

Multilineage Priming of Enhancer Repertoires Precedes Commitment to the B and Myeloid Cell Lineages in Hematopoietic Progenitors

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SUMMARY

Recent studies have documented genome-wide binding patterns of transcriptional regulators and their associated epigenetic marks in hematopoietic cell lineages. In order to determine how epigenetic marks are established and maintained during developmental progression, we have generated long-term cultures of hematopoietic progenitors by enforcing the expression of the E-protein antagonist Id2. Hematopoietic progenitors that express Id2 are multipotent and readily differentiate upon withdrawal of Id2 expression into committed B lineage cells, thus indicating a causative role for E2A (*Tcf3*) in promoting the B cell fate. Genome-wide analyses revealed that a substantial fraction of lymphoid and myeloid enhancers are premarked by the poised or active enhancer mark H3K4me1 in multipotent progenitors. Thus, in hematopoietic progenitors, multilineage priming of enhancer elements precedes commitment to the lymphoid or myeloid cell lineages.

INTRODUCTION

In the adult bone marrow, long-term hematopoietic stem cells (LT-HSCs) have the ability to both self-renew and reconstitute the entire immune system for the life of the organism (Spangrude et al., 1988). LT-HSCs have the ability to differentiate into short-term HSCs (ST-HSCs), which give rise to multipotent progenitors (MPPs) and then differentiate into the lymphoid-primed multipotent progenitors (LMPPs) (Adolfsson et al., 2005). LMPPs differentiate into common lymphoid progenitors (CLPs) or granulocyte-macrophage progenitors (GMPs) that give rise to either a lymphoid- or myeloid-restricted pathway, respectively (Inlay et al., 2009; Kondo et al., 1997).

During the past decade, transcription factors have been identified that play critical roles in early B cell development. These

include the E2A proteins, EBF1, FOXO1, and PAX5 (Bain et al., 1994; Zhuang et al., 1994; Urbánek et al., 1994; Lin and Grosschedl, 1995; Dengler et al., 2008; Amin and Schlissel, 2008; Treiber et al., 2010). The E2A proteins are members of the E-protein transcription factor family that also include HEB, E2-2, and E2A. The E-protein transcription factors are characterized by the presence of a helix-loop-helix (HLH) dimerization domain and a DNA binding region. The E2A locus encodes for two proteins, E12 and E47, which arise from differential splicing of exons that encode for their DNA binding and dimerization domains. E2A proteins were originally identified based on their abilities to bind the immunoglobulin kappa chain enhancer and have since been implicated at multiple stages of lymphoid and B cell development (Bain et al., 1994; Beck et al., 2009; Dias et al., 2008; Murre et al., 1989; Semerád et al., 2009; Zhuang et al., 2004). E-proteins are antagonized by the Id proteins, which contain an HLH motif but lack a DNA binding region (Benezra et al., 1990). In mice deficient for E2A, B cell development is blocked at the CLP cell stage (Bain et al., 1994, 1997; Zhuang et al., 1994). A similar block in B cell development is observed in EBF-, FOXO1-, and PAX5-deficient mice (Dengler et al., 2008; Lin and Grosschedl, 1995; Nutt et al., 1997).

When cultured in B cell-supportive conditions in vitro, E2A-deficient bone marrow cells self-renew indefinitely without losing multipotent differentiation potential (Ikawa et al., 2004). B cell differentiation in E2A-deficient cells can be rescued by forced EBF expression, suggesting that the E2A proteins act upstream of EBF in B cell development (Hagman and Lukin, 2006; Lazorchak et al., 2005; Seet et al., 2004). Recent studies have directly linked these and other transcriptional regulators into a common framework (Ghisletti et al., 2010; Heinz et al., 2010; Treiber et al., 2010; Lin et al., 2010; Natoli, 2010). E2A initiates a program of B-lineage-specific gene expression by inducing the expression of *Ebf1*, *Bcl11a*, *Irf4*, *Irf8*, and *Foxo1* (Lin et al., 2010). E2A, EBF1, FOXO1, IRF4, and IRF8 act in concert to activate the expression of the *Pax5* locus (Decker et al., 2009; Lin et al., 2010). E2A, EBF1, and Foxo1 as well as Pax5 then act to induce the expression of a large subset of genes including signaling components, survival factors, and regulators that modulate cell cycle progression (Lin et al., 2010; McManus et al., 2011).

Here, we have generated a cytokine-dependent cell line, named Id2-HPC, which allowed for the expansion of a progenitor cell line that maintains multipotency both in vivo and in vitro. We then harnessed the differentiation potential of these cells to monitor how enhancer repertoires are established at the very earliest stages of B and myeloid cell development. We found that a subset of poised enhancer elements are marked at the uncommitted progenitor cell stage. However, H3K4me1 marks at a subset of enhancers were elevated during developmental progression, resulting in evolving active enhancer repertoires that we propose orchestrate the myeloid and B cell fates.

