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HOXA4 Provides Stronger Engraftment Potential to Short-Term Repopulating Cells Than *HOXB4*

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Genes of the *HOX4* paralog group have been shown to expand hematopoietic stem cells (HSCs). Endogenous expression of *HOXA4* is 10-fold higher than *HOXB4* in embryonic primitive hematopoietic cells undergoing self-renewal suggesting a more potent capacity of *HOXA4* to expand HSC. In this study, we provide evidence by direct competitive bone marrow cultures that *HOXA4* and *HOXB4* induce self-renewal of primitive hematopoietic cells with identical kinetics. Transplantation assays show that short-term repopulation by *HOXA4*-overexpressing multilineage progenitors was significantly greater than *HOXB4*-overexpressing progenitors in vivo, indicating differences in the sensitivity of the cells to external signals. Small array gene expression analysis showed an increase in multiple Notch and Wnt signaling -associated genes, including receptors and ligands, as well as pluripotency genes, for both *HOXA4*- and *HOXB4*-overexpressing cells, which was more pronounced for *HOXA4*, suggesting that both HOX proteins may assert their affects through intrinsic and extrinsic pathways to induce self-renewal of primitive hematopoietic cells. Thus, *HOXA4* increases short-term repopulation to higher levels than *HOXB4*, which may involve Notch signaling.

Introduction

Homeobox (*HOX*) transcription factors are key regulators of hematopoiesis (reviewed in Alharbi et al. [1]). *HOXB4* was the first *HOX* gene shown to play a role in the expansion of human and mouse hematopoietic stem cells (HSCs) by promoting self-renewal divisions [2,3]. Importantly, *HOXB4*-overexpressing HSC retained their full differentiation potential, but remained susceptible to external signals, as their numbers did not increase beyond those normally found in mice. The finding that exposure to HOXB4 recombinant protein also enhanced HSC numbers, made *HOXB4* an attractive candidate for clinical ex vivo expansion of HSC [4,5].

Our group recently demonstrated that overexpression of *HOXA4*, which is highly homologous to *HOXB4*, also led to a net expansion of functional mouse HSC in vitro [6]. This was accompanied with expansion of total bone marrow (BM) cultures by *HOXA4* that were up to 100-fold more important than control after 3 weeks of culture [6], and were comparable to earlier reported expansions with *HOXB4*-overexpressing progenitor cells [7]. The capacity to expand primitive hematopoietic cells appeared to be attributed to all paralog four members as evidenced in an embryonic stem cell overexpression model [8]. Moreover, coculture of human $CD34^+$ cells on stromal cells producing either HOXB4 or HOXC4 protein was shown to augment primitive cell numbers with similar magnitude [9]. These observations suggest that functional redundancy within the *HOX* network not only occurs in developmental programs [10,11], but also in hematopoiesis.

BM transplantation is dependent on the presence of long-term (LT) HSC, which can sustain the production of diverse blood cell types for extended periods (>20 weeks) in myeloablated mice [12]. The repopulation capacity of immediate descendants of LT-HSC, such as short-term HSC and early committed progenitors is limited in time (<20 weeks) and becomes more restricted with differentiation due to decreasing self-renewal potential.

The expression of *HOX* genes, predominantly the *A* and *B* clusters, also decreases with maturation [13–16], suggesting a role for *HOX* genes in the self-renewal potential of hematopoietic cells. Interestingly, quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis showed 10-fold higher expression of *HOXA4* than *HOXB4* in E14 fetal liver populations enriched for HSC [13]. The fact that fetal liver HSC are undergoing intensive self-renewal at that time of development to establish the HSC reservoir suggests that *HOXA4* may also be an important determinant of HSC self-renewal under physiological conditions. These observations indicate that *HOXA4* may be a more potent candidate for ex vivo expansion of HSC than *HOXB4* in a clinical context.

In this study, using retroviral overexpression, we have directly compared the effect of *HOXA4* and *HOXB4* on the capacity of primitive hematopoietic cells to expand in vitro and to engraft in vivo.

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Materials and Methods

Retroviral construction and transduction

B6SJL mice were intravenously injected with 150 mg/kg of 5-fluorouracil (5-FU; Mayne Pharma (Canada), Inc.) to recruit HSC into cell cycle, as previously reported [6]. After 4 days, BM cells were isolated from these mice and cocultured for 2 days on confluent layers of the GP+E-86 packaging cell line stably producing MSCV-*HOXA4*-GFP, MSCV-*HOXB4*-GFP, MSCV-*HOXA4*-YFP, or MSCV-GFP retrovirus [17] in Dulbecco's modified Eagle's medium (DMEM; Wisent, Inc.) supplemented with 15% heat-inactivated fetal bovine serum (FBS; PAA Laboratories, Inc.), 6 ng/mL interleukin (IL)-3, 10 ng/mL IL-6, 100 ng/mL stem cell factor (SCF), 10^{-5} M 2-mercaptoethanol (Mallinckrodt Baker, Inc.), 50 ng/mL gentamicin (Wisent, Inc.), 10 ng/mL ciprofloxacin (Wisent, Inc.), and 6 ng/mL polybrene (TekniScience, Inc.).

Animals and transplantation assays

For transplantation assays, congenic C57BL/6 (CD45.2) and B6SJL (CD45.1) mice (The Jackson Laboratories) were used. Mice were bred and maintained in a specific pathogen-free animal facility of the HMR Research Center. Transduced (*HOXA4*, *HOXB4*, *HOXA4/B4*, or control GFP) BM cells from B6SJL mice treated or not with 5-FU were intravenously injected into sublethally irradiated (800 cGy) C57Bl/6 mice together with 2×10^5 fresh BM cells. Peripheral blood (PB) repopulation was monitored every 2 weeks by flow cytometry for the presence of fluorescent cells. After 20 weeks posttransplantation, mice were sacrificed. All mouse experiment protocols were approved by the Animal Care Committee of the HMR Research Center.

