Context-Dependent Regulation of Hematopoietic Lineage Choice by HEBAlt

Duncheng Wang, Carol L. Claus, Paula Rajkumar, Marsela Braunstein, Amanda J. Moore, Mikael Sigvardsson and Michele K. Anderson

*J Immunol* 2010; 185:4109-4117; Prepublished online 8 September 2010;
doi: 10.4049/jimmunol.0901783
http://www.jimmunol.org/content/185/7/4109

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/09/07/jimmunol.0901783.DC1

Why *The JI*?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References

This article cites 70 articles, 36 of which you can access for free at:
http://www.jimmunol.org/content/185/7/4109.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Context-Dependent Regulation of Hematopoietic Lineage Choice by HEBAlt

Duncheng Wang,*†1 Carol L. Claus,*‡ Paula Rajkumar,* Marsela Braunstein,*† Amanda J. Moore,*† Mikael Sigvardsson,‡ and Michele K. Anderson*†

Hematopoietic development is controlled by combinatorial interactions between E-protein transcription factors and other lineage regulators that operate in the context of gene-regulatory networks. The E-proteins HEB and E2A are critical for T cell and B cell development, but the mechanisms by which their activities are directed to different genes in each lineage are unclear. We found that a short form of HEB, HEBAlt, acts downstream of Delta-like (DL)-Notch signaling to promote T cell development. In this paper, we show that forced expression of HEBAlt in mouse hematopoietic progenitors inhibited B cell development, but it allowed them to adopt a myeloid fate. HEBAlt interfered with the activity of E2A homodimers and with the expression of the transcription factor Pax5, both of which are critical for B cell development. However, when combined with DL-Notch signaling, HEBAlt enhanced the generation of T cell progenitors at the expense of myeloid cells. The longer form of HEB, HEBCan, also inhibited E47 activity and Pax5 expression, but it did not collaborate with DL-Notch signaling to suppress myeloid potential. Therefore, HEBAlt can suppress B cell or myeloid potential in a context-specific manner, which suggests a role for this factor in maintaining T lineage priming prior to commitment. The Journal of Immunology, 2010, 185: 4109–4117.

E-box binding transcription factors (E-proteins) are critical regulators of lymphoid lineage choice (1). Three E-protein–encoding gene loci are present in mammals: E2A (Tcf3/ITF1/Tcf2E2A), HEB (Tcf2/Alf-1/HTF4), and E2-2 (Tcf4/ITF2) (2). The E2A locus generates E47 or E12 proteins by mutually exclusive alternative splicing of two tandemly duplicated DNA-binding domains, whereas the HEB and E2-2 gene loci encode only one basic helix-loop-helix (bHLH) domain each. E-protein function as obligate dimers, and they can form homodimers or heterodimers with each other, as well as with tissue-specific bHLH factors, such as MyoD (3). Although it is known that E-protein homodimers and heterodimers are the primary bHLH complexes that act during lymphocyte development, their specific roles in hematopoietic development are not well understood.

HEB and E2-2 are more closely related to each other than either is to E2A at the amino acid level and in terms of their genomic loci structures (2). HEB and E2-2 can each give rise to two distinct proteins: a canonical form (HEBCan or E2-2Can) and an alternative (Alt) form (HEBAlt or E2-2Alt) (4–6). The Alt forms of HEB and E2-2 are generated by alternative transcriptional initiation sites and alternative splicing. However, the E2A gene locus does not contain an Alt domain. The 23-aa Alt domain, which is encoded in one exon between exons 8 and 9, is highly conserved within vertebrates, but it has not been found outside the vertebrates (6). Given the importance of E-proteins in cellular growth and development (7–9), it is remarkable that little is known about the functions of the Alt forms of HEB and E2-2.

All of the canonical E-proteins are important for lymphoid development (1). E2A-deficient mice exhibit a complete block in B cell development prior to the B220+ pre–pro-B cell stage, and they have aberrant T cell development (10–14). E2A is required for the upregulation of key regulatory factors, such as EBF, Pax5, and IL-7Rα, and it is needed for the expression of proteins required for differentiation and mature B cell function, such as Rag-1, Rag-2, mb-1, A5, and Igk (15, 16). By contrast, HEB and E2-2 have been thought to contribute to the total dosage of E-proteins required for B cell development, because the absence of either one of these factors led to a decrease in the numbers of pro-B cells but no obvious developmental block (17). Indeed, a human HEB cDNA inserted into the E2A locus was able to partially rescue B cell development in E2A−/− mice (18). However, the cDNA used in this study was HEBCan, leaving open the question of whether HEBAlt influences early B cell development.