RESULTS

Self-Renewal Activity in Hematopoietic Progenitors

Previous experiments demonstrated that multipotent progenitor cells can self-renew in vitro in the absence of E2A and retain pluripotency in vivo (Ikawa et al., 2004). To determine whether overexpression of Id proteins would effectively inhibit E2A and thus allow in vitro expansion of multipotent progenitor cells, we created lentiviral vectors utilizing a Tet-Off promoter that expresses human *ID2* (*hId2*) and GFP in the absence of doxycycline (see Figure S1A available online).

Lineage-depleted bone marrow from CD45.1 congenic C57BL/6 mice was infected with lentivirus carrying Id2 (TetOff_*hId2*) or empty vector control (TetOff_Empty). The transduced cells were cultured in the presence of cytokines IL-3, IL-6, WEHI supernatant, and SCF. Initially, both the control (C-HPCs) and *hId2*-infected (Id2-HPCs) cells rapidly expanded. However, upon culture for 4 weeks, C-HPCs had ceased growing whereas the Id2-HPCs continued to divide (data not shown). Id2-HPCs were then cultured in IL-7, Flt3L, and SCF on subconfluent S17 feeder cells. In these conditions, the cells continued to expand over time. The Id2-HPC cells overexpressed *hId2* mRNA, which could be robustly turned off with the addition of doxycycline to the culture media (Figure 1A).

Id2-HPCs did not express markers characteristic for T cell and NK cell lineages and expressed intermediate amounts of B220 and high abundance of CD43 and were Ly6D, IgM, and IgD negative (Figure 1B). A small percentage of the cells (1%–5%) expressed CD11b, CD19, or CD25, but the majority of cells remained lineage negative even after being cultured in vitro for relatively long periods of time. Id2-HPCs carried D_HJ_H joints, but lacked $V_H-D_HJ_H$ rearrangements, suggesting that they represent cells arrested at the CLP or pre-pro-B cell stage (Figure 1C; Busslinger, 2004).

To further characterize Id2-HPCs, we performed microarray analysis with RNA derived from freshly isolated LT-HSCs, ST-HSCs, CLPs, LMPPs, pre-B cells, pro-B cells, as well as cultured E2A-deficient cells, EBF-deficient cells, and Id2-HPCs. Nine expression patterns were identified from the 11,367 changed genes (Figure 1D). Genes encoding for proteins required for B cell development such as *Foxo1*, *Pou2af1*, *IgL*, *Ly6d*, and *Vpreb1* showed equivalent expression in the Id2-HPCs relative to HSCs and LMPPs but lower expression as compared to pro- and pre-B cells. In contrast, genes that are expressed in pluripotent progenitors such as *Cd34*, *Kit*, *Tie1*, *Slamf1*, and *Tal1* were upregulated in HSCs and CLPs when compared to Id2-HPCs. Vertical clustering of expression patterns showed that Id2-HPCs

resemble the transcription signatures of *Tcf3*(E2A)^{−/−} and *Ebf1*^{−/−} cells (Figure 1E). Taken together, these data indicate that forced Id2 expression in hematopoietic progenitors promotes the self-renewal of cells with a similar phenotype as observed in *Tcf3*^{−/−} and *Ebf1*^{−/−} pre-pro-B cells.

Id2-HPCs Are Multipotent

To determine whether Id2-HPCs maintain pluripotency and repopulating ability in vivo, cells were injected into irradiated CD45.2 recipients in a competitive repopulating assay. In the absence of E2A, B cells do not develop beyond the pre-pro-B cell stage and alternate lineage development is perturbed, so we administered doxycycline via food pellets to recipient animals to turn off Id2 expression and thus allow restoration of E-protein activity (Bain et al., 1994). The mice received doxycycline food pellets 24 hr before injection and throughout the remainder of the study. Recipient mice were lethally irradiated 24 hr before tail vein injection of Id2-HPCs (CD45.1) mixed (1:1) with freshly harvested CD45.2 bone marrow cells. At 6 week posttransplantation, Id2-HPCs had successfully reconstituted the bone marrow, thymus, and spleen of irradiated recipients to varying degrees, albeit at reduced ability compared to wild-type bone marrow (Figure 2; Figure S1). The large majority of the CD45.1 cells were GFP negative, showing the robust sensitivity of the tetracycline transactivator to doxycycline in vivo (Figure 2).

In the bone marrow, Id2-HPCs reconstituted the B and myeloid compartments successfully (Figure 2A). There were increased numbers of CD45.1 B220⁺ cells and decreased numbers of CD45.1 CD11b⁺ cells relative to their CD45.2 counterparts, suggesting a propensity for Id2-HPCs to commit to the B cell lineage (Figure 2A). The Id2-HPCs also successfully migrated to the peripheral lymphoid organs (Figures 2B and 2C). IgM expression was readily detectable on approximately 20% of CD45.1 splenocytes, demonstrating that Id2-HPCs have the ability to successfully progress beyond the pre-pro-B cell stage (data not shown). Id2-HPCs also reconstituted the T cell and myeloid compartments in the spleen (Figure 2B). In the thymus, Id2-HPCs reconstituted the double-negative, double-positive, and single-positive populations (Figure 2C). Thus, Id2-HPCs can be grown long term in culture and have the ability to reconstitute multiple hematopoietic cell lineages in vivo.