In vitro cultures

Transduced BM cells were sorted on a fluorescence-activated cell sorting (FACS) Aria III with DiVa software (BD Bioscience) and seeded at different doses in duplicate in BM expansion medium (DMEM, 15% FBS, 6 ng/mL IL-3, 10 ng/mL IL-6, 100 ng/mL SCF, 10^{-5} M 2-mercaptoethanol, 50 ng/mL gentamicin, and 10 ng/mL ciprofloxacin). Doubling times were calculated using the Doubling Time website [18].

Flow cytometry analysis

BM cells from in vitro cultures were stained once a week for primitive hematopoietic population markers using the following conjugated antibodies: B220-biotin (bio), Gr1-bio, CD11b (MAC1)-bio, CD3-bio, TER119-bio, CD48-bio, c-Kit-APC, and Sca1-PE/Cy7. PB repopulation of the chimeras was monitored every 2 weeks by flow cytometry for the presence of GFP^+ and/or YFP^+ cells, and their contribution to the myeloid, B cell and T cell lineages (LINs), was determined using the following conjugated antibodies: B220-APC, CD11b (MAC1)-A700, and CD3-bio. More than 20 weeks posttransplantation, mice were sacrificed and analyzed by flow cytometry for the contribution of GFP^+ and/or YFP^+ cells to the myeloid, lymphoid, and erythroid LINs using the following conjugated antibodies: CD11b (MAC1)-Pacific Blue, Gr1-bio, B220-APC/Cy7, TER119-APC, CD4-APC/Cy7, and CD8α-APC. Biotinylated antibodies were stained with PerCP/Cy5.5-conjugated streptavidin. (All conjugated antibodies and streptavidin were purchased at BioLegend or BD Pharmingen.)

Clonogenic progenitor assays

Clonogenic progenitor assays for myeloid progenitors were performed by plating cells from *HOXA4*, *HOXB4*, or both (*HOXA4/B4*)-overexpressing BM cultures or from long-term repopulated *HOXA4*-, *HOXB4*-, *HOXA4/B4*-, or GFP-overexpressing BM chimeras in DMEM containing 1% Methocel MC (Sigma-Aldrich) supplemented with 10% FBS, 5.7% bovine serum albumin (TekniScience, Inc.), 10^{-5} M 2-mercaptoethanol, 5 U/mL erythropoietin, 10 ng/mL IL-3, 10 ng/mL IL-6, 50 ng/mL SCF, 2 mM glutamine (Life Technologies), and 200 mg/mL transferrin (Wisent). Colonies were scored as previously described [19].

Western blot analysis

Cells were lysed in cell lysis buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1% Triton X-100, 5 mM EDTA (Mallinckrodt Baker, Inc.) with protease inhibitor cocktail (BD Bioscience). Proteins were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with a monoclonal anti-HOXB4 antibody (1:1,000 dilution; Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA) followed by a secondary polyclonal anti-rat antibody coupled with horseradish peroxidase (Santa Cruz Biotechnologies, Inc.) or a monoclonal anti-Flag antibody (1:1,000 dilution; Sigma-Aldrich) followed by a secondary polyclonal anti-rabbit antibody coupled with horseradish peroxidase (Jackson Immuno Research Laboratories, Inc.). For actin detection, membranes were incubated with a monoclonal anti-actin antibody (1:1,000 dillution; Millipore) followed by a secondary polyclonal anti-mouse antibody coupled with horseradish peroxidase (Jackson Immuno Research Laboratories, Inc.).

HSC purification and transduction

For HSC purification, BM cells from wild-type (C57BL/6 and/or B6SJL) mice were harvested and LIN-negative cells were purified using the Mouse Hematopoietic Progenitor Cell Enrichment Kit (STEMCELL Technologies). LIN^- cells were stained using conjugated antibody c-Kit-APC, Sca1-PerCPCy5.5, and CD150-PECy7 (BioLegend) and $c\text{-}Kit^+Sca1^+CD150^+$ HSC were sorted on a FACS Aria III with DiVa software (BD Biosciences). After overnight culture in prestimulation media (DMEM, 15% FBS, 6 ng/mL IL-3, 40 ng/mL IL-6, 100 ng/mL SCF, 10^{-5} M 2-mercaptoethanol, 50 ng/mL gentamicin, and 10 ng/mL ciprofloxacin), HSC were subjected to two rounds of retroviral infection by spinoculation at 2,250 rpm for 90 min using MSCV-*HOXA4*-GFP, MSCV-*HOXB4*-GFP, or MSCV-GFP retrovirus. Two days after the last round of infection, GFP^+ cells were sorted on a FACS AriaIII.

RNA isolation and amplification

Total RNA was isolated from $HOXA4^+$, $HOXB4^+$, or control-transduced HSC using TRIzol reagent (Life Technologies), treated with DNase-I (Life Technologies),

and purified using the RNeasy MinElute Kit (Qiagen). RNA was amplified using the MessageAmp II aRNA Amplification Kit (Life Technologies). cDNA was prepared from 500 ng of amplified RNA or 1 μg of total RNA using MMLV-RT (Life Technologies) and random primers (Life Technologies) according to the manufacturer's protocols.

Quantitative reverse transcription–polymerase chain reaction

Primers for human and mouse *HOX* genes were used according to previously validated sequences [20,21]. Primers for candidate target genes were selected using the Primer Bank [22]. qRT-PCR was performed on an ABI 7500 Thermal Cycler (Applied Biosystems) using SYBR Green (Applied Biosystems). Triplicates were accepted in a 0.5 CT range. Relative quantification was achieved using the ΔΔCt method.

Results

HOXA4 and HOXB4 BM cells have equally strong proliferation potential

To compare the effect of *HOXA4* or *HOXB4* overexpression on the growth of primitive hematopoietic cells, cultures were initiated with primary BM cells overexpressing *HOXA4*-IRES-YFP, *HOXB4*-IRES-GFP, or both (Fig. 1A). qRT-PCR analysis confirmed the overexpression of *HOXA4* and/or *HOXB4*, which were up to 1,000-fold higher than the endogenous levels (Fig. 1B). All BM cultures underwent massive proliferation resulting in average expansions of 5×10^6-fold over the initial numbers after 4 weeks for all three conditions (Fig. 1C), which is 100-fold over the expansion of control cultures as reported before [6]. Co-overexpression of *HOXA4* and *HOXB4* did not further enhance proliferation.