E2A and HEB factors are also important during T cell development. E2A−/− and HEB−/− mice, which lack HEBAlt and HEBCan, exhibit partial blocks at the earliest stage of T cell development and at the pro–T to pre–T transition (19). Delta-like (DL)-Notch signaling is also critical for T cell development from the earliest stages (20–24), and it may help to direct E-protein activity toward T lineage genes and away from B lineage genes. Moreover, the low basal levels of MAPK activity in T cell precursors protect E2A from DL-Notch–induced degradation (25, 26). HEB also collaborates with E2A to control IL-7–mediated pro–T cell expansion, thus linking cell-cycle control to differentiation (27, 28). Interestingly,
the third E-protein, E2-2, is not expressed at high levels in developing B cells or T cells; instead, it seems to be a critical determinant of plasmacytoid dendritic cell development (29).

By contrast, myeloid development depends on the inhibition of E-protein activity, in part by an increase in Id factors (30, 31), which sequester E-proteins in inactive heterodimers. Id-2 is induced by PU.1, and high-level expression of PU.1 promotes myeloid development, whereas low levels of PU.1, enforced by Ikara-s induced Gfi1 expression, are necessary for B cell development (32). PU.1 is also necessary for the earliest stages of T cell development (33, 34), but it must be downregulated in pro-T cells (35–37). Interestingly, forced expression of Id factors alone in precursors placed in the fetal thymic environment diverts cells to the NK lineage, rather than to the myeloid lineage, indicating that context is critical for the developmental outcome imposed by E-proteins and Id factors (38, 39).

We first identified HEBAlt while screening an arrayed cDNA library constructed from SCID thymocytes, which represent a genetically enriched source of pro-T cells (40). Additional work in our laboratory confirmed that it was expressed in pro-T cells, and it could promote the entry of uncommitted precursors into the T cell lineage (6). Because pro-T cells retain some myeloid potential (41), we evaluated the ability of HEBAlt and HEBCan to influence B cell development and myeloid development in the presence and absence of DL-Notch signaling. We found that HEBAlt inhibits B cell development in a DL-Notch–independent manner, but it requires DL-Notch signaling to inhibit myeloid development. Thus, our results suggest that HEBAlt collaborates with DL-Notch signals to promote early T cell development by suppressing alternative lineage potentials.

Materials and Methods

Animals

The mice used for the RT-PCR studies were CD1 (E14.5 fetal liver) and C57BL/6 mice (bone marrow). For OP9 cocultures, embryos were obtained from National Institutes of Health Swiss timed matings (National Cancer Institute, Frederick, MD), CD1 timed matings (Charles River Laboratories, Wilmington, MA), or from C57BL/6 wild-type or C57BL/6 HEB+/+ × C57BL/6 HEB−/− timed matings (Sunnybrook Research Institute animal facility). The studies described were reviewed and approved by institutional review committees at Sunnybrook Research Institute.

Isolation of hematopoietic progenitors from fetal liver and bone marrow

Fetal liver E14.5 lineage-negative (Lin−) cells were obtained by staining cells with biotin-conjugated anti-Gr1, anti-Ter119, anti-CD19, and anti-F4/80 Abs, followed by binding to streptavidin-conjugated microbeads (Miltenyi Biotec, Auburn, CA). MACS was performed to obtain the anti-F4/80 Abs, followed by binding to streptavidin-conjugated microbeads with biotin-conjugated anti-Gr1, anti-Ter119, anti-CD19, and Lin−Retroviral transduction of fetal liver precursors

Although Lin− fetal liver cells were MACS enriched and transduced by coculture with GP+E.86 packaging cells that had been stably transduced with MIGR1-control or MIGR1-HEBAlt overnight, GFP+ LSK cells were sorted directly into 96-well round-bottom plates containing OP9-GFP stromal cells by a FACS Aria (BD Biosciences, San Jose, CA). Three sets of cultures were performed, each consisting of 30 wells at 10 cells/well, 30 wells at 3 cells/well, and 30 wells at 1 cell/well, for a total of 90 replicates of each dilution. After 6 d of culture, cells were harvested and analyzed by flow cytometry using a FACSCalibur for expression of GFP, CD45, and CD19 using anti–CD45-allophycocyanin and anti–CD19-PE. All wells containing CD45+GFP+CD19+ cells were scored as positive. Frequencies of precursors able to give rise to GFP+CD19+ cells were calculated by the maximum likelihood method applied to the Poisson model, using the Poisson9 program, as previously described (44).

