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Hoxa9 Regulates Flt3 in Lymphohematopoietic Progenitors

Kimberly Gwin, Elena Frank, Ayoko Bossou, and Kay L. Medina

Early B cell factor (EBF) is a transcription factor essential for specification and commitment to the B cell fate. In this study, we show downregulation of a developmentally regulated cluster of hoxa genes, notably hoxa9, coincides with induction of EBF at the Pro-B cell stage of B cell differentiation. Analysis of the hematopoietic progenitor compartment in Hoxa9−/− mice revealed significantly reduced frequencies and expression levels of Flt3, a cytokine receptor important for lymphoid priming and the generation of B cell precursors (BCPs). We show that Hoxa9 directly regulates the flt3 gene. Chromatin immunoprecipitation analysis revealed binding of Hoxa9 to the flt3 promoter in a lymphoid progenitor cell line. Knockdown of Hoxa9 significantly reduced Flt3 transcription and expression. Conversely, forced expression of Hoxa9 increased Flt3 transcription and expression in a Pro-B cell line that expressed low levels of Flt3. Hoxa9 inversely correlated with ebfl in ex vivo-isolated bone marrow progenitors and BCPs, suggesting that EBF might function to silence a Hoxa9 transcriptional program. Restoration of EBF function in an EBF−/− cell line induced B lineage gene expression but did not directly suppress hoxa9 transcription, revealing alternate mechanisms of Hoxa9 regulation in BCPs. These data provide new insight into Hoxa9 function and regulation during lymphoid and B cell development. Furthermore, they suggest that failure to upregulate Flt3 provides a molecular basis for the lymphoid/early B cell deficiencies in Hoxa9−/− mice. The Journal of Immunology, 2010, 185: 6572−6583.

The generation of B cell precursors from hematopoietic stem cells (HSCs) is orchestrated through complex genetic networks that function to instruct lymphoid lineage and B cell fate specification, commitment, and differentiation into naive B cells. Much progress has been made in the identification of regulatory proteins that play key roles in these networks (1). However, a comprehensive understanding of the genetic circuits and their components that direct the generation of B cell precursors from hematopoietic stem cells is far from complete.

Immunophenotypic fractionation and functional analysis studies have identified various developmental intermediates between HSCs and committed Pro-B cells. Well-characterized subsets include long-term repopulating cells (lineage negative/low [Lin−] c-kithi Sca-1+ [LSK+CD34−CD27−Flt3−], short-term repopulating cells (LSK+CD34+FLT3−), multipotential progenitors (MPPs; LSK+CD27−CD27+Flt3+), lymphoid-biased MPPs (LMPPs; LSK+CD27−Flt3hiVCAM1−), common lymphoid progenitors (CLPs; Lin+c-kithiSca-1+IL-7R+), Pre-Pro-B cells (CD45R/B220−CD43+Flt3hi), and Pro-B cells (CD45R/B220+CD43−CD19+) (2). Flt3+hi LMPPs are the earliest subset to evidence lymphoid priming (3, 4). Expression of the IL-7R is concomitant with transition to the CLP stage wherein IL-7 signaling promotes B cell fate specification by upregulating the B cell fate specification factor early B cell factor (EBF) (5, 6).

The transcription factors E2A, EBF, and Pax5 play sequential, synergistic, and nonredundant roles in orchestrating lymphoid lineage and B cell differentiation. E2A is required for generation of LMPPs and regulates a subset of genes expressed in this population, including igh, rag1, tdt, and ebfl (7–9). EBF and E2A synergize to induce the early program of B lineage gene expression, including the B lineage commitment factor Pax5 (10). Together, these factors coordinate critical early B lineage differentiation events and restrict alternative developmental programs. The importance of E2A, EBF, and Pax5 in regulation of B lymphopoiesis is underscored by the retention of developmental plasticity in cell lines derived from mice deficient in any of these B lineage regulators (11–13). Currently, a comprehensive understanding of the genetic networks these factors regulate that facilitate B cell fate specification and commitment is far from complete.

Cell lines derived from gene-targeted mice are valuable tools for the identification and characterization of genetic circuits that regulate cellular differentiation pathways (11–13). Importantly, they circumvent limitations imposed by molecular manipulation of rare populations and the developmental heterogeneity inherent to ex vivo isolated immunophenotypically defined subsets. Long-term expanded EBF−/− and Pax5−/− cell lines exhibit considerable developmental plasticity and retain many molecular and cellular features of their in vivo counterparts (12–14). The goal of this study was to identify novel genetic events that accompany B cell fate specification through comparative analysis of EBF−/− and Pax5−/− cell lines. We found differences in expression of a progenitor-associated gene program that tracked with expression of Hoxa9. Analysis of hematopoietic progenitors from Hoxa9−/− mice revealed significant reductions in surface expression, as well as frequencies and numbers, of Flt3+ cells. This observation prompted us to investigate if Hoxa9 regulates Flt3. Indeed, we determined Hoxa9 regulation of flt3 is direct. Chromatin immunoprecipitation
(ChIP) analysis revealed binding of Hoxa9 to the βt3 promoter in vivo, and data obtained from knockdown and ectopic expression studies revealed that modulation of Hoxa9 levels altered Flt3 transcription and expression. Although EBF and Hoxa9 inversely correlate during B cell differentiation, EBF does not directly regulate hoxa9 transcription. These data provide new information regarding the role of Hoxa9 in regulation of lymphopoiesis and B cell development and address the role of EBF in silencing a Hoxa9-driven progenitor program.

