematopoietic populations derived from Cdx4-induced EBs protected only a minority of mice (8 of 30) from radiation-induced bone marrow aplasia. Donor chimerism in surviving mice was low (average <1%, Fig. 9B), suggesting that the transplanted population contained only small numbers of definitive HSCs or was comprised of progenitors with limited self-renewal potential. We noted that Cdx4 induction in EBs increased HoxB4 expression only 2-fold and that OP9 cocultured cells expanded by Cdx4 induction (or retroviral transduction of HoxB4) expressed significantly more HoxB4 than cells expanded by Cdx4 expression alone (Fig. 2 and data not shown). The weak enhancement of HoxB4 expression by Cdx4 appears inadequate to maintain or expand transplanted HSCs on OP9 stromal cultures. Given that HoxB4 is a major factor in the self-renewal and expansion of ESC-derived HSCs, we examined whether combining the hematopoietic specification ability of Cdx4 with the self-renewal potential of HoxB4 could improve engraftment of hematopoietic populations derived from differentiated ESCs. EBs were formed from the conditional Cdx4 cell line. Some cultures were left uninduced, whereas others were induced by doxycycline during days 6 of EB development. At day 6, EB cells from both sets of cultures were transduced with a retroviral vector expressing HoxB4 linked via internal ribosomal entry site (ires) to green fluorescent protein (GFP) and subsequently cultured on OP9 stromal cells for 10–14 days (under our present conditions, OP9 coculture with the enhanced percentage of B220+ cells in the peripheral blood of transplanted animals showed high-level GFP+ cells in all hematopoietic tissues tested and showed characteristic splenic hematopoietic colonies (CFU-S, Fig. 3). Although not the equivalent of the long-term HSC (35), the CFS-U reflects a primitive multipotent hematopoietic progenitor that previously has not been demonstrated reliably in animals engrafted from ESCs differentiated in vitro. The frequency of CFU-S detectable in stromal coccultures (14.7 ± 3 in 10⁶ cells) is 10-fold less than whole bone marrow (data not shown). These data suggest that EB cells expanded on OP9 stromal cocultures produce hematopoietic progenitors that support rapid engraftment after radiation-induced marrow aplasia.

In data from three independent transplantation experiments with cells genetically modified by either HoxB4 alone, or both Cdx4 and HoxB4, survival due to the radioprotective effect of transplanted cells was close to 100% at 8 weeks (12 of 13 for HoxB4; 18 of 18 for Cdx4/HoxB4). Flow cytometric monitoring of GFP+ cells in the peripheral blood of transplanted animals showed high-level donor chimerism that was stable over at least 6 months (Fig. 4A). Moreover, myeloid, lymphoid, and erythroid lineages were reconstituted in the peripheral blood, spleen, lymph nodes, bone marrow, and thymus of engrafted mice (Fig. 4B; see also Fig. 10A–C, which is published as supporting information on the PNAS website; see also ref. 36). Interestingly, when compared with mice transplanted with cells treated with HoxB4 alone, mice engrafted with Cdx4/HoxB4-treated cells consistently showed a higher degree of lymphoid reconstitution (Fig. 4B and 10A–C), a result that correlated with the enhanced percentage of B220+ cell formation in OP9 cultures (Table 1). Bone marrow from primary animals engrafted with Cdx4/HoxB4-expressing cells successfully reconstituted multiple lineages of hematopoietic cells when transplanted into lethally irradiated mice.
irradiated secondary mice (Fig. 4 C and D and 10 D and E). Moreover, the thymus from both primary and secondary engrafted animals was reconstituted with CD4+CD8+ cells for >4 months after transplantation (Fig. 10 B and E), indicating stable and long-term engraftment of the lymphoid lineage. Taken together, the existence of CD4+/CD8+ double-positive cells in the thymus of both primary and secondary engrafted mice and the detection of the expected blood lineages in the peripheral blood, spleen, lymph nodes, bone marrow, and thymus suggested stable hematopoietic reconstitution with self-renewing, multipotential HSCs.