In Vitro Differentiation of Id2-HPCs into Myeloid and B Cell Lineages

The transplantation experiments revealed that Id2-HPCs possessed multilineage potential. To exclude the possibility that the multipotency of Id2-HPCs was caused by a heterogeneous populations of lineage-restricted cells, single-cell cultures were initiated to determine the clonal properties of Id2-HPCs. Individual cells from the highest 4%, lowest 4%, or intermediate 10% of GFP mean fluorescence were sorted onto preseeded S17 cells in 96-well plates and cultured as described above. Clones from each of the conditions (L, low GFP; I, intermediate GFP; H, high GFP) were expanded, and one clone from each group was chosen for further analysis (Figure S2A). To examine their myeloid lineage potential, three Id2-HPC monoclonal cell lines (L1, I2, and H1) were cultured in the presence of IL3, Flt3-L, GM-CSF, and M-CSF. Within 48 hr after initiation of the

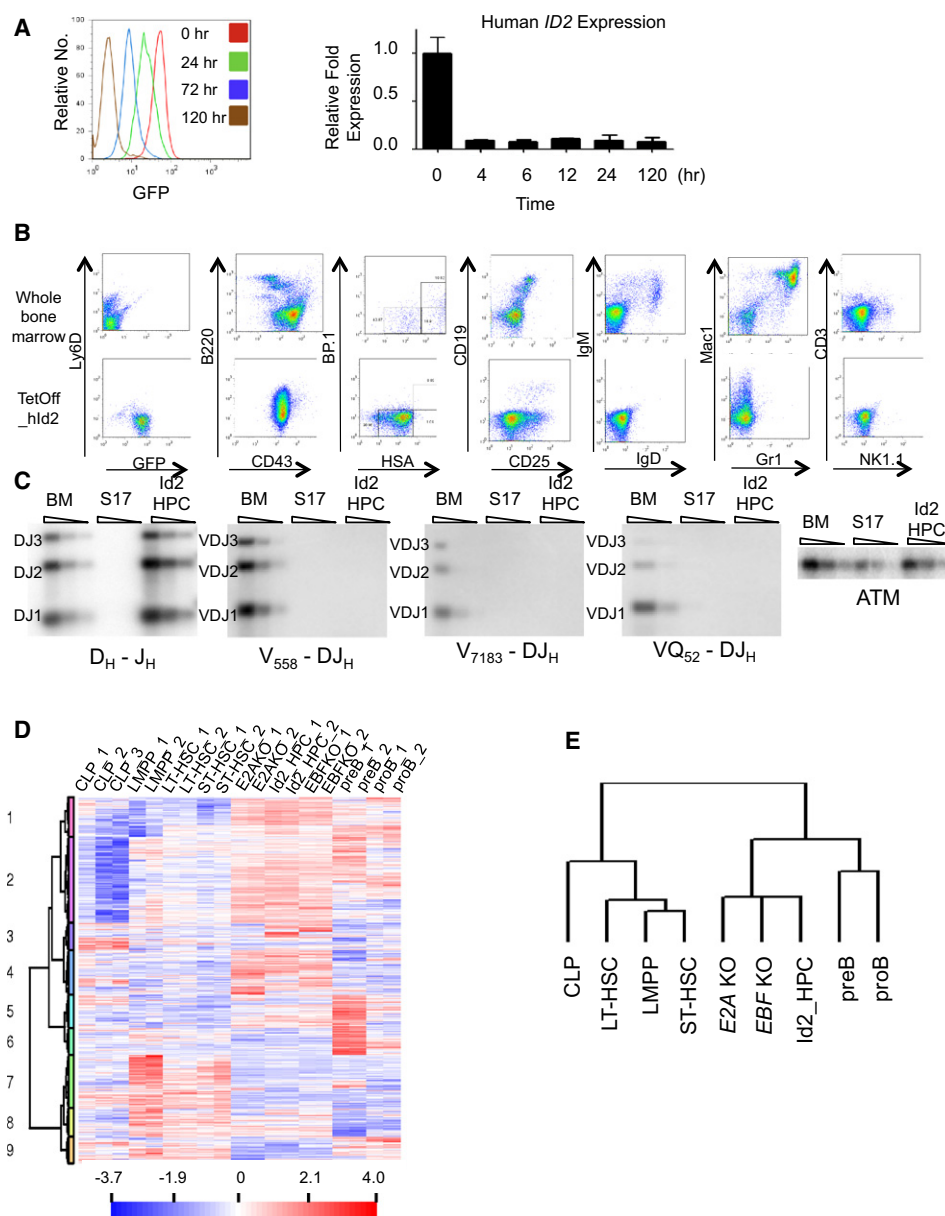


Figure 1. Establishment of a Long-Term Culture of Multipotent Hematopoietic Progenitors

(A) Rapid and robust downregulation of GFP and human *ID2* after doxycycline addition in vitro. FACS plots of GFP (left) at 0, 24, 72, and 120 hr after doxycycline addition are shown. Real-time PCR analysis of human *ID2* expression at 0, 4, 6, 12, 24, and 120 hr after doxycycline addition. Error bars refer to standard deviation.

