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HoxB4 Confers Definitive Lymphoid-Myeloid Engraftment Potential on Embryonic Stem Cell and Yolk Sac Hematopoietic Progenitors

Michael Kyba,[1] Rita C.R. Perlingeiro,[1] and George Q. Daley[1,2,3]

[1]Whitehead Institute for Biomedical Research
9 Cambridge Center
Cambridge, Massachusetts 02142

[2]Division of Hematology/Oncology
Massachusetts General Hospital
Harvard Medical School
Boston, Massachusetts 02115

Summary

The extent to which primitive embryonic blood progenitors contribute to definitive lymphoid-myeloid hematopoiesis in the adult remains uncertain. In an effort to characterize factors that distinguish the definitive adult hematopoietic stem cell (HSC) and primitive progenitors derived from yolk sac or embryonic stem (ES) cells, we examined the effect of ectopic expression of HoxB4, a homeotic selector gene implicated in self-renewal of definitive HSCs. Expression of HoxB4 in primitive progenitors combined with culture on hematopoietic stroma induces a switch to the definitive HSC phenotype. These progenitors engraft lethally irradiated adults and contribute to long-term, multilineage hematopoiesis in primary and secondary recipients. Our results suggest that primitive HSCs are poised to become definitive HSCs and that this transition can be promoted by HoxB4 expression. This strategy for blood engraftment enables modeling of hematopoietic transplantation from ES cells.

Introduction

Blood development in embryoid bodies (EBs) differentiated from ES cells recapitulates yolk sac hematopoiesis (Keller et al., 1993). Like yolk sac progenitors, ES derivatives are ineffective at repopulating hematopoiesis in lethally irradiated adults, a property believed to reflect developmental immaturity or a defect in homing to the bone marrow (Müller and Dzierzak, 1993; Yoder, 2001). Using the EB differentiation system, we have shown that primitive progenitors can generate definitive lymphoid, myeloid, and erythroid lineages when engraftment is driven by transformation with the *Bcr/Abl* oncogene (Perlingeiro et al., 2001). Likewise, yolk sac progenitors can contribute to hematopoiesis in the adult when engrafted into neonates (Yoder et al., 1997) or when cultured on stroma taken from the paraaortic region of the embryo, where definitive HSCs are first detected (Matsuoka et al., 2001). These data argue that primitive embryonic blood progenitors can be induced to become definitive lymphoid-myeloid hematopoietic stem cells if exposed to the proper microenvironment.

The molecular mechanisms that distinguish primitive and definitive hematopoiesis are largely unknown. Earlier studies have identified several homeotic selector genes that are expressed in definitive HSCs but not in yolk sac, including HoxB3, B4, A4, and A5 (Sauvageau et al., 1994; McGrath and Palis, 1997). We tested HoxB4 as a candidate gene to promote definitive potential for the following reasons: (1) HoxB4 had been shown to enhance hematopoietic repopulation when overexpressed in adult bone marrow, without inducing leukemia or interfering with hematopoietic differentiation (Sauvageau et al., 1995); (2) HoxB4 had been implicated in self-renewal of the definitive HSC (Sauvageau et al., 1995); and (3) HoxB4 had previously been shown to enhance the formation of mixed hematopoietic colonies from differentiating ES cell cultures (Helgason et al., 1996). In this report, we demonstrate that ectopic expression of HoxB4 endows two types of embryonic hematopoietic progenitors (precirculation yolk sac and ES-derived progenitors) with the potential to engraft and contribute to multilineage lymphoid-myeloid hematopoiesis in irradiated adult mice.

[3]Correspondence: daley@wi.mit.edu

Results

HoxB4 Transduction of Yolk Sac Cells

We expressed the HoxB4 cDNA in cells isolated from precirculation murine yolk sac (E8.25, 2–4 somite pair embryos) using a retrovirus that coexpressed GFP (Van Parijs et al., 1999). Cells were grown on an OP9 stromal cell layer, previously shown to support maintenance of hematopoietic progenitors derived from ES cells in vitro (Nakano et al., 1994). HoxB4-infected cultures gave rise to abundant colonies of semiadherent cells with hematopoietic blast morphology, while control cultures showed no growth (Figure 1A). Cultured cells were injected into four lethally irradiated syngeneic adult recipients, which were assayed over time for GFP-positive cells in the peripheral blood (Figure 1C, left). Bone marrow from one primary mouse was examined for donor-derived GFP-positive cells counterstained with antibodies specific for myeloid and lymphoid hematopoietic lineages. Recipients showed donor-derived engraftment of myeloid (Gr-1$^+$ and Mac-1$^+$), B lymphoid (B220$^+$), and T lymphoid (CD4$^+$ and CD8$^+$) cells (Figure 1B), demonstrating that HoxB4 expression confers on precirculation yolk sac cells the capacity for engraftment and multilineage differentiation in irradiated adults. Donor-derived bone marrow cells from primary animals were transplanted into secondary recipients, where they contributed to multilineage hematopoiesis for at least 5 months, the longest time point analyzed in this study (Figures 1C and 1D). However, lymphoid engraftment waned over time in secondary animals, an observation we have linked to the inhibitory effects of constitutive HoxB4 expression on lymphoid differentiation, as discussed below. Our data demonstrate that HoxB4 expression induces definitive hematopoietic stem cell potential in primitive yolk sac-derived hematopoietic precursors isolated prior to the onset of circulation.