**Clonal Analysis of Engrafted Mice.** Clonal analysis of marked donor cells is the accepted standard for documenting the BM-HSC (37, 38), and the introduction of HoxB4 via retrovirus into the ESC-derived hematopoietic populations allowed us to use proviral integration sites as unique genetic markers (Fig. 5A; see also Fig. 11, which is published as supporting information on the PNAS website). Genomic DNA was isolated from either spleen or bone marrow cells of primary and secondary mice. In some cases, genomic DNA was extracted from populations of Gr-1+ myeloid cells and B220+ and CD3+ lymphoid cells that were purified by antibody-conjugated magnetic beads or flow-cytometric sorting to >99% homogeneity. Isolated DNA was digested with EcoRI and NcoI and analyzed by Southern hybridization with probes that reflected either the unique proviral integration site (GFP) or the fragment of the HoxB4 cDNA common to all proviruses (Fig. 5A), as well as endogenous HoxB4, which served as an internal DNA loading control. In essentially all samples tested, we detected multiple comigrating fragments (bands), representing shared proviral integration sites, in cells from spleen and bone marrow, and from fractionated myeloid and lymphoid cell populations from primary and secondary mice (Fig. 5B and C). Importantly, several comigrating fragments were seen in paired primary and secondary mice after long-term engraftment (>17 weeks), indicating that multiple clones carried extensive self-renewal capacity (Fig. 5B and C). Moreover, by comparing the hybridization intensity of the endogenous and proviral HoxB4 fragments, we calculated that most tissues harbored one to three proviral copies per cell and showed engraftment with 3–15 prominent clones (Fig. 5B and C). Although most tissues harbor comigrating bands, not all clones are represented among all tissues in paired samples. Some fragments were seen only in primary recipients (Fig. 5B, #), others were unique to secondary engrafted animals (Fig. 5B, *), and some were seen predominantly in one lineage (Fig. 5 B and C, *). Such clonal extinction, clonal succession, and lineage restriction is an expected feature of HSC dynamics (39).

**Discussion**

In the present study, we demonstrate that Cdx4 expression can stimulate hematopoietic development in differentiating cultures of ESCs, as documented by increased numbers of hemangioblasts and multipotential hematopoietic progenitors within EBs, expansion of definitive hematopoietic and lymphoid progenitors in stromal co-cultures, and improved lymphoid engraftment of irradiated recipient mice. We also employ clonal analysis of retroviral integration sites in hematopoietic populations of engrafted mice to demonstrate our derivation of self-renewing, multipotential HSCs from ESCs. Thus, our culture conditions enable the directed differentiation of ESCs into hematopoietic progenitors with the cardinal features of definitive HSCs.
ing ESCs or primitive yolk sac progenitors enabled engraftment of posterior tissues during embryogenesis, we conclude that Cdx4 is acting to enhance mesodermal commitment to hematopoietic fates through modulation of the Hox code. No significant defects in hematopoiesis were observed in Cdx1 and/or Cdx2 knockout mice, with the exception that yolk sac circulation is abnormal in Cdx2-deficient embryos (14, 15). However, given the reports of Cdx2 involvement in human and murine leukemogenesis (17, 18), it is likely that there are overlapping and, perhaps, redundant roles of the Cdx genes in hematopoiesis.

Previously, we showed that expression of HoxB4 in differentiating ESCs or primitive yolk sac progenitors enabled engraftment of irradiated mice, but the recipient animals showed only low levels of lymphoid reconstitution (12). Despite initial reports that retroviral transduction of bone marrow with HoxB4 produced HSC expansion and enhanced competitive engraftment without distortion of hematopoietic differentiation (5–7), several groups have now observed alterations in the lympho-myeloid differentiation program (9, 11). This indicates that HoxB4 can compromise lymphoid engraftment, because the predominant lymphocyte populations in our engrafted animals lack GFP expression, which we have shown correlates with the transcriptional silencing of the HoxB4 provirus (see Fig. 12, which is published as supporting information on the PNAS web site). Current efforts are underway to derive HSCs from ESCs without ectopic HoxB4 gene expression, and evidence exists from one study that HoxB4 is dispensable for generating hematopoiesis from human (48).

The self-renewing, multipotential nature of the HSC was demonstrated definitively in the mid-1980s in experiments that used retroviruses as unique genetic markers to trace HSC fates following bone marrow transplantation (37, 38). The demonstration that highly purified lymphoid and myeloid blood cells in engrafted mice show common sites of proviral integration established that multiple blood lineages derived from single precursor cells. Some of these clones were detected again in the hematopoietic tissue of secondary recipient mice (38, 39). The evidence that single clones can reconstitute the lympho-myeloid system of both primary and secondary recipients established the paradigmatic definition of stem cells as self-renewing multipotential progenitors. In this study, we applied classical Southern hybridization analysis of proviral integration sites in engrafted blood lineages of primary and secondary mice to demonstrate the clonal derivation of HSCs from murine ESCs. Long-term reconstitution of primary and secondary mice with common clones demonstrates self-renewal, whereas evidence that myeloid and lymphoid cells derive from common clones demonstrates multineage differentiation potential. Taken together, our data validate the classical definition of a self-renewing, multilineage hematopoietic stem cell and indicate the successful derivation of long-term HSCs from ESCs in vitro. The application of similar principles to the derivation of HSCs from human ESCs, coupled to methods to generate genetically matched ESCs by nuclear transfer, provides an important theoretical foundation for combined cell and gene therapy for the treatment of genetic and malignant disorders of the blood (3).

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