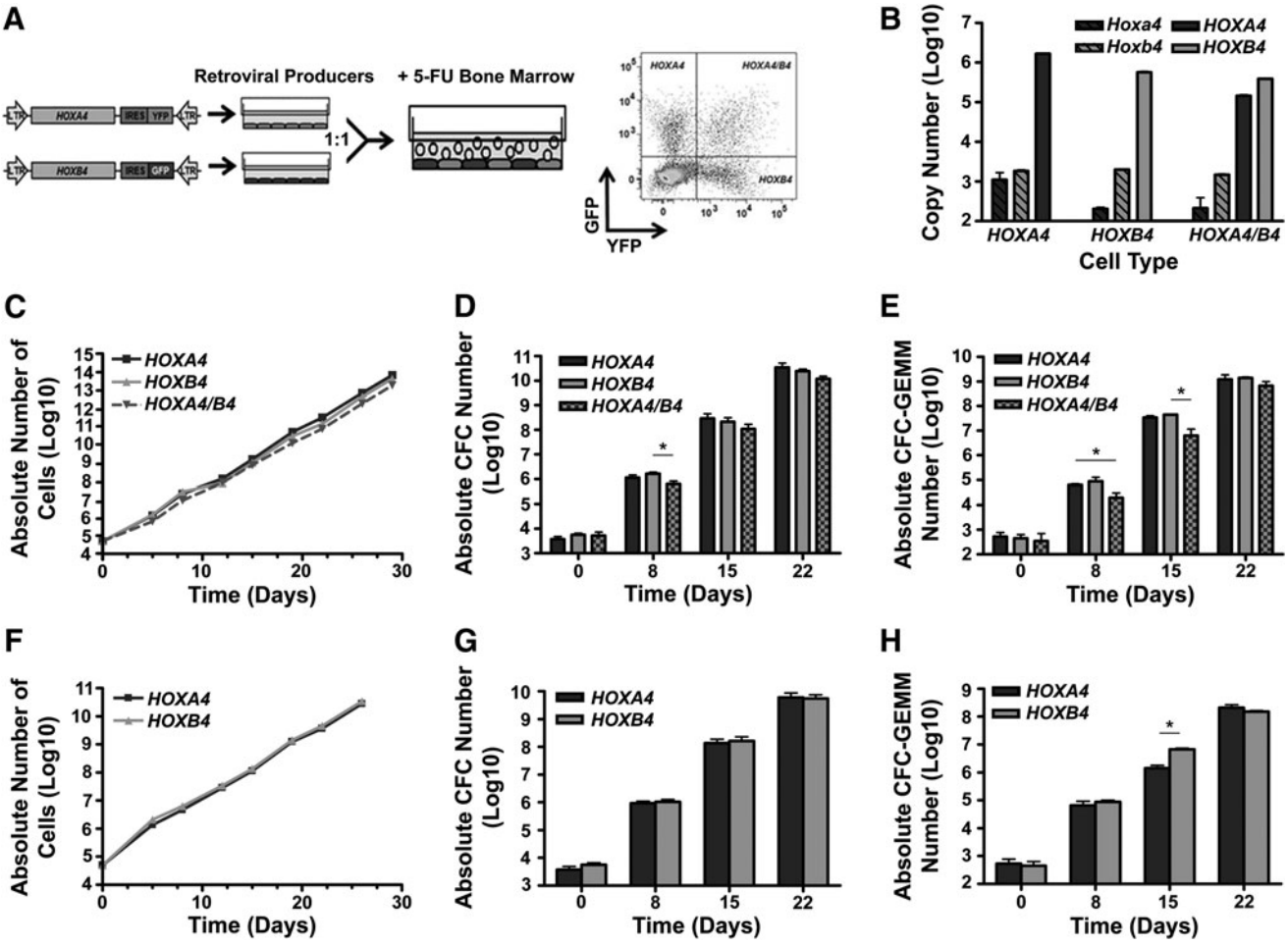

FIG. 1. In vitro BM cultures. **(A)** Overview of the in vitro experimental strategy, including a representative flow cytometry profile showing cells expressing *HOXA4*-YFP, *HOXB4*-GFP, or both. **(B)** qRT-PCR analysis for endogenous and overexpressed *HOXA4* and *HOXB4* genes in total BM cells transduced with *HOXA4*-YFP, *HOXB4*-GFP, or both vectors. **(C)** Representative growth curves of individual cultures of total BM cells transduced with *HOXA4*-YFP, *HOXB4*-GFP, or both ($n = 3$). Number of **(D)** total myeloid progenitors and **(E)** primitive GEMM progenitors in individual BM cultures transduced with *HOXA4*-YFP, *HOXB4*-GFP, or both vectors ($n = 2$). **(F)** Representative growth curve of *HOXA4*-YFP and *HOXB4*-GFP BM cells in cocultures ($n = 3$). Number of **(G)** total myeloid progenitors and **(H)** primitive GEMM progenitors in *HOXA4*-YFP and *HOXB4*-GFP in coculture ($n = 2$). $^*P \leq 0.05$ two-tailed Student's *t*-test. BM, bone marrow; CFC, colony-forming cell; GEMM, granulocytic–erythroid–megakaryocyte–monocyte; 5-FU, 5-fluorouracil; qRT-PCR, quantitative reverse transcription–polymerase chain reaction.

The numbers of myeloid progenitor cells, assessed by colony-forming cell (CFC) assays, were also comparable with few significant transitory differences (Fig. 1D, E), resulting in a net expansion up to 15×10^6-fold. Importantly, the number of granulocytic–erythroid–megakaryocytic–monocytic (GEMM)-CFC was equally increased by *HOXA4* and *HOXB4* (Fig. 1E), indicating self-renewal of primitive hematopoietic cells in vitro. Consistently, flow cytometry analysis showed an increase of $LIN^-CD48^-c\text{-}Kit^+Sca1^+$ primitive cells (data not shown). In competition cultures, the proportions of *HOXA4* and *HOXB4* cells fluctuated around 50% and contained comparable numbers of progenitors, indicating equal proliferative potentials for *HOXA4* and *HOXB4* on hematopoietic cells in vitro (Fig. 1F-H and data not shown).