(B) Phenotypic analysis of Id2-HPC cells. TetOff_hld2-infected cells were cultured on S17 feeder cells with IL-7, SCF, and Flt3L. Cells were analyzed by FACS for the expression of B220, Ly6D, CD43, CD25, CD19, Mac1, Gr1, CD3, and NK1.1 after 3 months in culture. The upper panel shows staining from wild-type bone marrow as controls and the lower panel shows staining from expanded Id2-HPCs in the absence of doxycycline.

(C) IgH gene rearrangement analysis of Id2-HPCs. DNA was isolated from wild-type bone marrow, S17 feeder cells, and TetOff_hld2 cells and analyzed by Southern blot for the presence of IgH DJ and V-DJ rearrangements. ATM was used as a loading control.

(D) Microarray analysis of LT-HSCs, ST-HSCs, LMPPs, CLPs, pre-B cells, pro-B cells, *Tcf3*^{-/-} cell line, *Ebf1*^{-/-} cell line, and Id2-HPC cell line. Duplicate genes have been removed. The color scale is shown below the diagram.

(E) Vertical clustering of cell types from microarray experiment in Figure 1D.

myeloid culture, Id2-HPCs expressed significant levels of CD11b (Figure 3A; Figures S2B and S2C). Interestingly, the mean fluorescence intensity of B220 was increased in the myeloid cultures in all three clones, similar to that seen in other studies (Xie et al., 2004). Additionally, *Id2* expression was higher in the myeloid

cultures than in progenitor cells prior to differentiating conditions (Figure 3D). Id2-HPCs possess similar myeloid potential as the *Tcf3*^{-/-} cell line (Figure S1B). These data indicate that cells expressing high amounts of Id2 are favored to commit to the myeloid cell lineage.

