Hoxa9 Regulates Flt3 in Lymphohematopoietic Progenitors

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

*J Immunol* published online 22 October 2010
http://www.jimmunol.org/content/early/2010/10/22/jimmunol.0904203
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 ebf1 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: 000–000.

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-kit+ Sca-1− [LSK−] CD34−/CD27− Flt3−), short-term repopulating cells (LSK+CD34+Flt33), multipotential progenitors (MPPs; LSK+CD27+CD34+Flt3+), lymphoid-biased MPPs (LMPPs; LSK+CD27+Flt3+VcAM1+), common lymphoid progenitors (CLPs; Lin−c-kit+Sca-1+IL-7R+), Pre−Pro-B cells (CD45R/B220+CD43+Flt3+CD19−), and Pro-B cells (CD45R/B220+CD43+CD19+) (2). Flt3hi 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 ebf (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 Hox9a to the flt3 promoter in vivo, and data obtained from knockdown and ectopic expression studies revealed that modulation of Hox9a levels altered Flt3 transcription and expression. Although EBF and Hox9a inversely correlate during B cell differentiation, EBF does not directly regulate hox9a transcription. These data provide new information regarding the role of Hox9a in regulation of lymphopoiesis and B cell development and address the role of EBF in silencing a Hox9a-driven progenitor program.

**Materials and Methods**

**Mice**

C57Bl/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Hox9a-/- and Hoxa7-/- have been described (15, 16). Animals were bred and maintained at the Mayo Clinic animal care 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 on 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-/-). 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 FACScalibur or Canto flow cytometers (BD Biosciences, San Jose, CA) using the following combinations of Abs: PE-cy7, APC-cy7, E8-5, 124, 5M5, IFN-γ, 7B2, 9G8, and 129. Samples were processed for molecular and cellular analysis.

**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 RT-PCR was performed using the MX3005 system (Stratagene). The RT-PCR reactions consisted of first-strand cDNA, gene-specific primers, Rox reference dye, and Brilliant SYBR Green 2 reaction mixtures consisted of first-strand cDNA, gene-specific primers, Rox reference dye, and Brilliant SYBR Green 2 reaction mixtures. RT-PCR was performed using the Mx3005 system (Stratagene). The RT-PCR reactions were performed in triplicate. The primers used were *(rt)*:

- **E2A** (F) 5'-CCTTCCAGACTACACACACC-3' and (R) 5'-CACTAATTGAGTACATTTGTT-3';
- **EBF** (F) 5'-AAGGGGACATATACTGGTTG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **Hoxa9** (F) 5'-GCGTCAGACGTTACACAG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **Hoxa7** (F) 5'-GCGTCAGACGTTACACAG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **RAG2** (F) 5'-GCGTCAGACGTTACACAG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **Stat1** (F) 5'-GCGTCAGACGTTACACAG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **Stat3** (F) 5'-GCGTCAGACGTTACACAG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **Stat5** (F) 5'-GCGTCAGACGTTACACAG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **TCTCCTCAACTACACC-3' and (R) 5'-CACTAATTGAGTACATTTGTT-3';
- **cd34** (F) 5'-AAGGGGACATATACTGGTTG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **cd7** (F) 5'-AAGGGGACATATACTGGTTG-3' and (R) 5'-CCCTCTTCCTTTTTACACAGTA-3';
- **gapdh** using the 2^(-ΔΔCT) method.

**RNA purification and microarray analysis**

RNA was prepared from three independent flasks of RAG2-/-, Pax5-/-, and EBF-/- cells and integrity determined by Agilent testing (Palo Alto, CA). Total RNA was converted to cDNA and double-stranded cDNA purified by phase-lock gel with phenol-chloroform extraction. Purified cDNA was in vitro transcribed into biotinylated cRNA using the Affymetrix RNA transcript-labeling reagent (Affymetrix, Santa Clara, CA). Labeled cRNAs were fragmented and hybridized to individual Affymetrix Mouse Genome 430 2.0 Arrays and scanned using the Affymetrix GeneChip Scanner 3000 (Affymetrix). Robust multiarray coupled with the GC content of the probes was performed to normalize the data and generate background adjusted intensity values. ANOVA was performed on the normalized data from robust multiarray coupled with the GC content of the probes to identify statistically significant differentially expressed genes (parametric test, variances not assumed equal by Welch t test, p value cutoff 0.05). The microarray data were deposited in the Gene Expression Omnibus database under accession number GSE16002 (http://www.ncbi.nlm.nih.gov/geo) and the analysis used in the study provided in Supplemental Tables I and II.