HOXA4 promotes better short-term hematopoietic repopulation than HOXB4

To assess the potential of *HOXA4* and *HOXB4* HSC to repopulate lethally irradiated recipients, *HOXA4* or *HOXB4*-transduced BM cells were transplanted at low doses (10,000–30,000 BM cells, corresponding to 5–15 HSC) along with 200,000 total BM cells (Fig. 2A). All mice that received *HOXA4* or *HOXB4*-transduced BM cells were repopulated at higher levels in the periphery than control chimeras (Fig. 2B). Interestingly, compared to *HOXB4*, the short-term repopulation was significantly superior for *HOXA4* BM cells (Fig. 2B). Flow cytometry showed that this elevated short-term repopulation by *HOXA4* was associated with an increased repopulation of B cells (8 weeks, Fig. 2C, left panel), but was changed to higher myeloid contributions compared to control at long-term repopulation (20 weeks, Fig. 2C, right panel). Only transient fluctuations in myeloid and T cell reconstitution were observed for *HOXB4* (Fig. 2C, left panel).

Higher levels of engraftment were also observed in *HOXA4* and *HOXB4* hematopoietic organs (Fig. 2D), which were sustained by 2-fold higher frequencies of myeloid progenitors (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd).

Competitive transplantation (Fig. 3A) assays also demonstrated an early transient higher reconstitution by *HOXA4* compared to *HOXB4* cells in the periphery, and similar long-term repopulation (Fig. 3B), but in contrast to single *HOX* chimeras, no significant differences between *HOXA4* and *HOXB4* were observed in LIN distribution ($B220^+$, $CD3^+$, and $MAC1^+$) (Fig. 3C). However, in the hematopoietic organs a significant higher contribution was observed in the BM for *HOXB4*-transduced cells (Fig. 3D). Concurrently, more

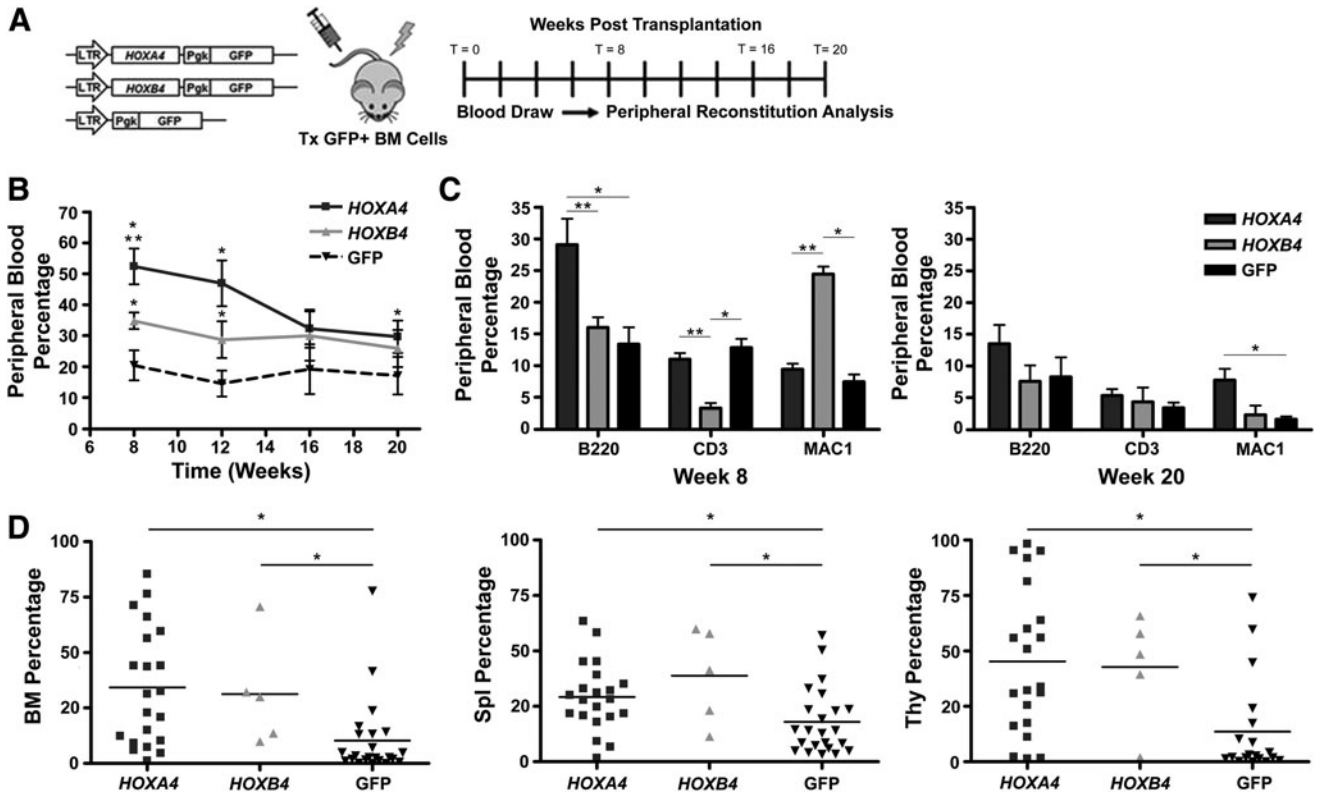

FIG. 2. Hematopoietic reconstitution in vivo. **(A)** Overview of the in vivo experimental strategy used in this study. **(B)** Engraftment (in %GFP) of *HOXA4* ($n = 15$), *HOXB4* ($n = 5$), and control chimeras ($n = 13$) in the periphery over time. **(C)** PB analysis of *HOXA4*, *HOXB4*, and control-transduced BM chimeras for myeloid and lymphoid LIN contribution at 8 weeks (*left panel*) and 20 weeks (*right panel*) reconstituted mice assessed by flow cytometry. B cells and T cells were detected using antibodies against B220 and CD3 surface markers, respectively. Antibody for MAC1 was used to detect myeloid cells. **(D)** Hematopoietic organ repopulation (in %GFP) of long-term (>20 weeks) *HOXA4*, *HOXB4,* and control GFP chimeras by flow cytometry analysis. For *HOXA4* and control chimeras the results are pooled from three independent experiments.*$P \leq 0.05$ (over control); **$P \leq 0.05$ (*HOXA4* vs *HOXB4)* one-tailed **(B)** and two-tailed **(C, D)** Student's *t*-test. LIN, lineage; PB, peripheral blood; Spl, spleen; Thy, thymus.