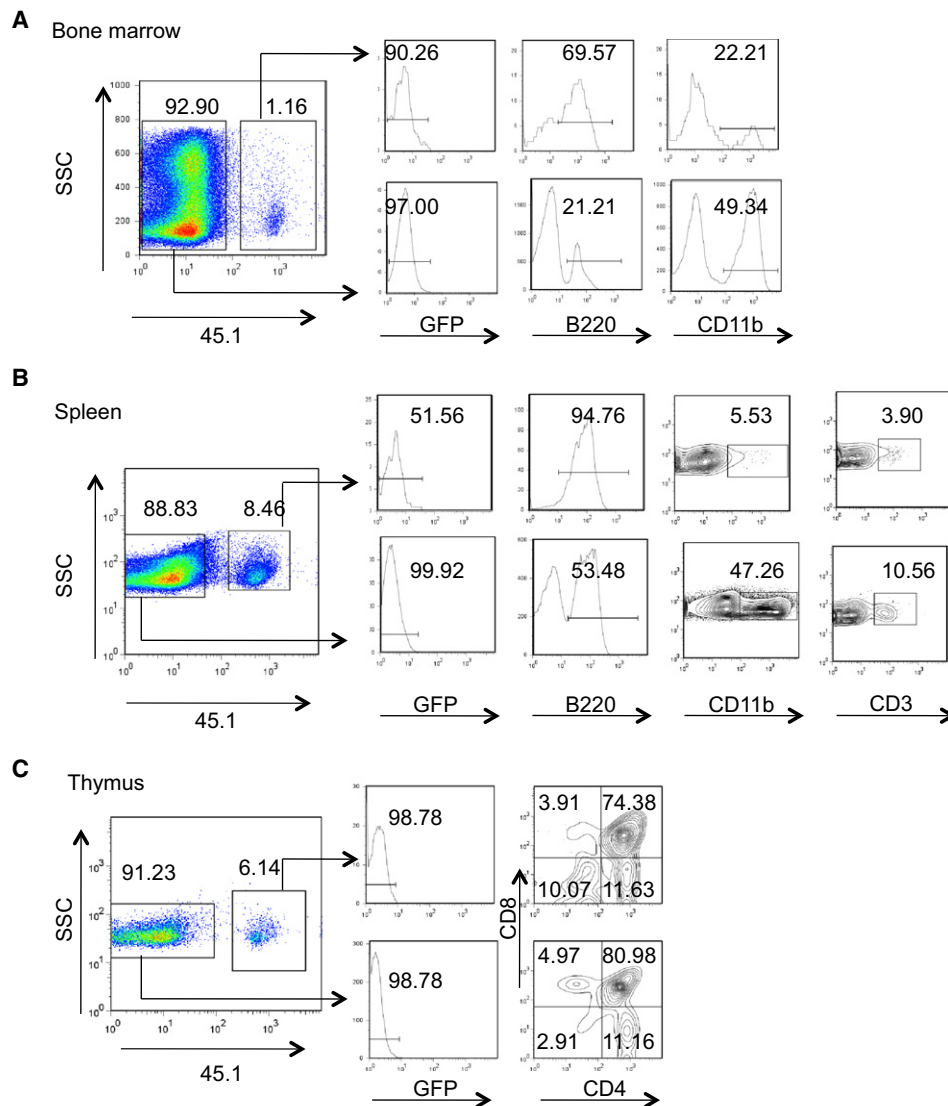


Figure 2. Id2-HPCs Reconstitute Multiple Lineages In Vivo and In Vitro

Id2-HPCs can reconstitute multiple lineages in irradiated recipients. A competitive reconstitution assay was used to assess the ability of Id2-HPCs to reconstitute irradiated recipients. 1,000,000 Id2-HPCs and 1,000,000 freshly isolated wild-type bone marrow cells were injected into lethally irradiated recipients receiving doxycycline feed. Reconstitution was analyzed 6 weeks later. The data are representative of two experiments.

(A) Id2-HPCs successfully reconstitute the bone marrow of recipients. Bone marrow cells were analyzed by FACS analysis for expression of GFP, B220, and CD11b.

(B) Id2-HPCs successfully reconstitute the spleen of recipients. Splenic cells were analyzed by FACS analysis for expression of GFP, B220, CD11b, and CD3.

(C) Id2-HPCs successfully reconstitute the thymus of recipients. Thymic cells were analyzed by FACS analysis for expression of GFP, CD4, and CD8.

To promote differentiation of Id2-HPCs into the B cell lineage, cells were cultured in the presence of S17 cells, IL-7, and SCF either with or without doxycycline. Id2-HPCs that were cultured in the absence of doxycycline continued to divide but maintained the pre-pro-B cell phenotype. Id2-HPCs that were grown in the presence of doxycycline rapidly downregulated GFP and showed a transient upregulation of the recently defined marker for B cell lineage-primed progenitors, LY6D (Inlay et al., 2009; Mansson et al., 2008). This was followed by a robust increase in the levels of CD19 expression (Figure 3B; Figures S2B and S2C). Differentiation into committed B cell lineage progenitors

was also supported by the presence of substantial levels of V_H-D_H-J_H joints (Figure 3C). Quantitative PCR analysis confirmed upregulation of the myeloid-specific genes, including *Itgam* and *Csfr1*, upon culture conditions that favor myeloid differentiation, and the upregulation of B cell-specific genes, including *Ebf1* and *Foxo1*, in conditions that favor B cell differentiation (Figure 3D). We note, however, that cultured Id2-HPCs do not retain their B cell developmental potential indefinitely. Taken together, these observations indicate that forced expression of Id2 in hematopoietic progenitors permits the establishment of a long-term, but not indefinite, culture of multipotent progenitors.