OP9-GFP and OP9-DL1 cocultures

Sorted transduced cells were placed on OP9-GFP or OP9-DL1 monolayers supplemented with 5 ng/ml IL-7, SCF, and Flt3L in OP9 medium (24) at ∼3000 sorted cells/well in six-well plates. Cultures were split every 3–5 d and placed on freshly plated subconfluent monolayers. Cultures were analyzed by flow cytometry using a FACSCalibur, FACS Diva, FACS Aria, or LSR II (BD Biosciences), and FACS data were analyzed using FloJo (Tree Star, Ashland, OR).

Immunoblotting

Fetal liver GFP+ LSK cells that had been transduced with MIGR1-control or MIGR1-HEBAlt were cultured in OP9-GFP cocultures for a total of 22 d. Cells were collected from cultures and subjected to immunoblotting with anti-HEB Ab (Santa Cruz Biotechnology, Santa Cruz, CA; clone A-20) or anti-HEB Ab kindly provided by D. Littman, Skirball Institute of Molecular Medicine, New York, NY). Blots were stripped and probed with an anti-tubulin Ab as a loading control. The anti-HEB Ab epitope is contained within the common portion of HEBAlt and HEBCan; thus, it detects both proteins, which are distinguishable by size.

Luciferase reporter assays

Luciferase assays were performed by cotransfection of 100 ng 8X E-box reporter construct with expression plasmids encoding mouse E47, HEBAlt, and/or HEBCan into HeLa cells using Lipofectin (Invitrogen), as previously described (45, 46). Expression of E2A, HEBAlt, and HEBCan were driven by CMV promoters in pCDNA3 and/or pBK-CMV constructs. HEBAlt and HEBCan sequences were identical to those used in the retroviral constructs. Control plasmid DNA (pCDNA3) was included where necessary to bring each amount of expression plasmid up to 300 ng total. Renilla plasmids encoding pRl0 were also added as transfection-efficiency controls, and the reporter activity was calculated and presented as relative units of luciferase to Renilla activity.

Results

HEBAlt inhibits the generation of pro-B cells from multipotential precursors

Our previous work showed that expression of HEBAlt in hematopoietic precursors promoted their entry into the T cell lineage. To determine whether this was due, in part, to inhibition of alternative lineage potential, we retrovirally expressed HEBAlt or HEBCan in fetal liver precursors and followed their development into the B lineage in the OP9-GFP coculture system (24, 47) (Fig. 1A, 1B). Although Lin− fetal liver cells do not normally express HEBAlt,
transduction with HEBAlt or HEBCan leads to an increase in HEBAlt mRNA levels, as previously shown (6) (Supplemental Fig. 1A). Because this cross-regulation complicates analysis of the specific roles of HEBAlt versus HEBCan, we initially used HEB−/− precursors, which are devoid of HEBAlt and HEBCan (17). The HEB-null allele on the C57BL/6 background is embryonic lethal, necessitating timed matings of HEB+/− mice to obtain HEB−/− fetal precursors. E14.5 Lin− fetal liver were transduced with retroviral constructs, cultured overnight, sorted for GFP+ LSK (Lin− Sca-1+c-kit+GFP+ or YFP+) populations, and placed in OP9-GFP coculture for 7 d. C. FACS analysis of CD45 and CD19 expression. CD45+ cells are B lineage primed, and CD19+ cells are B lineage committed. D. Total cell numbers after 7 d of OP9-GFP coculture. E. Scatter plot showing a compilation of the decreases in the percentage of B lineage cells in response to HEBAlt in six different experiments using wild-type precursors. IRES, internal ribosome entry site; MIGR1, MSCV-IRES-GFP vector backbone; MIY, MSCV-IRES-YFP; MSCV, murine stem cell virus; YFP, yellow fluorescent protein.

FIGURE 1. HEBAlt inhibits the appearance of CD19+ B cell precursors. A, Retroviral vector structures used in this study. Arrows indicate transcriptional start sites driven by MSCV viral long terminal repeat promoters. Blue segments are HLH DNA-binding and dimerization domains. The pink segment represents the Alt domain, and the gray segment represents the HEBCan-specific domain. The black segments are putative transactivation domains. B, Experimental design. Lin− (Gr-1, Ter119, CD19, F4/80) cells from E14.5 fetal liver were transduced with retroviral constructs, cultured overnight, sorted for GFP+ LSK (Lin− Sca-1+c-kit+GFP+ or YFP+) populations, and placed in OP9-GFP coculture for 7 d. C, FACS analysis of CD45 and CD19 expression. CD45+ cells are B lineage primed, and CD19+ cells are B lineage committed. D, Total cell numbers after 7 d of OP9-GFP coculture. E, Scatter plot showing a compilation of the decreases in the percentage of B lineage cells in response to HEBAlt in six different experiments using wild-type precursors. IRES, internal ribosome entry site; MIGR1, MSCV-IRES-GFP vector backbone; MIY, MSCV-IRES-YFP; MSCV, murine stem cell virus; YFP, yellow fluorescent protein.