**ChIP assays**

ChIP analysis was performed using the EZ-Chip kit (Millipore, Bedford, MA) according to the manufacturer’s instructions. Briefly, 2 × 10^5 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 lysate buffer and sonicated to shear DNA (400 l-kb fragments). Abs for ChIP (used at 1 μg/2 × 10^6 cell equivalents) included anti-HoxA9 (Upstate Biotechnology, Lake Placid, NY), anti-PU.1 (Cell Signaling Technology, 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-7Rα were described (26, 27).

**Retroviral transductions**

Individual MISSION lentiviral short hairpin RNA (shRNA) (Sigma-Aldrich, St. Louis, MO) constructs encoding shRNA’s targeting Hox9a were cotransfected with the ViraPower packing mix (Sigma-Aldrich) into 293T cells per the manufacturer’s instructions to generate viral supernatants. Viral supernatants were collected 2–3 d after transfection and assayed for viral supernatants. Viral supernatants were added to BM cells in the presence of 8 μg/ml polybrene. After overnight culture, viral supernatants were removed and the cells given fresh media and cytokines. Twenty-four hours later, puromycin selection was initiated (2 μg/ml). The cultures were maintained for 10–14 d under puromycin selection, then harvested and processed for flow and cytomometric analysis. To determine if ectopic expression of Hox9a altered Flt3 transcription and expression, we transduced a Flt3−/− cell line expressing low levels of Flt3 with retroviral constructs encoding an empty vector (MigR1-GFP) or encoding a full-length Hox9a cDNA (Mig-Hox9a-GFP, purchased from Addgene, Cambridge, MA). Retroviral supernatants obtained from Plat-E cells transfected with MigR1-GFP or Mig-Hox9a-GFP plasmid DNA were used to transduce Pax5-/- cells. Pax5-/- cells expressing MigR1-GFP or Hox9a-GFP were isolated based on GFP expression and maintained as cell lines under identical conditions as nontransduced Pax5-/- cells. An EBF-/- cell line stably expressing a tamoxifen-inducible EBF–estrogen receptor (ER) fusion construct was established by retroviral transduction of an EBF-/- line with EBF–ER-GFP–containing viral supernatants as previously described and transduced cells sorted based on GFP expression (20, 28). EBF activity was induced in the EBF-/-/EBF–ER line by addition of 4-hydroxytamoxifen (4-HT) (Sigma-Aldrich) or diluent (0.1% ethanol). Cells were harvested at 24–72 h posttreatment and processed for molecular and cellular analysis.

**Statistical analysis**

Statistics were done using the Student t test.

**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<sup>−/−</sup>, Pax5<sup>−/−</sup>, and RAG2<sup>−/−</sup> cell lines. First, we compared the expression patterns of the three essential B lineage transcription factors in the cell lines (Fig. 1A). All three cell lines showed relatively equivalent levels of E2A (cife2a) transcripts. Ebf1 transcripts are expressed in Pax5<sup>−/−</sup> 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<sup>−/−</sup> cells (20). To determine if the differential expression of EBF and Pax5 in the EBF<sup>−/−</sup> and Pax5<sup>−/−</sup> 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<sup>−/−</sup> cells compared with Pax5<sup>−/−</sup> cells, consistent with previous findings (20). Flt3 transcripts were high in EBF<sup>−/−</sup> cells, low in Pax5<sup>−/−</sup> cells, and below the level of detection in RAG2<sup>−/−</sup> 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<sup>−/−</sup> cells expressed high levels of CD34, CD27, and Flt3, compared with Pax5<sup>−/−</sup> or RAG2<sup>−/−</sup> cells (Fig. 1B). All three lines expressed the lymphoid lineage markers AA4.1 and the IL-7R (Fig. 1B). Only the RAG2<sup>−/−</sup> cell line expressed the B lineage-restricted Ag CD19. Long-term expanded cell lines may not exactly align with their in vivo developmental counterparts. Therefore, we evaluated expression of the markers differentially expressed on EBF<sup>−/−</sup> cell lines in BM CLPs through mature B cells. In particular, we focused on CD34 and CD27, because regulated expression of Flt3 as a function of B cell differentiation has been reported by others (30). CLPs are enriched in the Lin<sup>−</sup> c-kit<sup>hi</sup> IL-7R<sup>+</sup> fraction of BM (Fig. 1C) (31). Approximately 50% of Lin<sup>−</sup> c-kit<sup>hi</sup>IL-7R<sup>+</sup> defined CLPs express the lymphoid lineage-associated marker AA4.1, and the majority of Lin<sup>−</sup> c-kit<sup>hi</sup>IL-7RAA4.1<sup>+</sup> coexpress CD34 or CD27 (Fig. 1C) (32). Next, we examined the expression patterns of CD34 and CD27 on BCPs. Pre–Pro-B and Pro-B cells were gated as B220<sup>+</sup>CD43<sup>+</sup> and Pre-B/sIg<sup>+</sup> cells as B220<sup>+</sup>CD43<sup>−</sup> (Fig. 1D, boxed regions). The top right panels in Fig. 1D represent the staining pattern of CD34 and CD27 within the Pre–Pro-B/Pro-B–enriched fraction and the bottom panels within the Pre-B/sIg<sup>+</sup> subset. Expression of CD34 and CD27 is absent from the vast majority of BCPs. This in vivo analysis is consistent with our cell line data and shows that B cell differentiation is accompanied by downregulation of a cellular program preferentially expressed by primitive hematopoietic progenitors.