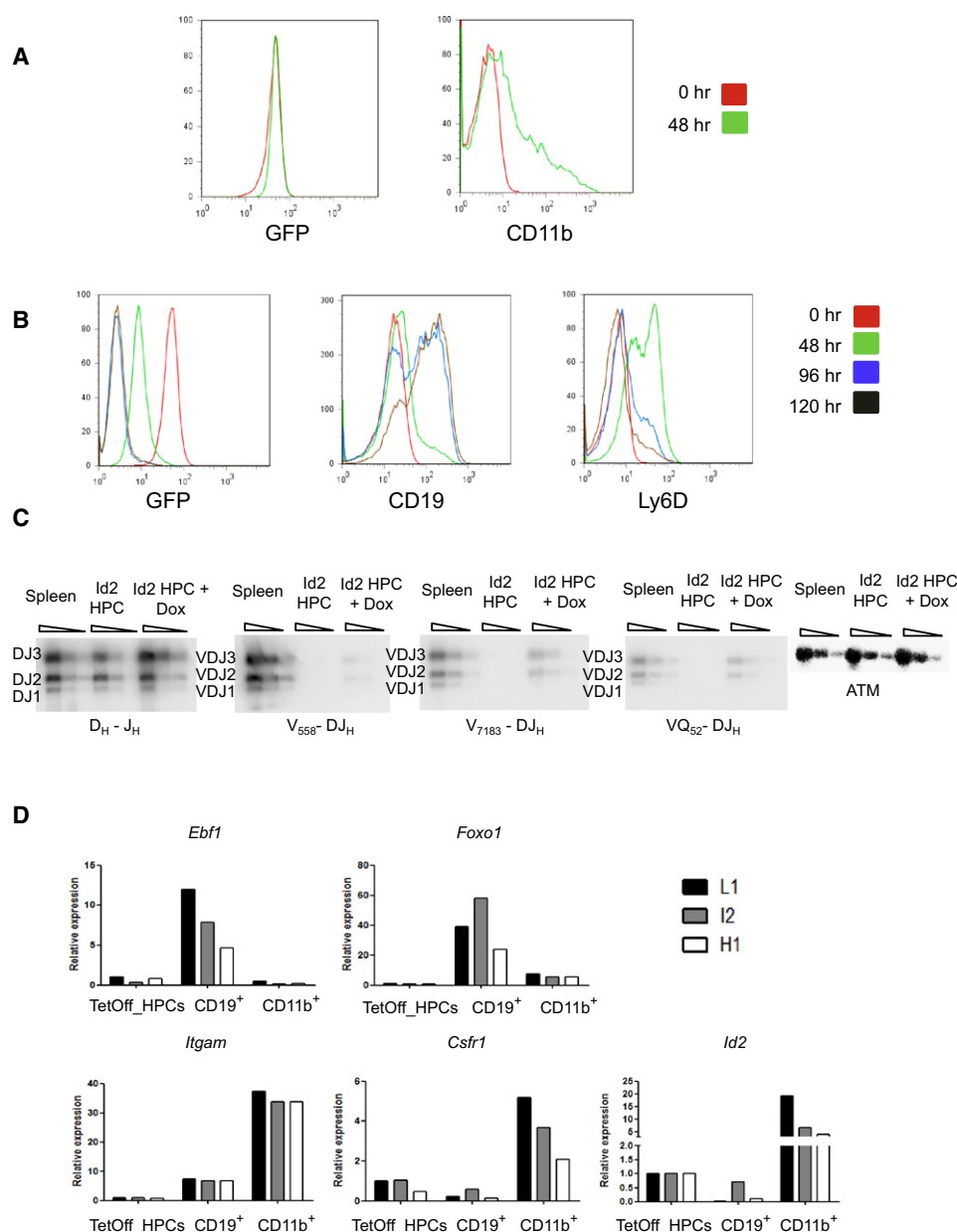


Figure 3. In Vitro Differentiation Potential of Id2-HPCs

(A) In vitro differentiation of Id2-HPCs into myeloid cells. Cells were cultured in IL-3, Flt3L, GM-CSF, and MCSF in the presence or absence of doxycycline for 2 days in vitro and analyzed by FACS at day 0 and day 2 for CD11b.

(B) In vitro differentiation of Id2-HPCs to pro-B cells. Cells were cultured in IL-7, SCF in the presence or absence of doxycycline for 6 days in vitro and analyzed at day 0, day 2, day 4, and day 5 for GFP, CD19, and Ly6D expression.

(C) IgH gene rearrangement in differentiated Id2-HPCs. DNA was isolated from wild-type bone marrow, day 0 Id2-HPCs and day 8 plus doxycycline Id2-HPCs and analyzed by Southern blotting for IgH DJ and V-DJ rearrangements. ATM was used as a loading control.

(D) Gene expression analysis of lineage-specific transcripts in undifferentiated Id2-HPCs and CD19⁺ or CD11b⁺ differentiated Id2-HPCs. RNA was isolated from monoclonal Id2-HPCs at the undifferentiated state or the CD19⁺ or CD11b⁺ differentiated state, and transcript levels were analyzed by real-time PCR analysis.

Gene Expression Signatures in B and Myeloid Cells Derived from Multipotent Progenitors

To compare the expression signatures from differentiated B and myeloid cells derived from hematopoietic progenitors that express Id2, Id2-HPCs were differentiated into either CD19⁺ B cells or CD11b⁺ myeloid cells. RNA was isolated from the cultures and

analyzed by microarray gene expression analysis. We hierarchically clustered the expression of genes with a 2-fold or more change in absolute expression levels between any two groups (Figure 4A). Several patterns emerged: genes whose transcripts became elevated in differentiated B cells, genes whose transcripts declined in differentiated myeloid cells, genes whose