transduction with HEBAlt or HEBCan leads to an increase in HEBAlt mRNA levels, as previously shown (6) (Supplemental Fig. 1A). Because this cross-regulation complicates analysis of the specific roles of HEBAlt versus HEBCan, we initially used HEB−/− precursors, which are devoid of HEBAlt and HEBCan (17). The HEB-null allele on the C57BL/6 background is embryonic lethal, necessitating timed matings of HEB−/− mice to obtain HEB−/− fetal precursors. E14.5 Lin− fetal liver were transduced with retroviral constructs, cultured overnight, sorted for GFP+ LSK (Lin− Sca-1+c-kit+GFP+ or YFP+) populations, and placed in OP9-GFP coculture for 7 d. C, FACS analysis of CD45 and CD19 expression. CD45+ cells are B lineage primed, and CD19+ cells are B lineage committed. D, Total cell numbers after 7 d of OP9-GFP coculture. E, Scatter plot showing a compilation of the decreases in the percentage of B lineage cells in response to HEBAlt in six different experiments using wild-type precursors. IRES, internal ribosome entry site; MIGR1, MSCV-IRES-GFP vector backbone; MIY, MSCV-IRES-YFP; MSCV, murine stem cell virus; YFP, yellow fluorescent protein.

Although HEBAlt expression led to a severe decrease in CD19+ cells, CD19− cells were still present. Myeloid development is supported from fetal liver-derived LSK cells by OP9-GFP cells during the first week of culture (50), suggesting that the CD19− cells could belong to the myeloid lineages. Therefore, we analyzed the expression of CD11b on transduced sorted LSK cells after 6 d of OP9-GFP culture. All cultures contained some myeloid cells, but HEBAlt-transduced cultures exhibited a markedly increased percentage of CD11b+ cells with high forward scatter, consistent with a myeloid identity (Fig. 2A). The ratio of myeloid cells to B lineage cells was increased in response to HEBAlt even more in HEB−/− cultures than in wild-type cultures, suggesting a dose-dependent inhibition of B cell development (Fig. 2A, 2B). Moreover, the paucity of CD19− cells was not likely due to a simple lack of CD19 expression, because there were no B220+ cells that were not also CD19− at this time point (Fig. 2B). There was a moderate increase in cell death in HEBAlt-transduced cultures relative to controls (Fig. 2D). However, overall cell numbers in HEBAlt-transduced cultures did not decrease relative to control-transduced cultures (Fig. 2C). Therefore, the decrease in B lineage cells did not seem to be primarily a result of increased cell death. Interestingly, HEBCan-induced inhibition of B cell development in wild-type precursors did not result in an increase in the percentages of myeloid cells, suggesting that the mechanisms of inhibition might not be identical.

HEBAlt expression in hematopoietic progenitors results in a reduced frequency of cells that can give rise to CD19+ committed B cell precursors

To better define the nature of the HEBAlt-induced B cell defect, we analyzed the frequency of precursors able to develop into CD19+ cells in HEBAlt-transduced cultures compared with controls using a limiting-dilution assay (Fig. 3) (44). Typical levels of overexpression were between 5- and 10-fold in B lineage cells (Sup-
If B-primed progenitors overexpressing HEBAlt were simply inhibited in their proliferation potential, then equal numbers of cells would be expected to become GFP+CD19+ in HEBAlt-transduced and control cultures, with individual wells producing lower numbers of cells. However, if commitment or maturation of progenitors was indeed impeded, wells containing precursors capable of giving rise to GFP+CD19+ cells (Fig. 3B) would occur at a lower frequency in HEBAlt-expressing cultures compared with control-transduced cultures. To distinguish between these possibilities, we transduced LSK cells with HEBAlt-encoding virus or control virus and sorted GFP+ LSK cells into 96-well plates containing OP9-GFP stromal cells. The cultures were analyzed 6 d later. We found that although HEBAlt-expressing cells were capable of producing GFP+CD19+ cells, they were significantly less likely to do so than control cells (Fig. 3A). These results are consistent with a defect in B lineage commitment.