Downregulation of HoxA transcription accompanies B cell fate specification

To identify novel genetic events that accompany B cell fate specification, we took a functional genomics approach. The EBF<sup>−/−</sup> cell line exhibits a block at the lymphoid, but not B lineage-specified stage, the Pax5<sup>−/−</sup> cell line at the specified Pro-B cell stage, and the RAG2<sup>−/−</sup> cell line at the committed Pro-B cell stage. RAG2<sup>−/−</sup> 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<sup>−/−</sup> transcriptome subtracted functional genomics comparison of EBF<sup>−/−</sup> and Pax5<sup>−/−</sup> 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<sup>−/−</sup> cells compared with Pax5<sup>−/−</sup> 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<sup>−/−</sup> cells compared with Pax5<sup>−/−</sup> 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<sup>−/−</sup> cells (Table I). The levels of hoxa7, -9, and -10 transcripts in EBF<sup>−/−</sup> cells were striking (ranging from >37- to >1500-fold over levels expressed in Pax5<sup>−/−</sup> cells). In addition to hoxa transcripts, EBF<sup>−/−</sup> cells expressed transcripts for cd34, consistent with the surface expression of CD34 on this cell line (Fig. 1B). Other differentially expressed genes between EBF<sup>−/−</sup> and Pax5<sup>−/−</sup> cells included those involved in cell signaling (tyrobp, fcgr2b, ccl3, cink, igfbp4, gpr105, gpr34), signal transduction (prkar2a, ptpre, selempl1, selemp2), transcriptional regulation (fosf1a, ankrf3), and immune responses (cd7).

Differential expression of hoxa7–10 transcripts in EBF<sup>−/−</sup> 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<sup>−/−</sup> cell lines. Hoxa7–10 transcript abundance was evaluated by real-time RT-PCR in three nonclonal EBF<sup>−/−</sup> cell lines and the clonal line used in the GEP (Fig. 2B). Transcripts corresponding to the three hoxa genes were expressed in all EBF<sup>−/−</sup> cell lines analyzed. Thus, expression of hoxa transcripts is a shared feature of EBF<sup>−/−</sup> cell lines. The EBF<sup>−/−</sup> 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<sup>−/−</sup> cell lines. However, all three HoxA transcripts were detected in an E2A<sup>−/−</sup> BM-derived cell line (Fig. 2B) (11). We conclude, therefore, that expression of hoxa transcripts likely reflects the developmental stage represented by EBF<sup>−/−</sup> 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 transcription 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 transcription 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$^{+}$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 and *flt3* 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 within the Lin− hematopoietic progenitor compartment (39). Previous studies reported that Hoxa9−/− mice had normal numbers and frequencies of HSCs and MPPs (16, 40). The gating strategy used in the previous phenotypic analysis excluded Flt3, precluding analysis of differential expression of Flt3 within the hematopoietic progenitor compartment. Differential expression of Flt3 and CD34 discriminates HSC and MPP subsets (41). Therefore, we also included an Ab to CD34 in the analysis as a further marker of hematopoietic differentiation.

Hematopoietic progenitors are enriched within the Lin− compartment (Fig. 3A, left panels, gated region). LSK+ cells (Fig. 3A, middle panels, boxed region) are a subset of Lin− cells enriched for HSCs and MPPs (Fig. 3A, left panels, gated region). As previously reported, Hoxa9−/− mice have a reduction in numbers of BM nucleated cells (17.1 ± 3.2 × 10^6 versus 23.2 ± 3.2 × 10^6, *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 × 10^4 versus 3.3 ± 1.2 × 10^4, *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 cd34 (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. 3, 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+CD150^{−}CD48^{+}Flt3^{+} (p = 0.0069), but not LSK+CD150^{−}CD48^{+}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+ CD150^{−}CD48^{+} 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-kit^{lo}IL-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-kit^{lo}IL-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-kit\(^{hi}\)IL-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 finding 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\(^-\) BCPs 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\(^+\) 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\(^{hi}\) cells (left panels). LSK\(^+\) cells are a subset of Lin\(^-\)c-kit\(^{hi}\) 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\(^{lo}\)IL-7R\(^+\) fraction (left panels, boxed region). Lin\(^-\)c-kit\(^{lo}\)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 Flt3 in vivo**