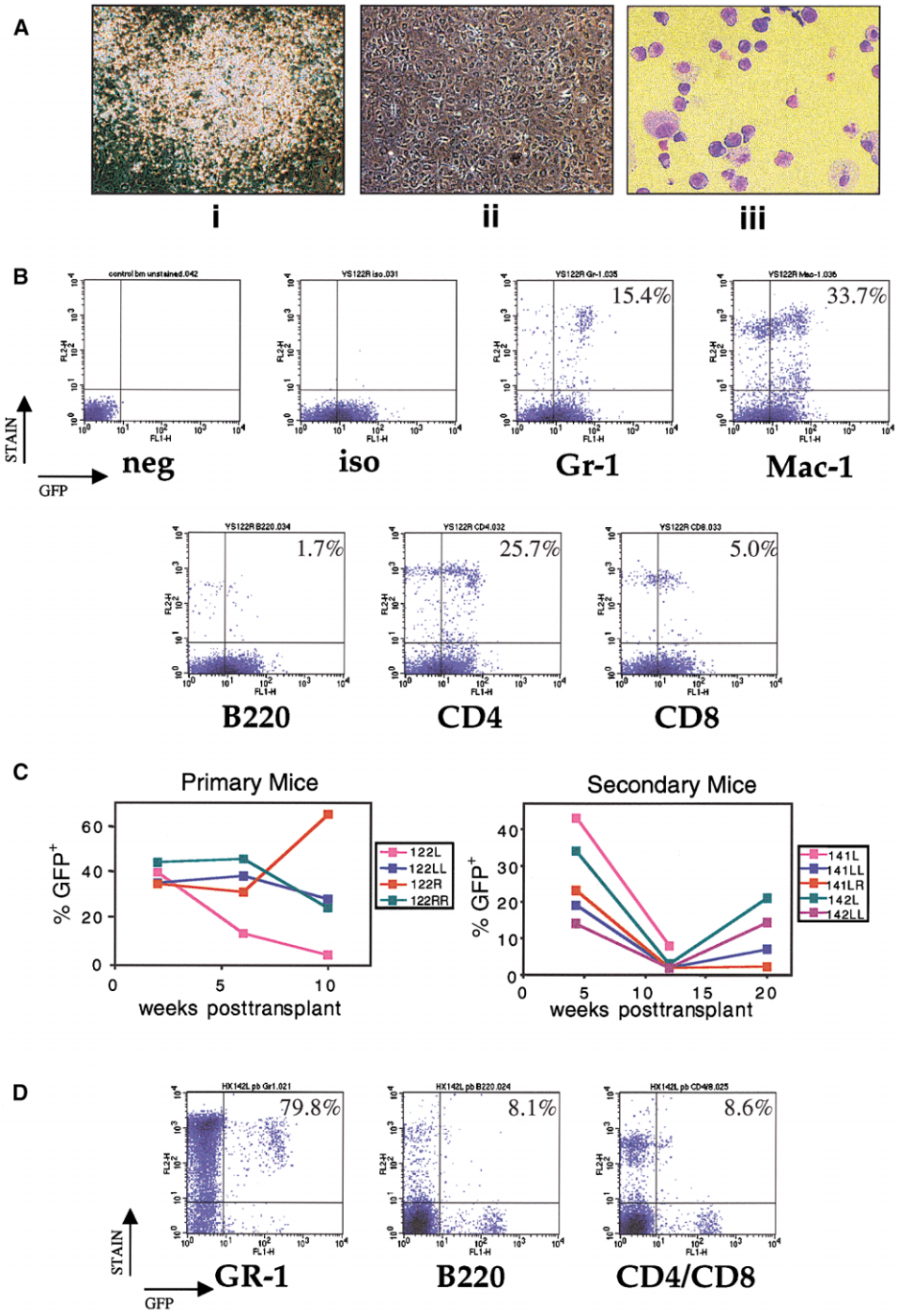

Figure 1. HoxB4-Transduced Yolk Sac Cells In Vitro and In Vivo

(A) (i) A typical colony from HoxB4-infected yolk sac cells grown on OP9 stroma. (ii) A control culture infected with empty vector, showing only the background of OP9 cells. (iii) Leukostain of cytospin preparation of HoxB4-infected yolk sac cells grown on OP9.

(B) FACS analysis of bone marrow of a representative YS-HoxB4-transplanted mouse. GFP intensity (marking donor cells) is plotted on the *x* axis, and intensity of counterstain with lineage-specific markers of hematopoietic differentiation is plotted on the *y* axis. Negative graph shows autofluorescence pattern of a nonengrafted mouse; iso graph shows isotype-matched control nonspecific antibody staining of the engrafted mouse. The host cells, which serve as internal controls for antibody staining, are represented in the left upper and lower quadrants of all graphs. Percentages represent the fraction of GFP^+ donor cells that express a given differentiation antigen (right upper quadrants, all graphs).

(C) Percent GFP^+ donor-derived cells in the peripheral blood of primary and secondary recipients as detected at indicated time points posttransplant. (Left) Four primary mice followed over 10 weeks. (Right) Five secondary mice injected with GFP^+ bone marrow from a primary mouse followed over 5 months.

(D) FACS analysis of peripheral blood from one secondary recipient stained with the myeloid differentiation marker Gr-1, the B cell marker B220, and a mixture of CD4 and CD8 to stain T cells.