**HEBAlt does not perturb the development of committed B cell precursors**

To overcome the severe block in early development and examine the influence of HEBAlt on committed B cell precursors, we cultured E14.5 Lin− fetal liver cells with IL-7, SCF, and Flt3L for 9 d to allow initiation of the B lineage gene-expression program. These cells were transduced with MIGR1-control or MIGR1-HEBAlt and analyzed for the expression of B220 and CD43 to further characterize the stages at which HEBAlt inhibited development. At an early time point, there clearly was a lower percentage of B220+ cells and a higher percentage of B220− cells.}

**FIGURE 3.** B lineage commitment frequency analysis of HEBAlt-transduced precursors by limiting-dilution assay. A, Chart showing the frequency of precursors capable of giving rise to CD19+ cells within control or HEBAlt-transduced precursors. E14.5 FL Lin− cells were transduced, cultured, and sorted into 96-well plates at 1, 3, and 10 cells/ well onto OP9-GFP stromal cells. After 6 d, the cells were analyzed by FACS. Wells containing CD45+GFP+CD19− cells were scored as positive, and the frequency of precursors with B cell potential was calculated by the maximum likelihood method applied to the Poisson model. B, Examples of FACS plots scored as positives.
CD43+ non-B lineage cells in the HEBAlt-transduced cultures, consistent with a very early block in development (Fig. 4A). However, after several additional days of culture, the B220+ CD43+ cells (and CD11b+ cells; data not shown) disappeared in control and HEBAlt-transduced cultures, consistent with the short lifespan of myeloid cells coupled with the absence of appropriate growth factors (Fig. 4B). In addition to B220 and CD43, the control and HEBAlt-transduced cells that expanded in culture expressed BP-1 (Fig. 4C), which, in combination with B220, marks B cell commitment independently of CD19, placing them at the late pro-B cell stage. To assess whether the recovery of HEBAlt-transduced cells was due to downregulation or degradation of HEBAlt protein in these cells, we performed an immunoblot on protein isolated from control and HEBAlt-transduced pro-B cells cultured and expanded for 3 wk. We probed the blot with an anti-HEB Ab that detects an epitope common to HEBAlt and HEBCan and differentiated them based on size. HEBAlt and HEBCan were expressed in control cultures, indicating that they were present at the protein level in committed B cell precursors, and levels of HEBAlt were elevated in HEBAlt-transduced cells (Fig. 4D, Supplemental Fig. 1C). Therefore, HEBAlt could no longer perturb B cell development once the cells had committed to the B cell lineage.

**HEBAlt is expressed at low levels in early B cell precursors**

Our results predicted that HEBAlt must be kept at low levels in early B cell precursors to allow commitment to the B cell lineage. Therefore, we examined the mRNA expression of HEBAlt at progressive stages of B cell development, using a sorting strategy that allowed precise isolation of multipotent precursors (MPPs), common lymphoid precursors (CLPs), and pre–pro-B cells (Supplemental Fig. 3A), as well as early pro-B and late pro-B cells (42) (Supplemental Fig. 3B). The mRNA expression levels of HEBAlt in these subsets was determined by Q-PCR, and they were compared with HEBAlt levels in postnatal thymocytes at the early T cell progenitor (ETP), DN2 (early pro-T), DN3 (late pro-T), and DN4 (pre-DP) stages of T cell development (Fig. 5). HEBAlt mRNA was nearly undetectable in MPPs, CLPs, and ETPs. Low levels of HEBAlt were present in pre–pro-B and early pro-B cells, and higher levels were found in DN2 (early pro-T) and DN3 (late pro-T) stages. Therefore, HEBAlt could no longer perturb B cell development once the cells had committed to the B cell lineage.

**HEBAlt and HEBCan interfere with E2A activity and Pax5 expression.** A, Luciferase assays were performed to assess the activation of an 8X E-box reporter construct by HEBAlt, HEBCan, E47, or coexpression of E47 with HEBAlt or HEBCan in epithelial HeLa cells. The amount and type of expression plasmid is indicated on the x-axis, and the y-axis shows the values of luciferase activity relative to Renilla activity, presented as the mean ± SD. B, Q-PCR analysis of E2a, HEBCan, and HEBAlt expression in early B cell precursors sorted from bone marrow. Pre–pro-B, early pro-B, and late pro-B cells as specified in Fig. 4. Values are normalized to β-actin and presented as the mean ± SD of triplicate readings. C, GFP+ LSK cells transduced with MIGR1-control, MIGR1-HEBAlt, or MIGR1-HEBCan were sorted and placed in OP9-GFP coculture for 4 d. The CD45<sup>hi</sup>CD19<sup>+</sup> cells and the CD45<sup>lo</sup>CD19<sup>+</sup> cells were harvested from the GFP<sup>+</sup> populations and analyzed by Q-PCR for expression of Pax5 mRNA. D, GFP+ LSK cells were sorted from MIGR1-control or MIGR1-HEBAlt–transduced precursors and placed in OP9-GFP coculture for 6 d. Pre–pro-B cells (B220<sup>−</sup>AA4.1<sup>−</sup>CD19<sup>−</sup>) were sorted from the GFP<sup>+</sup> populations and subjected to Q-PCR to measure Pax5 expression. Values are expressed relative to β-actin and are the mean ± SD of triplicate readings.
pro-T) cells, consistent with previous reports (6, 51). However, in late pro-B cells, HEBAlt expression levels were similar to those observed in pre-B cells. HEBCan was expressed in MPPs and CLPs and at higher levels than HEBAlt in pre–pro-B and early pro-B cells. However, in late pro-B cells, HEBAlt levels increased, and HEBCan levels decreased. Therefore, HEBAlt expression was higher in early T cell precursors than in early B cell precursors. Intriguingly, pre-B cells expressed higher levels of HEBAlt than pro-B cells at the mRNA (Supplemental Fig. 4B) and protein (Supplemental Fig. 4C) levels, whereas IgM+ bone marrow cells expressed very little. In addition, fetal liver CD19+ (pro-B and pre-B) mRNA levels were similar to those of early T cell precursors (Supplemental Fig. 4A). These observations suggest that HEBAlt plays a role during normal B cell development at the late pro-B to pre-B cell stages.