Materials and Methods

Mice

C57Bl6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Hoxa9+/− and Hoxa7+/− have been described (15, 16). Animals were bred and maintained at the Mayo Clinic animal facility and analyzed at 6–10 wk of age. All experiments were carried out in accordance with Mayo Clinic Institutional Animal Care and Use Committee guidelines.

Cell lines

EBF−/−, Pax5−/−, RAG2−/−, and E2A−/− cell lines have been described (11, 13, 17). These cell lines were maintained in irradiated OP42 stromal cells in previously defined culture medium containing recombinant human Flt3-ligand (10 ng/ml) and recombinant murine IL-7 (10 ng/ml) (EBF−/− and E2A−/−), IL-7 alone (5 ng/ml) (RAG2−/−), or 10 ng/ml of IL-7 and 5 ng/ml Flt3-ligand (Pax5−/−) (18). All cytokines were purchased from PeproTech (Rocky Hill, NJ).

Flow cytometry and isolation of hematopoietic progenitor populations

Methods for flow cytometry and progenitor isolation have been described (19, 20). Flow cytometric analysis was performed on the FACS Calibur or Canto flow cytometers (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, Ashland, OR). Abs used included: c-kit, FD4-TATTAATCGCTCGCCGACG (Stratogene); CLP, c-kit APC, CD27 PE, Sca-1 PE-Cy7, AA4.1, CD19, IgM, CD150, CD48, and Lineage mixture (CD45R/B220, Mac-1, Gr-1, CD3ε, and Ter119) (eBioscience, San Diego, CA, or BD Pharmingen, San Diego, CA). Expression of bovine-labeled Abs was revealed with Streptavidin-PerCP (BD Pharmingen) or eFluor Streptavidin-780 (eBioscience). Bone marrow (BM) cells were blocked with normal rat serum for 10 min at room temperature preaddition of primary Abs. For isolation of HSCs, MPPs, myeloid precursors, and CLPs, BM cells were harvested and depleted of Lin− cells using magnetic bead depletion, followed by sorting on the FACSAria (BD Pharmingen) or eFluor Streptavidin-780 (eBioscience). Bone marrow (BM) cells were blocked with normal rat serum for 10 min at room temperature, washed with 2× PBS, centrifuged, and resuspended in 1 ml PBS containing 0.1% BSA. Then 10 μl of PE-Cy5.5, IL-7R PE (Lin−, Sca-1+, AA4.1, CD19, IgM), CD150, CD48, and Lineage mixture (CD45R/B220, Mac-1, Gr-1, CD3ε, and Ter119) (eBioscience, San Diego, CA, or BD Pharmingen, San Diego, CA). Expression of bovine-labeled Abs was revealed with Streptavidin-PerCP (BD Pharmingen) or eFluor Streptavidin-780 (eBioscience). Bone marrow (BM) cells were blocked with normal rat serum for 10 min at room temperature preaddition of primary Abs. For isolation of HSCs, MPPs, myeloid precursors, and CLPs, BM cells were harvested and depleted of Lin− cells using magnetic bead depletion, followed by sorting on the FACSAria (BD Biosciences) using the following combinations of Abs: HSCs: Lin−PeCy7, c-kit APC, CD27 PE, Sca-1 PeCy5.5 (Lin− c-kit+/− Sca-1+/−CD27±); MPPs: Lin−PeCy7, c-kit APC, Sca-1 PeCy5.5, CD27 PE (Lin− c-kit−/− Sca-1−/−CD27±); myeloid precursors: Lin−PeCy7, c-kit APC, Sca-1 PeCy5.5, IL-7R PE (Lin− c-kit−/− Sca-1−/− IL-7R±); B cell precursor (BCP) populations were sorted after magnetic bead depletion of ACK-lysed BM cells using Abs to Ter119, Gr-1, Mac-1, and CD3ε, followed by staining with Abs to B220, CD3ε, and IgM. Pro-B cells were sorted as B220−CD43+IgM+ and Pre-B cells as B220+CD43+IgM−. Flow cytometric analysis of hematopoietic progenitor and BCPS was performed as described (19, 21).

Quantitative PCR

RNA was extracted using RNA isolation kits (Qiagen, Valencia, CA, or Stratagene, La Jolla, CA) and treated with DNAse I (Invitrogen, Carlsbad, CA) to eliminate genomic DNA. Total RNA was reverse transcribed with random hexamers and Superscript III reverse transcriptase (Invitrogen). Real-time PCR was performed using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Expression levels were normalized to Gapdh, and fold changes were calculated using the 2−ΔΔCt method.

Retroviral transductions

Individual MISSION lentiviral sh hairpin RNA (shRNA) (Sigma-Aldrich, St. Louis, MO) constructs encoding shRNA’s targeting Hoxa9 were cotransfected with the ViralPower packing mix (Sigma-Aldrich) into 293T cells along with the manufacturer’s instructions to generate viral supernatants. Viral supernatants were collected and subjected to formaldehyde cross-linking followed by incubation with 0.125 M glycine to stop the reaction. The cell pellet was resuspended in lysis buffer and sonicated to shear DNA (400 1-kb fragments). Abs for ChIP (used at 1 μg/2 × 106 cell equivalents) included anti-Hoxa9 (Upstate Biotechnology, Lake Placid, NY), anti-Mes1 (A<em>bscam, Cambridge, MA), anti-Pbx1 (Cell Signaling Techniques, Beverly, MA), rabbit IgG, anti-PU.1, and anti-Runx1 (all from Santa Cruz Biotechnology, Santa Cruz, CA). The ChIP primers used to amplify the Flt3 promoter or IL-7RS2 have been described (26, 27).