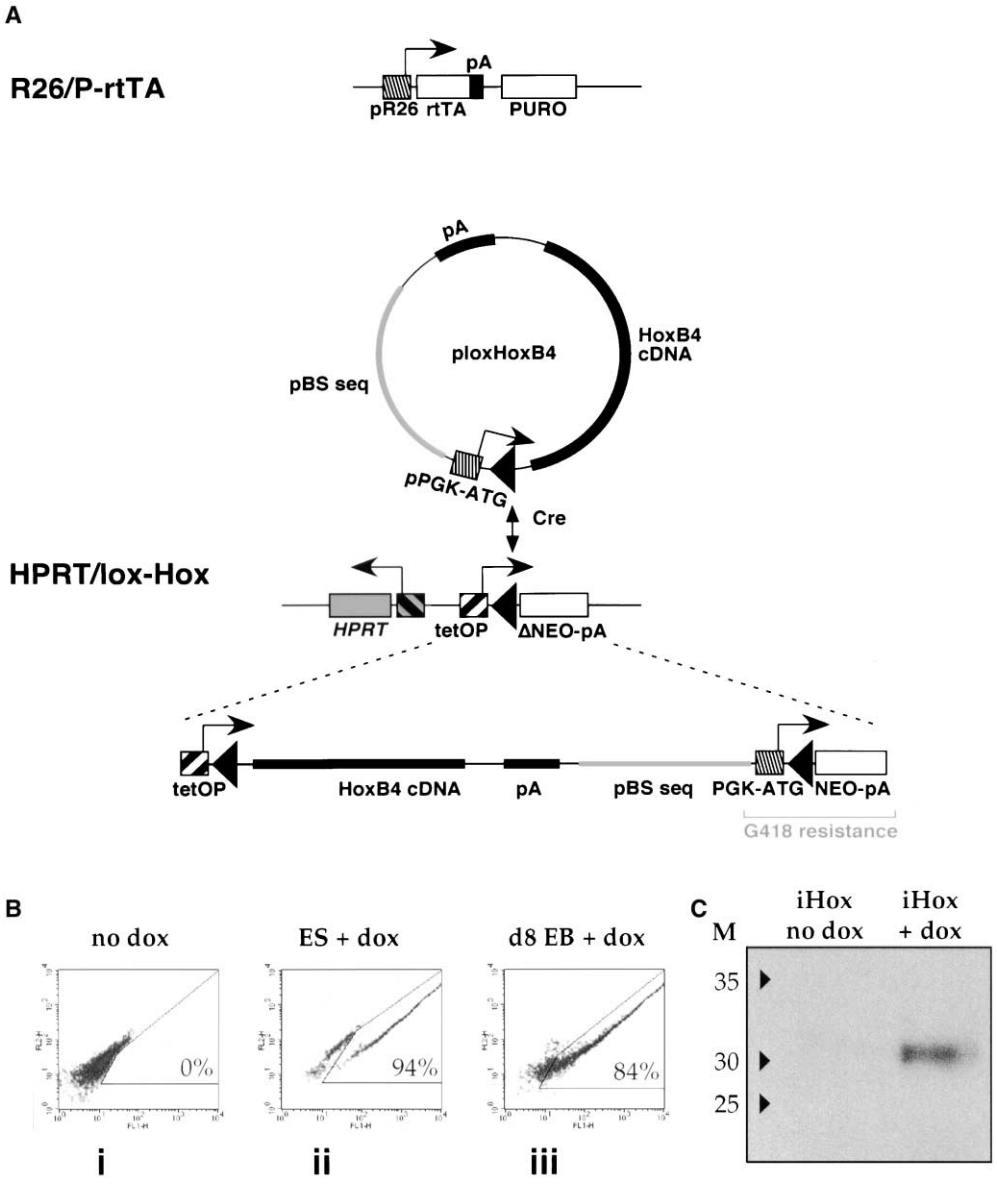

Figure 2. Generation of an ES Cell Line Specifying Inducible Transgene Expression

(A) Schematic representation of integrated expression cassettes. The rtTA is integrated into the constitutive ROSA26 locus on chromosome 6. Cre-mediated recombination of targeting vectors into the homing site on the X chromosome restores resistance to the antibiotic G418 (NEO), thereby facilitating efficient isolation of transgenic cells. Abbreviations: tetOP, tetracycline response element; PGK, phosphoglycerokinase promoter; ATG, methionine initiation codon; black triangle, lox recognition sequence for Cre recombinase; GFP, green fluorescent protein; ΔNeo, truncated neomycin (G418) resistance gene; pA, polyadenylation sequence.

(B) FACS profiles of an ES cell line modified for inducible GFP expression. (i) Uninduced ES cells. (ii) ES cells cultured in media supplemented with 1 μg/ml doxycycline; (iii) ES cells differentiated for 8 days as embryoid bodies and grown for two more days in medium supplemented with 1 μg/ml doxycycline. Percentage of cells gated for GFP positivity is indicated. Channel detecting GFP is indicated on *x* axis, and autofluorescence on *y* axis. An uncompensated, two-dimensional analysis was used to define maximum separation between GFP-positive and -negative populations.

(C) Western blot of uninduced and induced iHoxB4 cells using a monoclonal antibody to HoxB4.

HoxB4 Induction in Embryoid Body-Derived Cells

We used the same retroviral construct to infect cells from day 6 EBs and found that HoxB4 expression produced a similar outgrowth of hematopoietic blast cells on OP9 stroma (not shown). However, our results with yolk sac cells suggested that constitutive retroviral expression might have undesirable effects on hematopoietic differentiation. To achieve more consistent and homogenous induction of HoxB4 and to enable reversible HoxB4 expression in vitro and in engrafted animals, we generated a tetracycline-inducible HoxB4 transgene in ES cells. First, we inserted the reverse tetracycline transactivator (rtTA; Gossen et al., 1995) by homologous recombination into the constitutively active ROSA26 locus