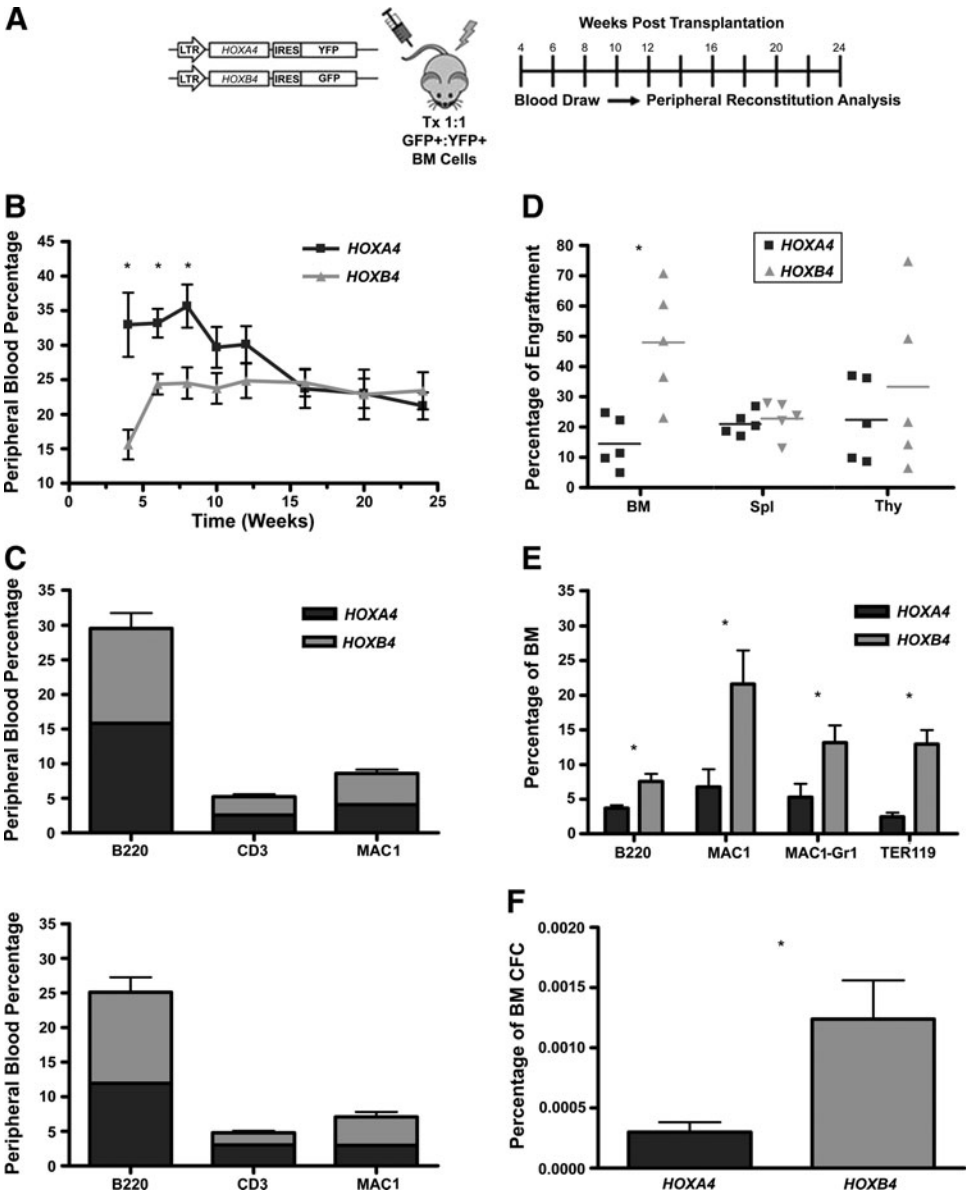

FIG. 3. *HOXA4* and *HOXB4* in vivo competition. **(A)** Overview of the in vivo experimental strategy used in this study. **(B)** Engraftment of *HOXA4* (%YFP) and *HOXB4* (%GFP) in competitive chimeras ($n = 5$) in the periphery over time. **(C)** PB analysis of *HOXA4*, *HOXB4,* and control chimeras for myeloid and lymphoid LIN contribution in short-term (8 weeks, *top panel*) and long-term (20 weeks, *lower panel*) reconstituted mice assessed by flow cytometry. B cells and T cells were detected using antibodies against B220 and CD3 surface markers, respectively. Antibody for MAC1 was used to detect myeloid cells. **(D)** Hematopoietic organ repopulation (in %GFP and %YFP) of long-term (>20 weeks) competitive chimeras by flow cytometry analysis. **(E)** Flow cytometry analysis of LIN repopulation in BM of long-term (<20 weeks) competitive chimeras using antibodies against B220, MAC1, Gr1, and TER119 surface marker. **(F)** Frequency of *HOXA4* and *HOXB4* myeloid progenitor in BM of long-term (>20 weeks) competitive chimeras. $^*P \leq 0.05$ two-tailed Student's *t*-test.

HOXB4 positive B, myeloid, and erythroid cells were observed in the BM, but not in other organs (Fig. 3E and data not shown). In agreement with this observation, more *HOXB4* progenitors were present in the BM, although frequencies of myeloid progenitors within the pool of either *HOXA4* or *HOXB4* cells were the same (Fig. 3F).