ChIP assays

ChIP analysis was performed using the EZ-ChIP kit (Millipore, Bedford, MA) according to the manufacturer’s instructions. Briefly, 2 × 106 EBF−/− or RAG2−/− cells were collected and subjected to formaldehyde cross-linking followed by incubation with 0.125 M glycine to stop the reaction. The cell pellet was resuspended in lysis buffer and sonicated to shear DNA (400 1-kb fragments). Abs for ChIP (used at 1 μg/2 × 106 cell equivalents) included anti-Hoxa9 (Upstate Biotechnology, Lake Placid, NY), anti-Mes1 (A<em>bscam, Cambridge, MA), anti-Pbx1 (Cell Signaling Technologies, Beverly, MA), rabbit IgG, anti-PU.1, and anti-Runx1 (all from Santa Cruz Biotechnology, Santa Cruz, CA). The ChIP primers used to amplify the Flt3 promoter or IL-7RS2 have been described (26, 27).

Results

Comparative analysis of EBF−/− and Pax5−/− cell lines

EBF is required for the early program of B lineage gene expression, including the B lineage commitment factor Pax5 (20). We
previously showed that forced expression of Pax5 cannot bypass the requirement for EBF in B cell development, suggesting Pax5-independent roles for EBF (20). To identify novel events that accompany EBF expression and B cell fate specification, we performed a cellular and molecular comparison of EBF−/−, Pax5−/−, and RAG2−/− cell lines. First, we compared the expression patterns of the three essential B lineage transcription factors in the cell lines (Fig. 1A). All three lines showed relatively equivalent levels of E2A (ctfe2a) transcripts. Ebf1 transcripts are expressed in Pax5−/− cells. Consistent with the critical role of EBF in regulation of the Pax5 gene and our previous findings, Pax5 transcripts were very low to undetectable in EBF−/− cells (20). To determine if the differential expression of EBF and Pax5 in the EBF−/− and Pax5−/− cell lines resulted in quantitative differences in B lineage gene expression, we compared transcripts for several early lymphoid/B lineage genes, including rag-1, b29, vpreB1, and cd79a (mb-1) (Fig. 1A). All were significantly reduced or undetectable in EBF−/− cells compared with Pax5−/− cells, consistent with previous findings (20). Flt3 transcripts were high in EBF−/− cells, low in Pax5−/− cells, and below the level of detection in RAG2−/− cells. The varying levels of Flt3 transcripts correlated well with surface expression of Flt3 in the cell lines (Fig. 1B).

Comparative analysis of the cell lines revealed significant differences in gene expression. Therefore, we hypothesized that there may be additional differences between the lines that could be informative with regard to differentiation-related events that accompany B cell fate specification. Lymphoid progenitors are enriched within hematopoietic progenitor subsets that express CD34 and CD27 (22, 29). Therefore, we compared surface expression of these markers on the cell lines. Flow cytometric analysis revealed that the EBF−/− cell line exhibited a block at the lymphoid, but not B lineage-committed stage, and the RAG2−/− cell line at the committed Pro-B cell stage. RAG2−/− cells represent a homogeneous population of IL-7–dependent Pro-B cells, as opposed to the variable mixture of Pro-B and Pre-B cells present in wild-type Pro-B cultures or ex vivo-isolated BCPs (33). We reasoned that a RAG2−/− transcriptome subtracted functional genomics comparison of EBF−/− and Pax5−/− cell lines would preferentially illuminate molecular events that accompany B cell fate specification. Consistent with our real-time RT-PCR analysis of the cell lines (Fig. 1A), gene expression profiling (GEP) of the cell lines revealed that a subset of genes, including phef1, lef1, cd79b, pou2af1, and vpreb1, was underexpressed in EBF−/− cells compared with Pax5−/− cells (Table I). Not surprising, two of the differentially expressed genes, cd79b and vpreb1, are established EBF targets (34, 35).

Next, we focused on transcripts differentially expressed in EBF−/− cells compared with Pax5−/− cells to distinguish genetic changes that accompany B cell fate specification. Interestingly, transcripts corresponding to a cluster of homeobox genes (hoxa9, hoxa10, and hoxa7) were highly expressed in EBF−/− cells (Table I). The levels of hoxa7, -9, and -10 transcripts in EBF−/− cells were striking (ranging from >37- to >1500-fold over levels expressed in Pax5−/− cells). In addition to hoxa transcripts, EBF−/− cells expressed transcripts for cd34, consistent with the surface expression of CD34 on this cell line (Fig. 1B). Other differentially expressed genes between EBF−/− and Pax5−/− cells included those involved in cell signaling (tyrobp, fcgr2b, ccl3, cehk, igfbp4, gpr105, gpr34), signal transduction (prkar2a, ptpre, selenbp1, selenbp2), transcriptional regulation (foxj1a, ankr3d), and immune responses (cd7).