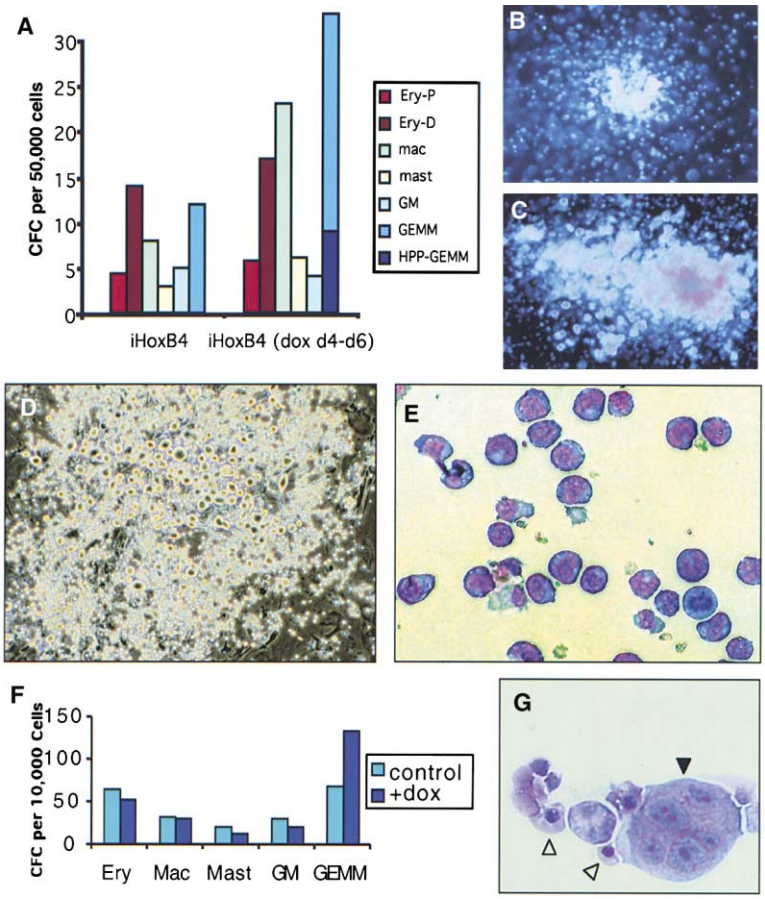

Figure 3. Effect of Doxycycline Induction of HoxB4 on EB Cells In Vitro

(A) Colony formation by cells from day 6 EB controls, and day 6 EBs treated with doxycycline from day 4 to day 6, in hematopoietic methylcellulose suspension culture. HPP-GEMM refers to dense GEMMs not normally seen at day 6 of EB development.

(B) Morphology of a typical day 6 GEMM.

(C) Morphology of a doxycycline-induced HPP-GEMM at the same magnification as in (B).

(D) A colony of semiadherent cells from HoxB4-induced day 6 EB cells plated on OP9 in the presence of doxycycline.

(E) Cytospin preparation of these cells growing on OP9.

(F) Colony-forming activity of doxycycline-induced cells grown on OP9 in methylcellulose with myeloid cytokines. Control indicates doxycycline not added to methylcellulose cultures; +dox indicates doxycycline induction maintained during methylcellulose culture.

(G) Cytospin preparation from a GEMM obtained from cultures described in (F). Open arrowheads show erythroblasts; filled arrowhead shows megakaryocyte.

(Zambrowicz et al., 1997). Then, we introduced a targeting site upstream of the HPRT locus such that site-specific integration of transgene constructs would regenerate a functional antibiotic resistance gene (NEO), thereby facilitating efficient selection of transgenic cells (Figure 2; Wutz et al., 2002).

FACS analysis of a transgenic ES cell line targeted with a GFP reporter construct demonstrated no detectable reporter expression in uninduced ES cells, thereby confirming the low basal rate of the conditional promoter (Figure 2B, i). Following induction with the tetracycline analog doxycycline, GFP was readily detected in undifferentiated cultures of ES cells (Figure 2B, ii). Robust expression was also seen when induction was started at day 8 of EB differentiation and maintained for 48 hr (Figure 2B, iii). Thus, the GFP reporter was free from the transgene silencing frequently seen in differentiated ES cells. We then targeted HoxB4 into the inducible locus and tested for expression of HoxB4 protein by Western blotting with a monoclonal antibody to HoxB4 (Gould et al., 1997). Expression was detectable only in doxycycline-induced cells (Figure 2C).

We tested the effect of HoxB4 induction on hematopoiesis by exposing EBs to doxycycline from day 4 to day 6 of differentiation, the time at which the hemangioblast undergoes commitment to the primitive HSC (Perlingeiro et al., 2001). At day 6 the EBs were dissociated and plated in methylcellulose suspension culture to score for hematopoietic colony-forming cells. HoxB4 induction had a marked stimulatory effect on the most immature multipotential myeloid progenitor detectable in this assay, the CFU-GEMM (Figure 3A). CFU-GEMM from uninduced EBs were sparse with a relatively limited erythroid burst, whereas HoxB4 induction generated larger, denser colonies that resembled CFU-GEMM from bone marrow (Figures 3B and 3C). We cultured cells from the day 6 EBs on OP9 stroma in media supplemented with cytokines and doxycycline to maintain HoxB4 expression. This yielded colonies of semiadherent cells with hematopoietic blast-like morphology (Figures 3D and 3E) that closely resembled the HoxB4-transduced yolk sac cells grown under comparable liquid culture conditions (Figure 1A). The expanded cells generated definitive myeloid colony types in methylcellulose media (Figures 3F and 3G). We characterized the cultured cells for surface antigen expression by FACS and found that the majority expressed the HSC markers c-kit and CD31 (Table 1). In addition, we noted minor populations of cells that expressed differentiation markers of the myeloid (Mac-1 and Gr-1) and, to a lesser extent, erythroid (Ter119) and lymphoid (B220) lineages. Thus, the cultured cells consist of immature hematopoietic progenitors undergoing substantial self-renewal and modest differentiation in culture.