Increased expression of Notch-related genes by HOXA4 and HOXB4

To gain insight into the molecular mechanisms by which *HOXA4* and *HOXB4* mediate the expansion of primitive hematopoietic cells, the expression levels of a panel of candidate genes were measured in *HOX* and control transduced $CD150^+LIN^-c\text{-}Kit^+Sca1^+$ (LKS) cells by qRT-PCR (Fig. 4A). Potential candidate target genes were selected based on previous screens with HOXB4 and/or HOXC4 (Supplementary Table S1) and based on their reported role in HSC biology, self-renewal, or cancer biology [3,9,23,24]. Ectopic expression levels for *HOXA4* and *HOXB4* were similar and about 100-fold above the endogenous levels (Fig. 4B, C). HOXA4 and HOXB4 modulated the expression of 51 of the 87 candidates tested (Supplementary Table S2). Among them, 23 were ≥2-fold upregulated by both HOXA4 and HOXB4, whereas 17 and 11 were ≥2-fold upregulated by either HOXA4 or HOXB4 only, respectively (Fig. 4D and Table 1). Differentially expressed genes were involved in processes such as regulation of transcription, cell adhesion, stem cell maintenance/differentiation, pluripotency, cell cycle, and proliferation (Supplementary Table S3).

Also, the expression of several genes of the Wnt and Notch signaling pathways were affected, more notably by HOXA4. Among 30 Notch-related genes tested, the levels of 24 were at least 1.5-fold changed by the overexpression of either *HOXA4* and/or *HOXB4* (Fig. 4E, F and Table 2). These included Notch receptors (*Notch1* and *Notch2*) and Notch ligands (*Dll1*, *Dll3*, and *Jag1*). The expression level of *Notch2* was higher than *Notch1* in primitive hematopoietic

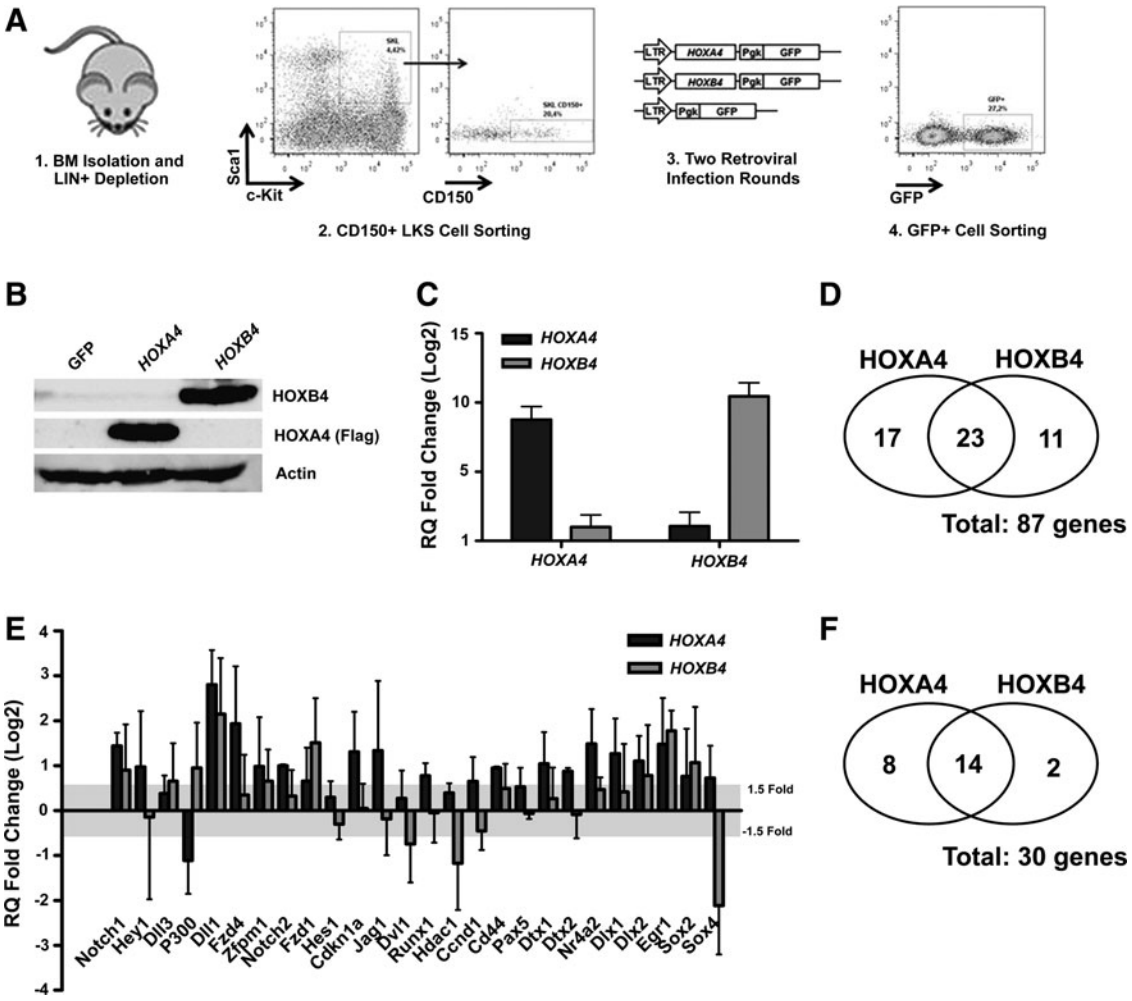

FIG. 4. Candidate gene expression in primary HSC. **(A)** Overview of the experimental strategy used in this study and representation of *HOXA4*-GFP, *HOXB4*-GFP, and control GFP retroviral vectors. **(B)** Western blot analysis for retroviral HOXA4 or HOXB4 expression in NIH-3t3 cells transduced with *HOXA4*, *HOXB4,* or control vectors. **(C)** qRT-PCR analysis for *HOXA4* and *HOXB4* genes in sorted HSC ($LIN^-c\text{-}Kit^+Sca1^+CD150^+$) transduced with *HOXA4*, *HOXB4*, or control vector. **(D)** Venn diagram showing the total number of genes that are ≥2-fold upregulated by HOXA4 and/or HOXB4 in HSC. **(E)** Log2 Fold change of candidate genes from the Notch signaling pathway in HSC overexpressing *HOXA4* or *HOXB4* compared to control HSC. *Dark region* corresponds to 1.5-fold change value. **(F)** Venn diagram showing the total number of the Notch signaling pathway genes that are ≥1.5-fold upregulated by HOXA4 and/or HOXB4 in HSC. HSC, hematopoietic stem cells; RQ, relative quantification; LKS, $LIN^-c\text{-}Kit^+Sca1^+$.

cells (data not shown), which corresponds with previous reports using reporter mice [25]. An upregulation of Notch target genes such as *Hey1* and *Runx1* indicates potential activation of the canonical Notch signaling pathway. Furthermore, in agreement with the larger B cell population the expression of *Pax5* was increased in *HOXA4*-transduced $CD150^+$LKS cells. Together these data suggest that the Notch signaling pathway plays a role in *HOX4*-mediated self-renewal of HSC.