**HEB factors can inhibit E2A activity**

Given the importance of E2A in the initiation of the B lineage gene-expression program, it was possible that HEBAlt and/or HEBCan inhibited early B cell development by disrupting E2A homodimer function. Therefore, we tested the ability of HEBCan and HEBAlt to inhibit E47 activity using an 8X E-box reporter construct (45). E47 alone activated expression of this construct by ∼90-fold in HeLa cells (Fig. 6A). HEBCan had only a limited ability to activate transcription (3-fold), whereas HEBAlt did not activate this reporter at all. However, cotransfection of HEBAlt or HEBCan with E47 decreased reporter expression to ∼10-fold over control, indicating strong inhibition of E47 function by either factor. These results suggested that initiation of B cell development might require a high ratio of E47 to HEBAlt and/or HEBCan. Therefore, we analyzed the relative levels of E2A, HEBAlt, and HEBCan mRNA side-by-side in pre–pro-B, early pro-B, and late pro-B cells; E2A was indeed expressed at much higher levels than HEBCan or HEBAlt during these stages (Fig. 6B). These results confirm that HEBAlt, HEBCan, and E2A do not play fully redundant roles in E-box activation and show that both HEB factors can negatively modulate the activity of E47 in this context.

**HEBAlt and HEBCan inhibit Pax5 expression in early B cell precursors**

Pax5 is one of the key regulators of B cell lineage commitment and maintenance of B lineage identity (52). Because E2A activity can be modulated by HEBAlt, and E2A activity is required for Pax5 expression (53), we set out to determine whether Pax5 mRNA levels could be perturbed in response to HEBAlt or HEBCan during B cell specification. GFP+ LSK cells were sorted from transduced Lin− progenitors and cultured for 4 d on OP9-GFP stroma, after which GFP+ cells from the CD45+ and CD45− fractions were sorted. Q-PCR analysis revealed that HEBAlt and HEBCan decreased the levels of Pax5 mRNA compared with controls (Fig. 6C). This occurred in the CD45+ fractions, which expressed low levels of Pax5, as well as in the CD45int fractions, which expressed higher levels of Pax5, consistent with specification toward the B cell lineage. To confirm that HEBAlt decreased expression of Pax5 specifically in B cell precursors, we next cultured transduced LSK cells for 6 d on OP9-GFP stroma and sorted out pre-pro-B cells (B220+AA4.1+CD19−). These cells also exhibited lower levels of Pax5 mRNA in the HEBAlt-transduced cultures than in the controls (Fig. 6D). Therefore, HEB factors can inhibit Pax5 mRNA expression in the context of early B cell precursors, possibly by interfering with E47 activity.