Markers of Definitive Hematopoiesis in HoxB4-Modified Progenitors

To determine whether HoxB4 expression in cultured primitive yolk sac and ES-derived progenitors might induce expression of genes linked to the primitive-defini-

Table 1. FACS Analysis of HoxB4-induced EB-Derived Cells Grown on OP9

Surface Antigen Expression of HoxB4-Induced ES-Derivatives		
Lineage	Antigen	Percent Positive Cells
Myeloid	Gr-1	5.6
	Mac-1	21.0
Erythroid	Ter119	0.7
Lymphoid	B220	0.6
	CD4	0.0
	CD8	0.0
Progenitor/Megakaryocytic	CD41	47.8
Pan-hematopoietic	CD45	17.0
HSC	c-kit	80.7
	Sca-1	5.4
HSC/Endothelial	CD31	78.0
	AA4	0.7
	CD34	0.5
	Flk-1	0.0

tive transition, we analyzed these populations by RT-PCR. The yolk sac from day 8.25 embryos expressed both embryonic β-H1 and adult-type β-major globins (Figure 4). In contrast, HoxB4-modified yolk sac and ES-derived populations all showed silencing of β-H1 globin in favor of expression of β-major, suggesting that HoxB4-expression and growth on OP9 stroma extinguished primitive erythroid potential (Figure 4). The small amount of β-H1 seen in the retrovirally transduced EB sample (EB:rv-HoxB4) is likely due to contamination with uninfected cells. We also examined two genes important for homing of the definitive hematopoietic stem cell to the adult bone marrow: CXCR4, required for stem cell homing after transplantation (Peled et al., 1999), and TEL, which plays a critical role in the transition of hematopoiesis from the fetal liver to the bone marrow (Wang et al., 1998). Neither CXCR4 nor TEL were detectable in yolk sac, but both were expressed in HoxB4-modified yolk sac and ES-derived hematopoietic populations (Figure 4). We conclude that HoxB4 expression, combined with expansion on OP9 stroma, confers markers of definitive hematopoiesis on these cells of primitive embryonic origin.

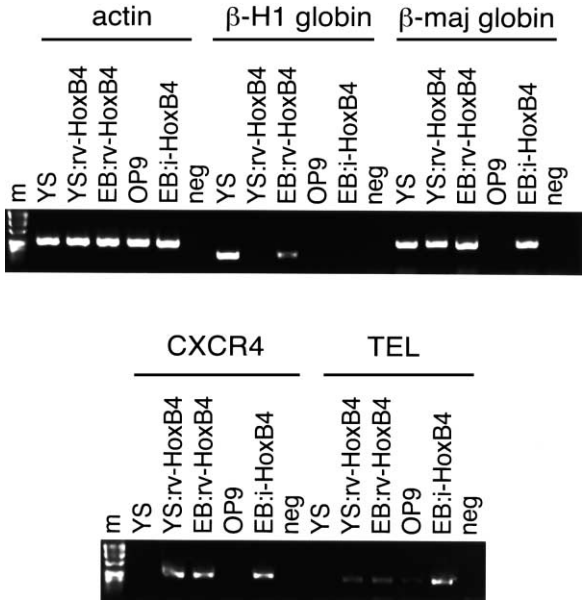

Figure 4. RT-PCR Analysis

Samples included day 8.25 yolk sac (YS), HoxB4-transduced yolk sac cells (YS:rv-HoxB4), HoxB4-transduced day 6 EB cells grown on OP9 (EB:rv-HoxB4), OP9 alone, HoxB4-induced day 6 EB cells grown on OP9 (EB-iHoxB4), and a no DNA control (neg). Samples were normalized by dilution to give equivalent signals for actin.

Engraftment of ES-Derived Hematopoietic Progenitors in Irradiated Mice

We transplanted HoxB4-induced ES-derived hematopoietic cells into irradiated syngeneic mice in order to assay their ability to engraft and differentiate in the adult environment. For this purpose, cells were first labeled by infection with the MSCViresGFP retrovirus and FACS sorted for GFP$^+$ cells before intravenous injection. Using GFP expression as a marker, we found that 5%–32% of bone marrow mononuclear cells were donor derived 2 weeks posttransplant, demonstrating that injected cells could home to the bone marrow. At 15 weeks posttransplant, substantial contributions to myeloid and lymphoid lineages were detected by simultaneous two-color detection of GFP and differentiation markers for myeloid (Gr-1, Mac-1), B lymphoid (B220), and T lymphoid (CD4, CD8) lineages (Figure 5A, right upper quadrants, all graphs). We monitored GFP$^+$ donor cells over time in engrafted mice by serial sampling of peripheral blood (Figure 5B). Despite exposure to 1000 cGy of γ irradiation, all animals showed mixed chimerism with donor and host-derived cells. Maintenance of HoxB4 induction in vivo was not necessary for sustained donor engraftment, suggesting that HoxB4 expression during in vitro culture was sufficient to confer definitive potential (Figure 5B, left). We detected donor-derived GFP$^+$ cells expressing the HSC markers c-kit, Sca-1, and AA4 in the bone marrow of engrafted mice, suggesting that the transplanted cells were represented in the hematopoietic stem cell pool (Figure 5A).

To assess whether long-term repopulating HSCs were generated, we transplanted donor-derived bone marrow cells from engrafted primary mice into secondary recipients and detected donor cells in secondary mice over 5 months (Figure 5B, right). The donor and secondary recipients were not exposed to doxycycline, allowing us to assess the intrinsic potential of the cells in the absence of HoxB4 transgene expression. FACS analysis of peripheral blood demonstrated multilineage donor contributions to both myeloid (GR-1$^+$) and lymphoid (B220, CD4$^+$, and CD8$^+$) compartments (Figure 5C). These data demonstrate long-term, multilineage, lymphoid-myeloid hematopoiesis in both primary and secondary animals engrafted with hematopoietic progenitors derived from ES cells by reversible HoxB4 expression.