Discussion

In this study, we demonstrate that *HOXA4* and *HOXB4* promote significant expansion of BM cells in vitro through maintenance and expansion of the progenitor pool. The magnitude of expansion induced by *HOXA4* and *HOXB4* was similar in vitro; however, when transplanted into mice, *HOXA4*-transduced cells clearly provided better short-term repopulation of BM than *HOXB4*. This was associated with a higher contribution of lymphoid cells compared to *HOXB4*-transduced cells, indicating that *HOXA4* is particularly important in restoring the B cell compartment, congruent with our previous observations [6].

In agreement with our data, it has been reported that other *HOX4* paralog genes confer similar potential expansion advantages to mouse or human primitive hematopoietic cells in vitro, when overexpressed [8] or cultured on engineered stromal cells (HOXB4 or HOXC4) [9]. Thus, in the absence of cues from the hematopoietic niche and its possible physical restraints, the proliferative potential of hematopoietic stem and progenitor cells is equally enhanced by *HOXA4* and *HOXB4.*

Whether the enhanced short-term repopulation in *HOXA4* BM recipients derives from a higher sensitivity of committed early progenitors, in particular those of the B cell LIN, to *HOXA4* or from a stronger response of HSC is not

Table 1. Relative Expression of ≥2-Fold Up- or Downregulated Gene Following *HOXA4* or *HOXB4* Overexpression in Primary HSC

		Log2 fold change	
Gene	*Name*	*HOXA4*	*HOXB4*
≥2-Fold upregulated genes by both HOXA4 and HOXB4			
Dll1	Delta-like 1 (Drosophila)	3.13	3.18
Zeb2	Zinc finger E-box binding homeobox 2	2.33	2.22
Pou5f1	POU class 5 homeobox 1	2.30	1.95
Egr1	Early growth response 1	2.20	1.93
Zic1	Zic family member 1	2.19	1.42
Sox2	SRY (sex determining region Y)-Box 2	2.12	1.67
Egr2	Early growth response 2	2.03	2.28
Dach1	Dachshund homolog 1 (Drosophila)	1.81	1.04
Col1a1	Collagen, type I, alpha 1	1.70	1.50
Zfpm1	Zinc finger protein FOG family member 1	1.69	1.02
Cxcr1	Chemokine (C-X-C motif) receptor 1	1.66	2.24
Dlx1	Distal-less homeobox 1	1.65	1.21
Nr2f2	Nuclear receptor subfamily 2 group F member 2	1.61	1.90
Cd38	CD38 molecule	1.54	2.24
Pax1	Paired box 1	1.53	2.19
Notch1	Notch 1	1.50	1.55
Lmx1b	LIM homeobox transcription factor 1 beta	1.48	3.57
Itga2	Integrin, alpha 2 (CD49B alpha 2 subunit of VLA-2 receptor)	1.44	1.78
Gpx8	Glutathioneperoxidase 8 (putative)	1.44	2.47
Lin28b	Lin-28 homolog B (*Caenorhabditis elegans*)	1.34	1.29
Dlx2	Distal-less homeobox 2	1.33	1.64
Thbs1	Thrombospondin 1	1.18	1.66
Fzd1	Frizzled family receptor 1	1.03	2.20
≥2-Fold upregulated genes by HOXB4 only			
Cdx2	Caudal type homeobox 2	0.78	3.80
Ccl3	Chemokine (C-C motif) ligand 3	0.38	2.03
Klf4	Kruppel-like factor 4 (gut)	–0.17	1.97
Slamf1	Signaling lymphocytic activation molecule family member 1	0.96	1.91
Timp3	TIMP metallopeptidaseinhibitor 3	–0.23	1.53
Zbtb16	Zinc finger and BTB domain containing 16	–0.46	1.50
Ebf1	Early B-cell factor 1	–0.28	1.49
EP300	E1A binding protein p300	–0.75	1.46
Pbx1	Pre-B-cell leukemia homeobox 1	0.75	1.25
Dll3	Delta-like 3 (Drosophila)	0.49	1.15
Hnf4a	Hepatocyte nuclear factor 4 alpha	0.75	1.12
≥2-Fold upregulated genes by HOXA4 only			
Fzd4	Frizzled family receptor 4	3.06	0.94
Jag1	Jagged 1	2.91	0.23
Nr4a2	Nuclear receptor subfamily 4, group A, member 2	1.93	0.52
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21. Cip1)	1.87	0.25
Cxcr4	Chemokine (C-X-C motif) receptor 4	1.77	–0.33
Hey1	Hairy/enhancer-of-split related with YRPW motif 1	1.73	0.79
Emcn	Endomucin	1.69	0.96
Mef2c	Myocyte enhancer factor 2C	1.48	0.70
Tgm2	Transglutaminase 2	1.43	–0.23
Cited2	Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2	1.39	0.22
Dtx1	Deltexhomolog 1 (Drosophila)	1.38	0.61
Nfyb	Nuclear transcription factor Y beta	1.38	–0.37
Ikaros	IKAROS family zinc finger 1 (Ikaros)	1.16	0.15
Trim28	Tripartite motif containing 28	1.15	0.76
Nsd1	Nuclear receptor binding SET domain protein 1	1.14	0.91
Dnmt1	DNA (cytosine-5)-methyltransferase 1	1.08	0.70
Notch2	Notch 2	1.00	0.52
Inverse regulation by HOXA4 and HOXB4			
Sox4	SRY (sex determining region Y)-box 4	1.11	–1.54

HSC, hematopoietic stem cells.