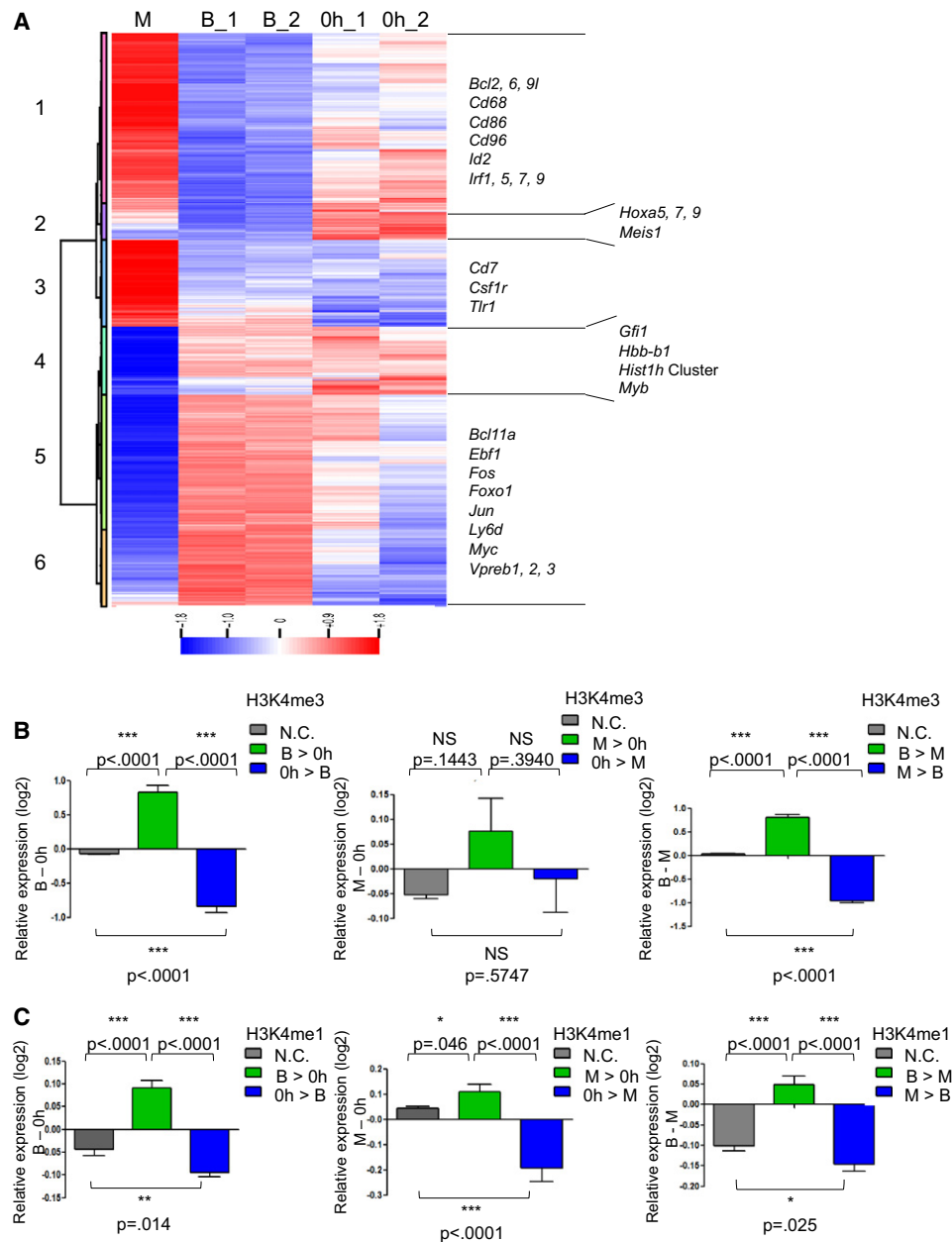


Figure 4. Establishment of Promoter and Enhancer Marks in Differentiating B and Myeloid Cells

(A) Multipotent progenitors carrying *Id2* were differentiated into either CD19⁺ B cells (upon exposure to doxycycline) or CD11b⁺ myeloid cells. Gene expression patterns of multipotent progenitors and differentiated progeny were analyzed by microarray.

(B) Changes in gene expression levels correlate to changes in proximal H3K4me3-mapped reads between HPCs, B, and myeloid cells. Changes in H3K4me3 were directly compared to gene expression levels. Expression from genes with 2-fold more mapped reads (tags) in one lineage versus another lineage are indicated in green (B > 0h or M > 0h or B > M) or blue (0h > B or 0h > M or M > B), and peaks with less than a 2-fold change in mapped reads (tags) are shown in gray (N.C. refers to no change). 0h refers to multipotent *Id2*-HPC cells. M refers to myeloid cells as characterized by CD11b expression that were differentiated from *Id2*-HPCs. B refers to B cells as characterized by CD19 expression that were differentiated from *Id2*-HPCs. p values are indicated. N.S. refers to not significant. Error bars refer to standard deviation.

(C) Changes in gene expression levels correlate to changes in distal H3K4me1-mapped reads between HPCs, B cells, and myeloid cells. Error bars refer to standard deviation.

transcript levels were elevated in undifferentiated cells, and genes that were activated upon myeloid differentiation (Figure 4A).