**DL-Notch signaling and HEBAlt collaborate to promote T cell development over myeloid development**

Although HEBAlt can inhibit E47 activity, it does not antagonize T cell development, indicating that it does not act as a context-independent inhibitor of all E-protein activity. Because B cell development is efficiently blocked by DL-Notch signaling, even in the presence of Id factors (38), we turned to an analysis of myeloid development in the presence of DL-Notch signaling. E14.5 Lin− fetal liver precursors were transduced with control, HEBAlt, or HEBCan-expressing retroviral constructs and cultured overnight to allow GFP expression. Sorted GFP+ LSK populations were placed on OP9-DL1 cells and analyzed after 4 d of coculture. As expected, the percentages and numbers of CD25+Thy-1+ (DN2/3; pro-T) cells were elevated over controls in the HEBAlt-transduced cultures but not in the HEBCan-transduced cultures (Fig. 7A, 7C). B cell progenitors did not arise in these cultures, as expected, but CD11b+ myeloid cells did. Under these conditions, unlike in OP9-GFP cocultures, fewer myeloid cells arose from uncommitted precursors in HEBAlt-transduced cultures (Fig. 7B, Supplemental Fig. 5). At this early time point in OP9-DL1 coculture, ∼60% of the control and HEBCan-transduced cells expressed CD11b compared with only 20% of the cells in the HEBAlt-transduced culture. Cell numbers were not appreciably different (Fig. 7C). Therefore, HEBAlt promoted T cell development at the expense of myeloid development in the presence of DL-Notch signaling. Importantly, HEBCan did not inhibit the appearance of CD11b+ cells (Fig. 7B, Supplemental Fig. 5), suggesting that DL-Notch signals collaborate uniquely with HEBAlt to enhance T cell development.

**FIGURE 7.** Increases in T cell precursors are accompanied by decreases in myeloid cells in HEBAlt-transduced OP9-DL1 cocultures. E14.5 Lin− fetal liver cells were transduced with retroviral constructs, cultured overnight, and then sorted to obtain GFP+ LSK cells, which were placed in OP9-DL1 cocultures. FACS analysis was performed 4 d later. A, Analysis of Thy-1 and CD25 expression. Thy-1+CD25+ cells are pro-T cells. B, Analysis of CD19 and CD11b expression. CD11b+ cells are myeloid. C, Total cell numbers at day 4 of LSK OP9-DL1 coculture are shown for three independent experiments.
development over myeloid development and that this function is not solely mediated by suppression of E47 activity.

Discussion
Our studies show that HEBAlt is able to repress alternative thymic lineage potential, as well as promote entry into the T cell lineage, and show that these functions are highly context dependent in a manner contingent on Notch signaling. HEBAlt expression in hematopoietic progenitors inhibited the appearance of CD19+ pro-B cells and allowed early progenitors to develop into myeloid cells, but only when DL-Notch ligands were not available. In the presence of DL-Notch signals, HEBAlt inhibited myeloid development and promoted T cell development. HEBAlt and HEBCan were able to inhibit E47 activity and Pax5 expression, providing a partial mechanistic explanation for their ability to inhibit B cell development. However, HEBCan did not inhibit myeloid development or enhance T cell development, suggesting that this activity was independent of E47 inhibition and was specific to HEBAlt. Taken together, our data indicate that the Alt domain is a powerful modulator of E-protein activity and that HEBAlt may play an important role in early hematopoietic lineage choice. Moreover, it is likely that the Alt forms of HEB and E2-2 can impact the functions of tissue-specific class B bHLH proteins, such as MyoD, NeuroD, and dHAND, and such interactions might be particularly important in processes that also depend on Notch signaling, such as neural crest development.

Our studies showed that several levels of context are critical for determining the output of HEBAlt activity. Environmentally, high levels of DL must be available for HEBAlt to inhibit myeloid development. It is likely that these signals change the context of the cell such that additional protein partners for HEBAlt become available. Signaling may also result in posttranslational modifications of HEBAlt itself that would allow interaction with different proteins. The importance of cellular context is also apparent in the differential ability of HEBAlt to influence B cell precursors at different stages of development. Our frequency assay results are consistent with a defect in B lineage commitment or in the early maturation of precursors expressing HEBAlt, suggesting that the protein can perturb the genetic networks that regulate B lineage cell fate. In contrast, we repeatedly observed that direct transduction of committed B cell precursors with HEBAlt did not decrease Pax5 expression or perturb cell-surface expression of CD19, even when inducible forms were used (data not shown). Coupled with the increase in HEBAlt mRNA during normal B cell development, these results suggest that HEBAlt is tolerated, and is likely to function normally during B cell development, in capacities that have yet to be determined.

Like HEBAlt, Id factors can disrupt E47 function and suppress B cell potential, whereas only Id factors enhance myeloid potential (38, 54–56). However, unlike Id factors, HEBAlt possesses a basic DNA-binding domain; we previously showed that HEBAlt is capable of specific binding to an E-box from the pre-Tα–regulatory region (6), and it can restore pre-Tα mRNA expression to HEB−/− precursors (57). Furthermore, although we found that HEBAlt and HEBCan are capable of inhibiting E47 activity, reminiscent of Id factors, other studies clearly showed that HEBCan and E47 are able to activate specific genes, indicating that the context of the regulatory DNA of each gene is critical in the correct interpretation of specific E-protein dimers (58, 59). Moreover, E2A−/− thymocytes display a partial block at the DN1 to DN2 transition, whereas HEBAlt promotes this transition, indicating that HEBAlt does not act simply as an inhibitor of E2A in all cellular contexts. Indeed, E47 and HEBCan are able to substitute for each other in at least some contexts (60), consistent with earlier reports in which HEBCan was able to partially compensate for the loss of E2A in B cell development (18). Additional studies are needed to determine the contexts in which E47, HEBAlt, and HEBCan work antagonistically or cooperatively with each other to drive the expression of lineage-specific genes.