Discussion

We have shown that expression of HoxB4 in primitive hematopoietic progenitors from yolk sac or differentiated ES cells, combined with culture on OP9 stroma, promotes the expansion of hematopoietic populations with definitive hematopoietic stem cell potential. The

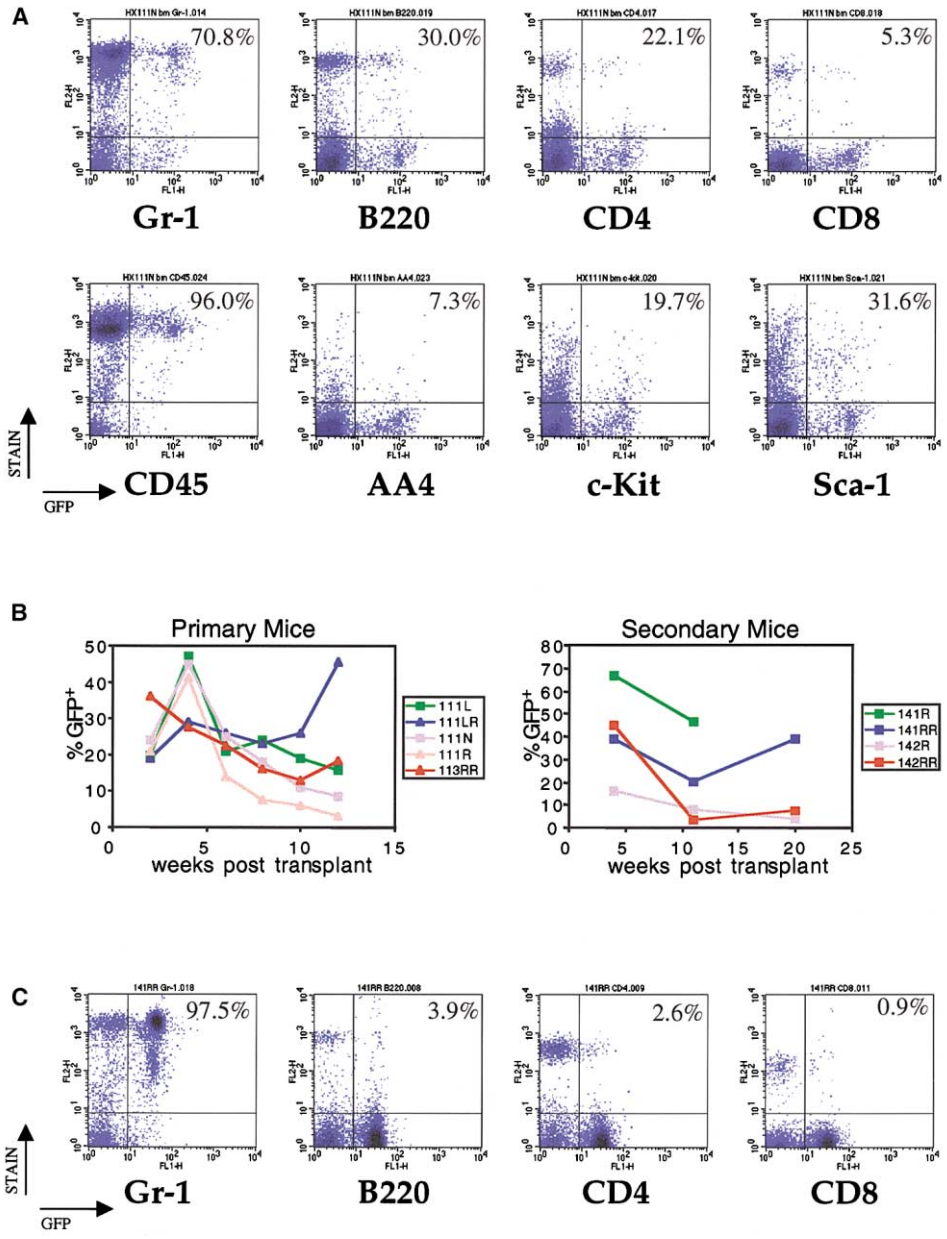

Figure 5. Engraftment of ES-Derived Hematopoietic Progenitors in Irradiated Primary and Secondary Mice

(A) FACS analysis of bone marrow of a representative recipient mouse (111N) 15 weeks posttransplant. Fluorescence intensity of GFP (marking donor cells) indicated on *x* axis; fluorescence intensity of differentiation markers, as indicated, on *y* axis. Percentages represent the fraction of GFP^+ donor cells that express a given surface antigen.

(B) Percent GFP^+ donor-derived cells in the peripheral blood of primary and secondary recipients as detected at indicated time points posttransplant. Triangles indicate mice provided with doxycycline in their drinking water. Squares indicate mice not treated with doxycycline. (Left) Five primary mice followed over 3 months. (Right) Four secondary mice injected with GFP^+ bone marrow from a primary mouse (111L, never exposed to doxycycline) followed over 5 months, without doxycycline.

(C) FACS analysis of peripheral blood from one secondary recipient stained for the myeloid differentiation marker Gr-1, the B cell marker B220, and two T cell markers, CD4 and CD8. GFP intensity indicated on *x* axis; intensity of differentiation markers indicated on *y* axis. The host cells, which serve as internal controls for antibody staining, are represented in the left upper and lower quadrants of all graphs. Percentages represent the fraction of GFP^+ donor cells that express a given differentiation antigen (right upper quadrants, all graphs).

cultured cells express several HSC markers, including c-kit, Sca-1, and CD31, and are rich in hematopoietic colony forming cells, particularly multipotent CFU-GEMMs. In contrast to the embryonic tissues from which they originated, the cultured cells exclusively express the adult isoform of β-globin as well as CXCR4 and TEL, suggesting that they have undergone a switch from primitive to definitive hematopoietic phenotype. Most importantly, they engraft and repopulate long-term lymphoid-myeloid hematopoiesis in irradiated primary and

secondary recipients, thereby satisfying the functional definition of the definitive hematopoietic stem cell.