Table 2. Relative Expression of Genes Implicated in the Notch Signaling Pathway in HSC Overexpressing *HOXA4* of *HOXB4*

Gene	Name	Log2 fold change	
		HOXA4	*HOXB4*
Notch receptors			
Notch1	Notch 1	**1.50**	**1.55**
Notch2	Notch 2	**1.00**	0.52
Notch ligands			
Dll1	Delta-like 1 (Drosophila)	**3.13**	**3.18**
Dll3	Delta-like 3 (Drosophila)	0.49	**1.15**
Jag1	Jagged 1	**2.91**	0.23
Regulators			
Dtx1	Deltex homolog 1 (Drosophila)	**1.38**	0.61
Dtx2	Deltex homolog 2 (Drosophila)	0.88	0.09
Dtx4	Deltex homolog 4 (Drosophila)	0.39	0.58
Dlx1	Distal-less homeobox 1	**1.65**	**1.21**
Dlx2	Distal-less homeobox 2	**1.33**	**1.64**
Numb	Numbhomolog (Drosophila)	–0.03	–0.09
Cofactors			
Hdac1	Histone deacetylase 1	0.43	–0.59
Hdac2	Histone deacetylase 2	0.46	–0.32
EP300	E1A binding protein p300	–0.75	**1.46**
Targets			
Hes1	Hairy and enhancer of split 1 (Drosophila)	0.39	–0.23
Hey1	Hairy/enhancer-of-split related with YRPW motif 1	**1.73**	0.79
Runx1	Runt-related transcription factor 1	0.83	0.22
Zfpm1	Zinc finger protein FOG family member 1	**1.69**	**1.02**
Kat2a	K(lysine) acetyltransferase 2A	0.21	–0.43
Ccnd1	Cyclin D1	0.75	–0.39
Cdkn1a	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	**1.87**	0.25
Cd44	CD44 molecule (Indian blood group)	0.95	0.59
Nr4a2	Nuclear receptor subfamily 4 group A member 2	**1.93**	0.52
Pax5	Paired box 5	0.59	–0.06
Genes related to the Wnt pathway that crosstalk with Notch			
Fzd1	Frizzled family receptor 1	**1.03**	**2.20**
Fzd4	Frizzled family receptor 4	**3.06**	0.94
Egr1	Early growth response 1	**2.20**	**1.93**
Sox2	SRY (sex determining region Y)-box 2	**2.12**	**1.67**
Sox4	SRY (sex determining region Y)-box 4	**1.11**	**–1.54**
Dvl1	Dishevelled segment polarity protein 1	0.50	–0.35

Bold indicates ≥2-fold change in expression.

clear, but a HSC subset with a greater proliferative short-term phenotype and better B cell reconstitution potential has been defined [26].

Competitive transplantation assays confirmed the advantage of *HOXA4* in short-term repopulation, but the dominant lymphoid reconstitution of *HOXA4* was absent in the presence of *HOXB4*-transduced cells. These data suggest that a paracrine effect between *HOXA4*- and *HOXB4*-transduced cells exist that could be direct or indirect through the hematopoietic niche as has been reported for HOXB4 [27].

qRT-PCR data on a panel of candidate genes demonstrated modulation of the Notch pathway, predominantly by HOXA4, in primitive hematopoietic cells. The Notch signaling pathway is known to play a critical role in HSC self-renewal [28–31] and some synergism between HOXB4 and Dll1 in the expansion of primate $CD34^+$ cord blood cells has been reported [32,33]. Although crosstalk between the Notch and HOX pathway have been reported [34,35], we show for the first time that Notch genes are common downstream targets of *HOXA4* and *HOXB4* in primitive hematopoietic cells, suggesting that HOX4-induced self-renewal may involve Notch signaling. The Notch-related target genes included those coding for receptors, ligands, and cofactors, indicating that HOXA4 and HOXB4 may regulate the responsiveness of hematopoietic cells to Notch ligands, and amplify the signal response through enhanced ligand production. However, the enhanced lymphoid repopulation by *HOXA4* BM cells could not be explained solely by higher *Notch1* expression, which is important for lymphoid differentiation [25,36,37], as equal levels were found in *HOXB4* cells.

In addition to the Notch pathway, the expression levels of Wnt-related genes were also increased by *HOXA4* and *HOXB4*. Notch and Wnt are major pathways in HSC self-renewal and embryonic studies demonstrated that *HOX* genes integrate their signals to establish segment identity [34,38]. Crosstalk between these signaling cascades has also been demonstrated in HSC [39] and modulation of genes in both

pathways maybe essential for the prolonged self-renewal of HSC induced by HOXA4 or HOXB4. Furthermore, the observation that activation of the Wnt signaling pathway by *Wnt3a* promotes short-term multilineage reconstitution of $LIN^-c\text{-}Kit^-Sca1^+$ BM cells in vivo [40] indicate that Wnt signaling might contribute to the enhanced HOXA4-induced short-term repopulation, as *HOXA4* overexpression induced higher expression of Wnt-associated genes.

qRT-PCR data also showed the increase in expression of several self-renewal and pluripotency genes, which indicate that HOXA4 and HOXB4 may activate intrinsic self-renewal pathways. Among these genes are *Lin28b*, a micro RNA-binding protein that, when overexpressed, increases self-renewal activity of adult HSC [41], and the transcription factors *Sox2* and Po*u*5f1 (*Oct4*), also known for their critical role in pluripotency. The co-overexpression of the latter has also been shown to enhance the proliferation and differentiation of human mesenchymal stem cells [42]. Thus, upregulation of those genes by HOXA4 and HOXB4 likely contributes to the induction of HSC self-renewal.

In conclusion, our results show that overexpression of *HOXA4* and *HOXB4* results in the activation of genes involved in both intrinsic and extrinsic pathways, and suggest a potential role for the Notch pathway in association with Wnt signaling downstream HOXA4 and HOXB4 in primitive hematopoietic cells. Moreover, in the absence of niche-derived signals delivered, HSC-overexpressing *HOX4* genes have the same potency in culture, but show paralog-specific differences in vivo. Together, based on our results, manipulation of the Notch pathway in conjunction with *HOX4* merits further exploration for the expansion of primitive hematopoietic cells for therapeutic strategies.

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Author Disclosure Statement

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