Because neither HEBAlt nor HEBCan is expressed at high levels in uncommitted progenitors, they are unlikely to be involved in mediating the initial T/B fate choice during normal hematopoiesis, although they may act to inhibit B lineage regulators at the pre-T cell stage. ETPs, which express very low levels of HEBAlt, have already lost most of their B cell potential, indicating that HEBAlt is not strictly necessary for this function. However, some myeloid potential is retained in fetal and adult DN2/3 thymocytes (61, 62), and our results are consistent with a role for HEBAlt in the suppression of myeloid development in developing T cell precursors. Candidate targets of HEBAlt activity include PU.1 and C/EBPα, which can work together to divert T cell precursors into the myeloid lineages (63–65). Preliminary studies in our laboratory also revealed latent myeloid potential in HEB−/− T cell precursors, and they showed that the addition of HEBAlt to myeloid progenitors could restore their ability to become T cells (M. Braunstein and M.K. Anderson, manuscript in preparation). Therefore, our results agree with other studies that support a close relationship between the T cell and myeloid lineages (49, 50, 61, 62, 66) and suggest that DL-Notch signals may suppress myeloid development, in part, by upregulation of HEBAlt.

Therefore, we propose that HEBAlt operates as a T lineage fidelity factor during early T cell development in a DL-Notch–dependent manner. PU.1, GATA3, and Notch1 are all legacy genes from the stem cell stage, whereas HEBAlt is upregulated as T cell specification occurs. This function is reminiscent of Pax5, which must be present to maintain B lineage fidelity in B cell precursors and mature B cells (52, 67). Removal of Pax5 from B lineage cells at any stage, including in mature peripheral B cells, leads to de-differentiation back to a primitive state, which is then able to be induced to develop into alternative lineages. By contrast, HEBAlt is not expressed past β-selection checkpoint during T cell development (6) and, therefore, it is not required to maintain lineage fidelity after the fundamental T lineage regulatory network has been established. Likewise, HEBAlt is not expressed at high levels prior to DN2/3 pro-T stages of T cell development, suggesting that it is unlikely to be involved in prethymic restriction of the B cell lineage (68–70). As one of the few transcription factors upregulated at the DN1 to DN2 transition (71), HEBAlt is uniquely positioned to collaborate with DL-Notch signals in a feed-forward loop that maintains T lineage identity, in part, by suppressing myeloid lineage potential prior to T cell commitment. Indeed, we found that HEB−/− precursors in OP9-DL1 cocultures undergo a progressive loss of T lineage identity as they transit through the DN2 and DN3 stages (M. Braunstein and M.K. Anderson, submitted for publication). Future work will focus on defining the network connections between HEBAlt and other hematopoietic lineage regulators and on understanding the mechanism by which the Alt domain confers unique functions to E-proteins.

Acknowledgments
We thank M. Ratcliffe and J. Rast for critical comments and helpful discussions. We also thank the Comparative Research Facility at Sunnybrook Research Institute for excellent animal care. G. Knowles and A. Khandani provided important sorting expertise. HEB−/− mice were kindly provided by Trang Hoang, and we thank J.C. Züniga-Pflücker for the OP9-GFP and OP9-DL1 stromal cell lines. We thank D. Littman for the anti-HEB Ab. We also thank Matthew Chui and Gianna Vaccarelli for help with sorting and Q-PCR.

Disclosures
The authors have no financial conflicts of interest.
References

5. Zhuang, Y., P. Cheng, and H. Weintraub. 1996. B-lymphocyte development is 
9. Pui, J. C., D. Allman, L. Xu, S. DeRocco, F. G. Karnell, S. Bakkour, J. Y. Lee, 
13. Nie, L., S. S. Perry, Y. Zhao, J. Huang, P. W. Kincade, M. A. Farrar, and 
22. Rothenberg, E. V., and D. D. Scripture-Adams. 2008. Competition and collabor- 
23. Fujimoto, S., T. Ikawa, T. Kina, and Y. Yokota. 2007. Forced expression of Id2 in 