Flow cytometric analysis of Hoxa9−/− mice suggested a role for Hoxa9 in regulation of 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 were detected in CLPs (Fig. 2C) suggested that Hoxa9 may similarly regulate flt3 in lymphoid progenitors. EBF−/− cell lines 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 Pbx1, directly regulates 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 flt3, was also bound to the Flt3 promoter (46). A previous study showed that flt3 transcripts are reduced by AML-1 deficiency (47). However, we did not observe direct binding of AML-1 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 ~20% of normal levels. qRT-PCR revealed reduced 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 flt3 transcripts in Pax5−/− cells expressing Hoxa9 (Fig. 4C). The increase in 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 flt3.

**EBF does not directly regulate hoxa9 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 μM 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).

\textbf{FIGURE 4.} Requirement for Hoxa9 in regulation of \textit{flt3}. A. In vivo cross-linking of Hoxa9, Meis1, and Pbx1 to the Flt3 promoter (top panel) in Flt3+IL-7R+ \textit{EBF}/2 cells but not or Flt3+IL-7R+ RAG2/2 Pro-B cells. PU.1 also regulates Flt3 and was included as a positive control for the assay. The formaldehyde cross-linked chromatin was analyzed using PCR primers that span the murine Flt3 promoter or an irrelevant site in the il-7ra gene. Control rabbit IgG and an Ab to Runx1 were included as controls for nonspecific binding of the Abs. Data are representative of three independent experiments. B, shRNA knockdown of \textit{hoxa9} reduces \textit{flt3} transcription and expression. \textit{EBF}/2 cells were transduced with lentiviral supernatants containing either a nontemplate control (NT shRNA) or Hoxa9-targeted shRNA (Hoxa9 shRNA) vector. Puromycin-resistant cells were harvested and analyzed by real-time RT-PCR for the indicated transcripts or for expression of Flt3 by flow cytometry (the black line represents Flt3 staining, and the gray line indicates untransfected cells). Data depict a representative analysis from three independent experiments. C, Ectopic expression of Hoxa9 in \textit{Pax5}/2 cells upregulates \textit{flt3} transcription and cell surface expression. Left panels indicate \textit{hoxa9} and \textit{flt3} transcript levels in the empty vector (MigR1) or Hoxa9-transduced Pax5/2 cell lines. The right panel indicates surface expression of Flt3 on the MigR1 (gray filled histogram), Hoxa9-transduced (hatched line) versus unstained cells (open histogram). Data are representative of three independent analyses of MigR1 and Mig-Hoxa9-transduced Pax5/2 cells.
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 \textit{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 \textit{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.

**FIGURE 5.** EBF does not directly inhibit \textit{hoxa} transcription. A. An \textit{EBF}^{−/−} cell line stably expressing a tamoxifen-inducible EBF–ER fusion protein were treated with 1 mM 4-HT for 24 h to induced nuclear localization of EBF. After 24 h, the cells were harvested, then analyzed by real-time RT-PCR for transcripts representing established EBF target genes (\textit{b29, mb1, pax5}), transcripts corresponding to cell surface markers differentially expressed on \textit{EBF}^{−/−} cells and downregulated as a function of B cell differentiation (\textit{flt3, cd34, cd27}), or \textit{hoxa} and \textit{meis1} transcripts. The labels indicate Ctr (media control), diluent (95% ethanol), and 4-HT. Data are plotted as the mean ± SD of normalized expression ratios obtained from three independent experiments. B. Downregulation of hematopoietic progenitor markers on \textit{EBF}^{−/−} cells treated with 4-HT. Three days posttreatment with 4-HT (hatched lines), the \textit{EBF}^{−/−} cells were harvested and analyzed by flow cytometry for the indicated cell-surface markers. The black line represents \textit{EBF}^{−/−} cells without 4-HT and the gray filled histogram unstained cells.
for B cell differentiation beyond the Pro-B cell stage (30, 54). Holmes et al. (30) have shown that Pax5 silences Flt3 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 HoxA 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. 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 or meis1/1 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 differentiation. Importantly, they set the foundation for future studies addressing mechanisms of HoxA regulation in BCPs as well as other EBF-regulated events that accompany B cell fate specification.

Acknowledgments
We thank L. Grimes for providing the Hoxa7-10 and Hoxa7-11 mice, M. Kondo for the EBF–ER plasmid, Umnikrishnan Gopinathan and the Mayo Advanced Genomics Technology Core Microarray Facility for processing and analysis of the microarray platform, Erin Maetzold and Meibo Chen for expert technical assistance, and Ginny Shapiro for critical review of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