Differential expression of hoxa7–10 transcripts in EBF−/− cells has not been reported. HoxA transcripts are enriched in primitive hematopoietic progenitors and downregulated during the course of cellular differentiation (16, 36). First, the differential expression of hoxa7, hoxa9, and hoxa10 transcripts was confirmed by real-time RT-PCR (Fig. 2A). Next, we determined if expression of the hoxa7–10 gene cluster was unique to the clonal line used in the GEP platform or a shared feature of ex vivo-expanded EBF−/− cell lines. Hoxa7–10 transcript abundance was evaluated by real-time RT-PCR in three nonclonal EBF−/− cell lines and the clonal line used in the GEP (Fig. 2B). Transcripts corresponding to the three hoxa genes were expressed in all EBF−/− cell lines analyzed. Thus, expression of hoxa transcripts is a shared feature of EBF−/− cell lines. The EBF−/− cell lines are fetal liver, as opposed to BM, derived. Therefore, the differences in expression of HoxA genes could reflect the tissue of origin of the EBF−/− cell lines. However, all three HoxA transcripts were detected in an E2A−/− BM-derived cell line (Fig. 2B) (11). We conclude, therefore, that expression of hoxa transcripts likely reflects the developmental stage represented by EBF−/− cell lines and is not a result of fetal liver derivation.

Previous studies evaluated hoxa7 and hoxa9 transcripts in select subsets of hematopoietic progenitors (36, 37). However, a comparative quantitative analysis of hoxa7–10 transcripts in HSCs through Pre-B cells has not been performed. In addition, comparative analysis of expression patterns of hoxa versus ebf1 transcript in hematopoietic progenitors and BCPs has not been done and would be informative as to whether these events might be coordinately regulated during B cell differentiation. Real-time RT-PCR revealed high levels of hoxa7 and -10 transcripts in HSCs and diminished expression upon differentiation into the B and myeloid lineages (Fig. 2C). Hoxa9, in contrast, although expressed in HSCs and MPPs, was elevated in CLPs, then dramatically downregulated at the Pro-B cell stage. Interestingly, hoxa9 trancription followed a similar pattern as Flt3. A dramatic down-
FIGURE 1. Comparative analysis of EBF\(^{+/−}\), Pax5\(^{+/−}\), and RAG2\(^{+/−}\) cell lines. A, Real-time RT-PCR analysis of transcript abundance. All data were normalized to gapdh, and the bar graphs represent the mean and SE of data pooled from two to three independent analyses per cell line per transcript. B, Cell-surface phenotype of EBF\(^{+/−}\), Pax5\(^{+/−}\), and RAG2\(^{+/−}\) cell lines. The filled histograms represent RAG2\(^{+/−}\) cells, the open histograms Pax5\(^{+/−}\) cells, and the dashed lines EBF\(^{+/−}\) lines. The dotted line represents the fluorescent pattern of unstained cells. The data are representative of 3 analyses per cell line.

C, Flow cytometric analysis of CD27 and CD34 expression on CLPs. CLPs were gated as Lin\(^−\)IL-7R\(^+\)c-kit\(^{lo}\). AA4.1. D, Flow cytometric analysis of CD27 and CD34 during B cell differentiation. The contour plot on the left depicts the two major gated populations, B220\(^+\)CD43\(^+\) and B220\(^−\)CD43\(^−\). The contour plots on the right depict the expression patterns of CD34 or CD27 on either B220\(^+\)CD43\(^+\) or B220\(^−\)CD43\(^−\) subsets (indicated by the label above the plots). Data are representative of three independent BM analyses and depict the staining patterns of 6-wk-old C57Bl6 mice.
regulation of the hoxa cluster andflt3 coincided with induction of ebf1 transcription. These data confirm and extend previous reports regarding hoxa expression patterns in hematopoietic progenitors and show that downregulation of hoxa transcription accompanies B cell fate specification (16, 36, 37).

**Hoxa9−/− hematopoietic progenitors exhibit deficiencies in Flt3**

Hoxa9 is important for hematopoiesis and the generation of BCP (15, 16). Hoxa9 has been implicated in regulation of flt3, and Flt3 signaling is critical for the maintenance of lymphoid progenitors from which BCPs are derived (26, 38). To determine if Hoxa9 is essential for expression of Flt3 in vivo, we examined Flt3 expression in the hematopoietic progenitor compartment of Hoxa9−/− mice compared with strain- and aged-matched controls. Analysis of Hoxa9−/− BM was included to control for genomic effects due to gene targeting, as Hoxa7 is a nearby gene. Flt3+ cells are enriched (15, 16). Hoxa9 has been implicated in regulation of Lin− hematopoietic progenitors and show that downregulation of hoxa7 transcription. These data confirm and extend previous studies reported that Hoxa9−/− versus control, respectively (40). Next, we evaluated whether Hoxa9 deficiency altered percentages of LSK+ cells that expressed Flt3. As shown in Fig. 3A (flow cytometry, right panels, and summarized in Fig. 3B), the percentages of LSK+ cells expressing Flt3 is dramatically reduced in Hoxa9−/− BM compared with wild-type BM (37.3 ± 4.1% versus 69.2 ± 3.2%, Hoxa9−/− versus wild-type, respectively), as well as the mean fluorescence intensity of Flt3 levels (525 versus 2208, Hoxa9−/− versus wild-type, respectively).