Previous attempts at stable long-term hematopoietic engraftment of adult mice using differentiated ES cells have proven ineffective. One study demonstrated only limited lymphoid engraftment for embryoid body-derived cell populations differentiated for 11–22 days in culture (Müller and Dzierzak, 1993). Another study claimed to demonstrate long-term hematopoietic engraftment from embryoid body populations differentiated for only 4 days (Hole et al., 1996). However, this latter report provided scant characterization of engrafted donor cell populations, relying upon forward and side scatter features of circulating cells rather than defined markers of hematopoietic differentiation. Injection of EB-derived cells from this early time point, when significant numbers of undifferentiated ES cells persist, can result in engraftment of circulating nonhematopoietic cells with high tumor-forming potential (Müller and Dzierzak, 1993; our unpublished observations). Transient lymphoid contribution has been observed with $B220^+$ cells from 20-day-old EBs and longer-term lymphoid contribution from $AA4^+/B220^-$ cells from the same EBs (Potocnik et al., 1997), but never robust lymphoid-myeloid engraftment that can be carried into secondary hosts. The fact that the stem cell for embryonic erythropoiesis is able to produce definitive lineages was established by transforming these cells with the oncogene *Bcr/Abl*, which enabled single cells from day 5 EBs to produce primitive erythroid colonies in vitro and definitive lymphoid and myeloid progeny in adult hosts (Perlingeiro et al., 2001). The present work represents a less disruptive way to target this cell population, by expressing genes that are normally active in the definitive HSC. HoxB4 is ideal in this regard because its expression confers a competitive advantage on transplanted bone marrow cells without giving rise to leukemia (Sauvageau et al., 1995).

The possible role of HoxB4 in promoting definitive hematopoiesis was suggested by a comparison of Hox gene expression studies, which identify HoxB3, A4, B4, and A5 expression in definitive HSC (Sauvageau et al., 1994) but not yolk sac (McGrath and Palis, 1997). As defined by extinction of embryonic globin gene expression and acquisition of adult engraftment and lymphoid-myeloid differentiation potential, our results suggest that HoxB4 can induce primitive embryonic progenitors to acquire properties characteristic of the adult hematopoietic stem cell. The fact that expression of a single selector gene can promote this switch in precirculation yolk sac cells suggests that such cells are poised to become definitive HSC, but whether HoxB4 regulates this fate decision in the embryo is unknown. There is considerable redundancy of function among Hox gene paralogs, and other Hox genes, like HoxA4, may be equally capable of promoting this switch. Besides Hox genes, other transcription factors such as CBFA2 may also play a role in specifying definitive hematopoiesis (North et al., 1999). Alternatively, HoxB4 may be acting in a nonphysiological way, by promoting proliferation or enabling the engraftment of a cell that is not normally fated to give rise to definitive hematopoiesis. However, attempts to drive long-term engraftment using other growth-promoting genes, like activated forms of STAT5 and the cytokine receptor c-mpl, were unsuccessful (our unpublished results), suggesting that this functional potential is specific to HoxB4.

Although in vitro-generated, ES-derived HSCs engraft productively in mice, they reconstitute with a mixture of endogenous and donor-derived hematopoiesis. Thus, additional work remains to understand the competitive profile of ES-derived HSCs compared to their counterparts in fetal liver and adult bone marrow. We have obtained superior lymphoid engraftment from ES cells using inducible HoxB4 expression from the tetracycline response element. In yolk sac cells, retroviral expression of HoxB4 seems to favor myeloid differentiation. A similar effect has been observed in cord blood cells overexpressing HoxB4 (Brun et al., 2001) and with other Hox genes (Buske et al., 2001; Sauvageau et al., 1997). Although transient conditional expression of HoxB4 is superior to constitutive retroviral expression for generating long-term hematopoietic repopulation, the latter is sufficient to enable complete donor hematopoietic chimerism and partial reconstitution of immune function in the immunodeficient mouse model of combined gene and cell therapy described in the accompanying paper (Rideout et al., 2002 [this issue of *Cell*]).

The classical view of mammalian hematopoietic development held that hematopoietic stem cells originate in the yolk sac, migrate to the fetal liver, and ultimately settle in the bone marrow. More recent work has shown that lymphoid potential and long-term adult-repopulating cells arise at a distinct intraembryonic locale (Cumano et al., 1996, 2001; Medvinsky and Dzierzak, 1996; Muller et al., 1994; Sanchez et al., 1996), leading to a revised view that primitive and definitive hematopoietic progenitors have distinct origins. Our work and others (Matsuoka et al., 2001; Toles et al., 1989; Weissman et al., 1978; Yoder et al., 1997), showing that primitive embryonic progenitors can contribute to definitive hematopoiesis, suggests that there may yet be some validity to the classical view. ES cell differentiation recapitulates aspects of both primitive and definitive hematopoiesis in vitro. With the demonstration of hematopoiesis from human ES cells (Kaufman et al., 2001) and a growing interest in therapeutic applications of differentiated cells for regenerative medicine, understanding the key features that distinguish primitive and definitive hematopoiesis may have future clinical significance.