Table 1. EBF−/− cells express a novel transcriptome

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
<th>Description</th>
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<tbody>
<tr>
<td>Phef1</td>
<td>0.414</td>
<td>Pre-B cell colony enhancing factor 1</td>
</tr>
<tr>
<td>Lef1</td>
<td>0.0204</td>
<td>Lyphoid enhancer binding factor 1</td>
</tr>
<tr>
<td>CD79b</td>
<td>0.00726</td>
<td>B29, Ig β</td>
</tr>
<tr>
<td>Pou2af1</td>
<td>0.0853</td>
<td>POU domain, class 2, associating factor 1</td>
</tr>
<tr>
<td>Vpoch1</td>
<td>0.00453</td>
<td>Pre-B lymphocyte gene 1, surrogate L chain</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ebf1</td>
<td>0.00002</td>
<td>Pre-B cell colony enhancing factor 1</td>
</tr>
<tr>
<td>Cd44</td>
<td>0.00905</td>
<td>Pre-B cell colony enhancing factor 1</td>
</tr>
<tr>
<td>Poul2af1</td>
<td>0.00853</td>
<td>POU domain, class 2, associating factor 1</td>
</tr>
<tr>
<td>Vpoch1</td>
<td>0.00453</td>
<td>Pre-B lymphocyte gene 1, surrogate L chain</td>
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Data reflect genes expressed >10-fold in EBF−/− but not Pax5−/− cell lines.

BM nucleated cells (17.1 ± 3.2 ± 10⁶ versus 23.2 ± 3.2 ± 10⁶; Hoxa9−/− [n = 4] versus control [n = 6], respectively) (15). Also, consistent with previous findings, we did not find any significant differences in frequencies (3.9 ± 0.61% versus 4.3 ± 0.96%, Hoxa9−/− [n = 4] versus control [n = 6], respectively) or numbers of LSK+ cells (4.4 ± 1.7 ± 10⁶ versus 3.3 ± 1.2 ± 10⁶, Hoxa9−/− versus control, respectively) (40). Next, we evaluated whether Hoxa9 deficiency altered percentages of LSK+ cells that expressed Flt3. As shown in Fig. 3A (flow cytometry, right panels, and summarized in Fig. 3B), the percentages of LSK+ cells expressing Flt3 is dramatically reduced in Hoxa9−/− BM compared with wild-type BM (37.3 ± 4.1% versus 69.2 ± 3.2%, Hoxa9−/− versus wild-type, respectively), as well as the mean fluorescence intensity of Flt3 levels (525 versus 2208, Hoxa9−/− versus wild-type, respectively).

CD34 is expressed prior to Flt3 in hematopoietic progenitors (41). The vast majority of LSK+ cells express CD34. Hoxa9 has also been implicated in regulation of cd44 (42, 43). Interestingly, we found a statistically significant reduction in percentages of LSK+ cells expressing CD34 (81.8 ± 3.8% versus 93.9 ± 0.8%, Hoxa9−/− versus control, respectively; p = 0.0008). We note that although there are reductions in percentages of CD34+ cells in the LSK+ compartment, surface expression of CD34 is not compromised by Hoxa9 deficiency, in stark contrast to Flt3.

The expression patterns of Flt3 and CD34 are altered in Hoxa9−/− mice. However, from this analysis, it is difficult to conclude if these alterations indicate a requirement for Hoxa9 in regulation of these genes or reflect a functional requirement for Hoxa9 in HSCs/MPPs that alters the composition of the compartments defined by these markers. To address the latter, we analyzed the HSC/MPP compartments using the SLAM markers CD150 and CD48 (21). As
shown in Fig. 3C, percentages of LSK+ HSC/MPP subsets discriminated by differential expression of CD150 and CD48 is unchanged between Hoxa9−/− and wild-type mice (n = 4 mice of each genotype). Because Hoxa9 deficiency does not appear to directly alter the composition of the hematopoietic progenitor compartment, the decreased frequency of CD34+ and Flt3+ cells we found could be due to a molecular requirement for Hoxa9 in regulation of the cd34 and/or flt3 genes. A molecular requirement for Hoxa9 in regulation of flt3 is supported by a statistically significant reduction in numbers of LSK+CD150CD48+Flt3+ (p = 0.0069), but not LSK+CD150CD48+Flt3− cells in Hoxa9−/− mice compared with wild-type (Table II). In contrast to flt3, although we observed a statistically significant reduction in percentages of LSK+CD150CD48+ that express CD34+ (24.5 ± 8.2% versus 63.6 ± 12.3%, Hoxa9−/− versus wild-type, respectively), the absolute numbers of this immunophenotypically defined subset were not significantly reduced by Hoxa9 deficiency (1276 ± 518 versus 881 ± 336, Hoxa9−/− versus wild-type, respectively; p = 0.13). These findings support a more stringent requirement for Hoxa9 in transcriptional regulation of flt3 than cd34.

Flt3 is critical for the maintenance of lymphoid progenitors from which BCPs are derived (38, 44). Hoxa9−/− mice have reductions in CLPs, similar to mice defective in Flt3-ligand (16, 38). We showed that Hoxa9−/− mice have significant reductions in LSK+Flt3+ cells. Next, we examined if the defect in Flt3 expression extended into the CLP and B lineage compartments. CLPs are enriched in the Lin−c-kitIL-7R subset (Fig. 3D), and cells expressing this combination of markers were reduced ~2-fold in Hoxa9−/− mice compared with wild-type mice, consistent with previous findings (31). Approximately 30% of Lin−c-kitIL-7R
cells express Sca-1 and Flt3 (Fig. 3D). Cell-surface levels of Flt3 did not change appreciably between the LSK+ and CLP compartments in wild-type mice (Fig. 3A,3D). However, we observed decreased surface expression of Flt3 on Lin-c-kitIL-7R+ cells from Hoxa9+/− mice as well as decreased percentages of Flt3+ CLPs (Fig. 3D and summarized in Fig. 3E). As Flt3+ CLPs are the primary precursors of BCPs, these data are consistent with the B cell deficiency in Hoxa9+/− animals (32).

In differentiating BCPs, Flt3+ cells are restricted to the Pre–Pro-B and Pro-B subsets in BM (30). Consistent with previous findings, we did not find a significant reduction in percentages or absolute numbers of B220+CD43+ IgM− BCPs (Fig. 3F and data not shown) (16). Pre–Pro-B and Pro-B cells are enriched in the B220+CD43+ IgM− fraction of BM and can be distinguished, in part, by differential expression of CD19 (Fig. 3F). We found a statistically significant increase in percentages and numbers of B220+CD43+Flt3−CD19− cells in Hoxa9+/− mice compared with controls (Fig. 3F, Table II). Further flow cytometric analysis of this population revealed that these cells uniformly expressed the NK marker, NK1.1 and thus were not BCPs (data not shown). Unexpectedly, percentages and numbers of B220+CD43+Flt3+CD19− Pre–Pro-B cells were not altered in Hoxa9+/− mice (Fig. 3F, Table II) (45). However, both B220+CD43+CD19−Flt3+ and B220+CD43+CD19−Flt3− BCPs were significantly reduced in Hoxa9+/− mice (Fig. 3F, Table II). These data indicate a specific reduction in BCPs acquiring expression of CD19 as a consequence of Hoxa9 deficiency. No significant alterations in LSK+, CLP, or BCP subsets were observed in Hoxa7+/− mice, consistent with

**FIGURE 3.** Reduction in Flt3+ hematopoietic progenitors in Hoxa9+/− mice. A, Wild-type and Hoxa9+/− BM cells were analyzed by multiparameter flow cytometry. BM hematopoietic progenitors were first gated as Lin−c-kit+/− cells (left panels). LSK+ cells are a subset of Lin−c-kit+/− cells. The LSK+ gate is shown as a boxed region in the middle panels. LSK+ cells were analyzed for differential expression of Flt3 and CD34 (right panels). B, Mean ± SD of percentages of LSK+Flt3+ cells in wild-type (n = 6) and Hoxa9+/− (n = 4). C, Fractionation of LSK+ cells by differential expression of CD150 and CD48. D, Diminished expression of Flt3 in CLPs. Lin− cells were analyzed for differential expression of c-kit and IL-7R. CLPs are enriched in the Lin−c-kit+/− IL-7R+ fraction (left panels, boxed region). Lin− c-kit−IL-7R− CLPs were further analyzed based on differential expression of Sca-1 and Flt3 (right panels). E, Mean ± SD of percentages of Flt3+ CLP in wild-type (n = 7) and Hoxa9+/− (n = 5). F, B lineage precursor subsets were analyzed using differential expression of CD45R/B220, CD43, and IgM. The boxed region in the left panels indicates BCP broadly defined as B220+CD43+ cells. The right panels show the percentages of B220+CD43+IgM− subsets identified by differential expression of Flt3 and CD19. The data reflect analyses of four to six mice per genotype. A minimum of one million events within the BM mononuclear cell gate was collected for analysis of LSK+, 750,000 for CLP, and 250,000 for BCP subsets.
previous findings (data not shown) (16). Taken together, these data indicate a crucial role for Hoxa9 in regulation of Flt3 in hematopoiesis.

Hoxa9 directly regulates \( \text{flt3} \) in vivo

Flow cytometric analysis of Hoxa9\(^{-/-}\) mice suggested a role for Hoxa9 in regulation of \( \text{flt3} \). A previous study showed Hoxa9 and Meis1 bound to the Flt3 promoter in vivo in myeloid cell lines (26). The high levels of hoxa9 and flt3 transcripts we documented in CLPs (Fig. 2C) suggested that Hoxa9 may similarly regulate \( \text{flt3} \) in lymphoid progenitors. EBF\(^{+/+}\) cells express high levels of Flt3, as well as transcripts corresponding to hoxa9, meis1, and pbx1 (Figs. 1B, 2A). To determine if Hoxa9, alone or in combination with Meis1 and/or Pbx1, directly regulates \( \text{flt3} \) in EBF\(^{+/+}\) cells, we performed ChIP assays. As shown in Fig. 4A, Hoxa9, Meis1, and Pbx1 are bound to the Flt3 promoter in EBF\(^{+/+}\) cells. PU.1, which has also been implicated in regulation of \( \text{flt3} \), was also bound to the Flt3 promoter (46). A previous study showed that \( \text{flt3} \) transcripts are reduced by AML-1 deficiency (47). However, we did not observe direct binding of AML-1 binding to the flt3 promoter in EBF\(^{-/-}\) cells. The binding of Hoxa9, Meis1, and Pbx1 to the Flt3 promoter was specific. We did not observe binding of Hoxa9 to the Flt3 promoter in RAG2\(^{-/-}\) cells that do not express Flt3 or binding of Hoxa9 to a regulatory element in the il-7ra locus in EBF\(^{-/-}\) cells (Fig. 4A). These data suggest a direct molecular connection between Hoxa9 and Flt3 in vivo.