Experimental Procedures

Cell Culture

ES cells were maintained on irradiated MEFs in DME/15% IFS, 0.1 mM nonessential amino acids (GIBCO), 2 mM glutamine, penicillin/streptomycin (GIBCO), 0.1 mM β-mercaptoethanol, and 1000 U/mL LIF (Peprotech). For EB differentiation, ES cells were trypsinized, collected in EBD (IMDM/15% IFS, 200 μg/mL iron-saturated transferrin [Sigma], 4.5 mM monothiolglycerol [Sigma], 50 μg/mL ascorbic acid [Sigma], and 2 mM glutamine), and plated for 45 min to allow MEFs to adhere. Nonadherent cells were collected and plated in hanging drops at 100 cells per 10 μl drop in an inverted bacterial petri dish. EBs were collected from the hanging drops at day 2 and transferred into 10 ml EBD in slowly rotating 10 cm petri dishes. At day 4, EBs were fed by exchanging half of their spent medium for fresh EBD. Cells were harvested at day 6 by collagenase treatment. Retroviral supernatants were produced in 293 cells by FUGENE cotransfection, according to the manufacturer's specifications, of viral plasmid with packaging-defective helper plasmid, pCL-Eco (Naviaux et al., 1996). 293 cells were grown in DME/10% inacti-

vated fetal calf serum (IFS), and medium was replaced the day after transfection. A total of 10^5 EB or 10^4 yolk sac cells were resuspended in 3 ml of retroviral supernatant with 4 μg/mL polybrene and cytokines (100 ng/mL SCF, 40 ng/mL VEGF, 40 ng/mL TPO, 100 ng/mL Flt-3 ligand), transferred to semiconfluent OP9 cells in 6-well dishes, and centrifuged at 2500 rpm for 90 min at 33°C. After spin-infection, cells were returned to 37°C for overnight incubation, and the next morning the medium was exchanged for IMDM/10% IFS and the same cytokines. When confluent, the cultures were passaged by pooling suspension and semiadherent cells (obtained by trypsinization) and replated onto fresh OP9. Colony assays were done in methylcellulose medium with IL3, IL6, Epo, and SCF (M3434, StemCell Tech.).

Generation of MSCV-HoxB4iresGFP Retrovirus

The HoxB4 cDNA was subcloned as an Eco RI-Xho I fragment from MSCV-HoxB4-Puro (Helgason et al., 1996) into MSCViresGFP (Van Parijs et al., 1999).

Generation of the lox-Targeting Plasmid

The lox-targeting plasmid, plox, was generated by subcloning the Sal I (blunted)-Hind III fragment of pPGK-loxP-Xist (Wutz et al., 2002) into Bgl II (blunted)-Hind III cut pNeoEGFP (Clontech). plox has a stuffer fragment (the EGFP gene) derived from pNeoEGFP, bounded by multiple cloning sites upstream and downstream. We replaced the stuffer by digesting with Eco RI and Sal I and inserting the HoxB4 cDNA from MSCV-HoxB4iresGFP on an Eco RI-XhoI fragment to generate ploxHoxB4.

Generation of the Doxycycline-Inducible HPRT Target ES Cell Line and the Inducible HoxB4 Cell Line

The Sal I-Mlu I fragment from pHPRT-pBI-EGFP-loxNEO (Wutz et al., 2002) was subcloned into Sal/Mlu cut pneoEGFP (Clontech) in order to place an Xho I site downstream of Mlu I. The resulting Sal I-Xho I fragment was subcloned into Sal I cut pBluescript in order to place a Not I site downstream of Mlu I. Digestion of this plasmid with Not I liberated a fragment containing the tet response element and the loxΔNEO gene. This fragment was ligated into the Not I site of the HPRT targeting vector (Bronson et al., 1996). Two orientations are possible: we selected the orientation in which the lox site is between the HPRT upstream sequence and the ΔNEO gene, the opposite orientation used by Wutz et al. The resulting plasmid was linearized with Sal I and then electroporated into E14-nlsrtTA-7 ES cells (Wutz et al., 2002). After 10 days of selection in ES medium with HAT (Sigma), colonies were picked and expanded, and proper integration was confirmed by Southern blotting. This cell line, named Ainv15, was targeted with ploxHoxB4 by coelectroporation of 20 μg each of ploxHoxB4 and the Cre recombinase expression plasmid, pSalk-Cre (generously provided by Stephen O'Gorman), followed by selection in ES medium with 300 μg/mL G418 (GIBCO) and isolation of clones to generate the inducible cell line, iHoxB4. Protein extracts from iHoxB4 ES cells were tested by Western blotting using the I12 anti-HoxB4 monoclonal antibody (Gould et al., 1997). Blots were probed with a 1:50 dilution of hybridoma supernatent in PBS/5% skim milk powder/0.05% Tween-20 and visualized with HRP-conjugated goat-anti-rat secondary antibody (Santa Cruz Biotechnology, sc2006).

Yolk Sac Isolation

Pregnant female 129SvEv mice (Taconic) were sacrificed 8.25 days post copulation (the appearance of a vaginal plug was taken as day 0.5). Yolk sacs were separated from the embryo proper (which were examined to exclude yolk sacs from embryos with 5 or more somite pairs) and disaggregated by collagenase treatment.

Transplantation

Two- to three-month-old 129SvEv females (isogenic to the yolk sac cells) and 129Ola/Hsd (Harlan; isogenic to the ES cells) were given 2×500 cGy doses of γ irradiation, separated by 4 hr, and injected with 2×10^6 cells in 500 μl IMDM/10% IFS via lateral tail vein.

RT-PCR

Primers: actin(f) 5′-GTGGGGCGCCCCAGGCACCA-3′; actin(r) 5′-CTCCTTAATGTCACGCACGATTTC-3′; β-H1(f) 5′-AGTCCCCA TGGAGTCAAAGA-3′; β-H1(r) 5′-CTCAAGGAGACCTTTGCTCA-3′; β-maj(f) 5′-CTGACAGATGCTCTCTTGGG-3′; β-maj(r) 5′-CACAAC CCCAGAAACAGACA-3′; CXCR4(f) 5′-TCAAGCAAGGATGTGACTT CGA-3′; CXCR4(r) 5′-AGGTCCTGCCTAGACGCTCATT-3′; TEL(f) 5′-CTGAAGCAGAGGAAATCTCGAATG-3′; and TEL(r) 5′-GGCAGG CAGTGATTATTCTCGA-3′. Cycle conditions: 2 min at 94°C; 30 cycles of (45 s at 95°C; 1 min at 60°C; 1 min at 72°C); 5 min at 72°C.

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