Next, we sought to determine if Hoxa9 was required for maintenance of Flt3 expression. Lentiviral supernatants encoding shRNAs specific to Hoxa9 were generated and used to transduce the EBF\(^{+/+}\) cell line used in the ChIP assay. EBF\(^{+/+}\) transduced cells were selected by puromycin resistance conferred by a puromycin-resistance cassette in the lentiviral vector. Ten to 14 d post-initiation of puromycin selection, the cells were harvested and subject to FACS for evaluation of surface expression of Flt3 and quantitative RT-PCR (qRT-PCR) for analysis of hoxa9 and flt3 transcripts. As shown in Fig. 4B, an shRNA was identified that reproducibly reduced Hoxa9 transcripts to \( \sim 20\% \) of normal levels.

qRT-PCR revealed reduced \( \text{flt3} \) transcripts, and flow cytometry showed a significant reduction in surface expression of Flt3.

**Pax5\(^{-/-}\)** cells lack Hoxa9 transcripts and express low levels of Flt3. The decreased expression of Flt3 could be the result of silencing of Hoxa9. Therefore, we determined if ectopic expression of Hoxa9 in Pax5\(^{-/-}\) cells would increase transcription and expression of Flt3. Pax5\(^{-/-}\) cells were transduced with the empty vector MigR1-GFP or Mig-Hoxa9-GFP retroviral supernatants. GFP\(^{+}\) cells were isolated by cell sorting and expanded in vitro. qRT-PCR confirmed Hoxa9 transcripts in the Hoxa9-transduced cells (Fig. 4C). Importantly, we documented a 3–5-fold increase in \( \text{flt3} \) transcripts in Pax5\(^{-/-}\) cells expressing Hoxa9 (Fig. 4C). The increase in \( \text{flt3} \) transcripts corresponded to an increase in surface expression of Flt3 (Fig. 4C, right panel, hatched line). These data, combined with the BM analysis of Hoxa9\(^{-/-}\) mice, ChIP data, and shRNA knockdown studies, provide compelling molecular evidence that Hoxa9 directly regulates \( \text{flt3} \).

**EBF does not directly regulate hoxa transcription**

Downregulation of HoxA transcription accompanies B cell fate specification. Hoxa7–10 transcription inversely correlates with ebf and is sustained in lymphoid progenitor cell lines deficient in EBF. Together, these data support the hypothesis that EBF might function to limit HoxA expression and/or function in developing BCP. To determine if EBF is a direct negative regulator of hoxa7–10 transcription, we established an EBF\(^{-/-}\) cell line expressing a 4-HT–inducible EBF–ER fusion construct (28). Twenty-four hours post-exposure to 1 \( \mu \)M 4-HT, the cells were harvested and analyzed for induction of EBF target genes, including b29, mb-1, and pax5. As shown in Fig. 5A, EBF induced high levels of expression of all three genes within 24 h of 4-HT induction. Flt3 transcription in BCPs is silenced by Pax5 (30). Although Pax5 transcripts were expressed at high levels in 4-HT–treated EBF\(^{-/-}\) cells within 24 h, we did not observe appreciable changes in Flt3 transcription over a 24–48 h time period (Fig. 5A and data not shown).

Consistent with the failure to downregulate flt3 transcription, we documented no change in transcript abundance for hoxa7, hoxa9, hoxa10, or meis1 upon induction of EBF. The failure of Pax5 to silence flt3 transcription in EBF\(^{-/-}\) cells is in contrast to that previously documented in Pax5\(^{-/-}\) cells and likely reflects the primitive developmental state of EBF\(^{-/-}\) cells (30). Consistent with that possibility, Flt3 expression was diminished within 72 h of 4-HT treatment (Fig. 5B). In contrast to flt3, we observed downregulation of cd34 and cd27 within 24 h of 4-HT administration and diminished surface expression with 72 h of 4-HT treatment (Fig. 5B).

Taken together, these findings suggest that induction of EBF initiates a series of events that culminate in downregulation of a Hoxa9-driven regulatory network.

**Discussion**

In this study, we sought to identify novel genetic events that accompany B cell fate specification and investigate the role of EBF in their regulation. Gene expression profiling of EBF\(^{-/-}\) and Pax5\(^{-/-}\) cell lines revealed downregulation of hoxa transcription accompanies B cell fate specification. Hoxa9 has been implicated in regulation of flt3 (26). We show reduced frequencies and numbers of Flt3\(^{+}\) cells in Hoxa9\(^{-/-}\) mice, suggesting that Hoxa9 may be a key component of the regulatory circuitry that regulates the flt3 gene. Using a variety of experimental approaches, we determined that Hoxa9 regulation of flt3 is direct.Experimental manipulation of Hoxa9 levels directly impacted flt3 transcription and expression. Hoxa9 transcription inversely correlated with EBF. However, EBF does not directly suppress hoxa9 or meis1 transcription, suggesting alternate mechanisms of HoxA regulation in BCPs. Taken together, these data provide new insight into the role of Hoxa9 in lymphoid/B cell development and reveal that suppression of a Hoxa9-driven transcriptional program in BCPs is not directly regulated by EBF.
Two different groups have described B lineage defects in \( \textit{Hoxa9}^{+/2} \) mice (15, 16). However, the molecular basis of these deficiencies has not been determined. In this study, we show dramatic deficiencies in frequencies, absolute numbers, and expression levels of Flt3 in \( \textit{Hoxa9}^{+/2} \) hematopoietic progenitors. Flt3 tracks with Hoxa9 in MPPs through the Pre-B stages of B cell differentiation. Hoxa9 is bound to the \( \textit{flt3} \) promoter in vivo, and knockdown of Hoxa9 reduced \( \textit{flt3} \) transcription and expression. Conversely, forced expression of Hoxa9 increased \( \textit{flt3} \) transcription and surface expression in a cell line expressing low levels of Flt3. Taken together, these data provide compelling molecular evidence that Hoxa9 is a critical component of the genetic circuitry that regulates the \( \textit{flt3} \) gene.

Flt3 is a receptor tyrosine kinase enriched in primitive hematopoietic progenitors, but not murine HSCs (39, 48). Defective expression of Flt3 or deficiencies in Flt3-ligand result in significant reductions in Flt3+ MPPs, CLPs, and BCPs (38, 44). Previous studies demonstrated reductions in CLPs and BCPs in \( \textit{Hoxa9}^{+/2} \) mice, but did not investigate if these reductions correlated with alteration in Flt3 (16). In this study, we show that \( \textit{Hoxa9}^{+/2} \) mice exhibit significant deficiencies in percentages and absolute numbers of Flt3+ cells from the earliest onset of Flt3 expression. Flt3 signaling has been shown to activate the serine-threonine kinase Pim-1 (49). \( \textit{Hoxa9}^{+/2} \) mice are deficient in Flt3 and Pim-1. Hu et al. (50) have shown that the hematopoietic progenitor defect in \( \textit{Hoxa9}^{+/2} \) mice could be restored, in part, by ectopic expression of Pim-1. Alterations in Pim-1 levels have been shown to influence the size of the B lineage progenitor compartment (51). Pim-1 stimulates c-Myb activity (52). c-Myb is required for normal expression of IL-7R \( \alpha \) as well as IL-7 signaling (53). We did not observe diminished surface expression of IL-7R in \( \textit{Hoxa9}^{+/2} \) CLPs or Pro-B cells. However, \( \textit{Hoxa9}^{+/2} \) mice have defective responses to hematopoietic...
cytokines (40). Importantly, Pim-1 is a limiting factor for IL-7 responsiveness (51). IL-7–dependent Flt3-independent proliferation is concomitant with expression of CD19, and we documented selective reductions in CD19+ Pro-B cells. Taken together, our current findings, together with published works by others, suggest that Hoxa9 is a key component of the regulatory circuitry that initiates lymphoid priming through transcriptional activation of flt3. In a subset of wild-type LSK+ cells, Hoxa9, in combination with PU.1, induces expression of Flt3 (46). We suggest that signaling via Flt3 induces Pim-1, which is important for c-Myb activation. c-Myb, in turn, is required for lymphoid progenitors and BCP to respond to IL-7. Thus, failure to upregulate Flt3 to initiate this cascade of events provides a molecular explanation for decreased lymphoid priming and numbers of CLPs and BCPs in Hoxa9 mice.

Dysregulated expression of Hoxa9 or Hoxa10 inhibits B lymphopoiesis in some developmental contexts (54, 55). Forced expression of Hoxa9 expands MPPs but impairs the generation of IL-7 responsive BCPs. Interestingly, the consequence of forced expression of Hoxa9 is strikingly similar to aberrant Flt3 signaling, reinforcing the necessity for strict control of Hoxa9 and Flt3.
B cell differentiation beyond the Pro-B cell stage (30, 54). Holmes et al. (30) have shown that Pax5 silences Fli3 transcription in BCPs. However, regulatory circuits that limit HoxA9 transcription and/or function during B lymphopoiesis have not been determined. Our observation that EBF deficiency correlates with sustained HoxA expression suggested that EBF might function in silencing HoxA9 transcription during B cell differentiation. There is experimental precedent for lineage determining factors in silencing HoxA gene expression. GATA-1 has been shown to regulate Hoxa10 during megakaryocyte differentiation and is a primary cell fate determinant for that lineage (56). Gfi-1 directly represses Hoxa9, Pbx1, and Meis1 during granulopoiesis (57). Our gain-of-function data revealed that EBF does not directly suppress hoxa7-10 and/or meis1 transcription, suggesting alternate mechanisms of regulation in the B lineage. HoxA proteins are also regulated by microRNAs (58). It is possible that EBF indirectly regulates HoxA function by inducing a microRNA that impairs HoxA, Meis1, or Pbx1 functions. Future experiments utilizing genome-wide microRNA expression profiling will determine if EBF limits a HoxA-driven transcriptional program by controlling expression of microRNAs that regulate HoxA proteins.

In summary, this study provides new insight into molecular and cellular events that accompany B cell fate specification. Importantly, they set the foundation for future studies addressing mechanisms of HoxA regulation in BCPs as well as other EBF-regulated genes and cellular events that accompany B cell fate specification.

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Disclosures

The authors have no financial conflicts of interest.

References