As expected, *Ebf1*, *Foxo1*, *Bcl11a*, and *Vpreb* transcript levels were substantially elevated upon differentiation into the B cell

lineage (Figure 4A). Interestingly, the pattern of *Ly6d* expression also falls into this cluster, supporting previous data implicating *Ly6d* as a marker that specifies the B cell fate (Figure 4A; Inlay et al., 2009; Mansson et al., 2010). Myeloid-specific genes

including *Cd68* and *Csf1r* were readily activated in culture conditions that favor myeloid development (clusters 1 and 3). A group of genes was also identified whose transcript levels declined upon differentiating into either the B or the myeloid cell lineage (Figure 4A, clusters 2 and 4). Interestingly, cluster 2 includes genes that are characteristic of the hematopoietic stem cell signature, including *Meis1* as well as several members of the *Hoxa* family (Figure 4A). Collectively, these data indicate that Id2-HPCs in differentiating conditions activate lineage-specific programs of gene expression and repress transcription of genes associated with the HSC fate as well as alternate differentiated cell lineages.

Establishment of Promoter and Enhancer Marks in Differentiating B and Myeloid Cells

The gene expression patterns uncovered by microarray analysis in differentiating multipotent progenitors (Id2-HPCs) indicate genome-wide changes in transcriptional activity. We next asked when and how these changes are initiated and established. Recent studies have demonstrated a tight correlation between transcriptionally active promoters and H3K4 trimethylation, whereas H3K4 monomethylation has been associated with enhancer activity (Pokholok et al., 2005; Heintzman et al., 2007). To determine whether the changes in gene expression patterns upon differentiation correlate with the presence of H3K4me3 as well as H3K4me1, cell lysates were derived from Id2-HPCs and differentiated into CD11b⁺ and CD19⁺ cells. Lysates were immunoprecipitated with antibodies directed against H3K4me1 and H3K4me3 and analyzed by ChIP sequencing (Barski et al., 2007). More than 15,000 regions were identified that showed H3K4me3 in all three cell types (Figure S3A). The number of H3K4me1 islands varied between the cell types (Figure S3A). As expected, the majority of the H3K4me3 peaks were promoter proximal, whereas most of the H3K4me1 peaks were promoter distal (Figure S3B). ChIP-Seq analyses for H3K4me1 were performed in duplicate with biological replicates. We note that the patterns of H3K4me1 were very similar between the duplicates (Figures S3, S4, and S6).

To determine how the presence of epigenetic marks relates to lineage-specific programs of gene expression, changes in the abundance of H3K4me3 and H3K4me1 were directly compared to relative levels of gene expression (Figure 4B). As a threshold we used a 2-fold change in the number of normalized reads mapped to promoter regions associated with one lineage versus another. We observed a tight correlation between changes in H3K4me3 and gene expression levels upon comparing multipotent progenitors (Id2-HPCs) to differentiated CD19⁺ B cells (Figure 4B, left). Surprisingly, there was no substantial distinction between Id2-HPCs and myeloid cells (Figure 4B; middle). Changes in the levels of H3K4me1 in regions adjacent to genes correlated very well with the dynamics of gene expression abundance for both myeloid and B lineage cells when compared to multipotent progenitors (Figure 4C; left and middle). Thus, during the transition from the multipotent progenitor to the differentiated cell stage, changes in H3K4 monomethylation are tightly associated with the activation of either a B cell or myeloid cell-specific program of gene expression.

Islands of H3K4me1 Mark Lineage-Specific Enhancers in Multipotent Progenitors

To explore the possibility that enhancer regions are primed in Id2-HPCs prior to differentiation, we examined H3K4me1 enrichment for a subset of cell-type-specific genes including the B cell lineage-specific genes *Foxo1*, *Ebf1*, and *Vpreb3*. We also analyzed myeloid cell-specific loci such as *Csf1r* and *Cebpa*, and we examined the patterns of H3K4me1 in genes that are repressed upon specification into either the B or myeloid cell lineages, such as *Thy1* (Figure 5). We note that the patterns were normalized to a total number of 10,000,000 tags. H3K4me1 islands are already present in Id2-HPCs prior to differentiation but are markedly elevated in CD19-positive cells at the *Foxo1*, *Ebf1*, and *Vpreb3* loci, whose expression is activated upon commitment to the B cell lineage but not upon commitment to the myeloid cell lineage (Figure 5). Similarly, the relative abundance of H3K4 monomethylation is elevated in loci *Cebpa* and *Csf1r*, whose expression increases in myeloid cells but declines upon differentiating into the B cell lineage (Figure 5). Correspondingly, abundance of H3K4 monomethylation at the T cell-specific *Thy1* locus decreases upon differentiation into either the B or myeloid cell lineage (Figure 5). Potential enhancer elements in the *Foxo1*, *Ebf1*, *Thy1*, and *Csf1r* loci were cloned by PCR and inserted upstream of a basal promoter in a modified pGL3 vector and transfected, with control renilla luciferase, into the pro-B cell line 22D6, the double-positive T cell line 166, and the T cell line A12. Luciferase activity was quantitated after 24 hr. As expected, regions in the *Foxo1* and *Ebf1* loci that gained H3K4 monomethylation upon differentiation to the B cell state had greater luciferase activity in the B cell line as opposed to the T cell lines (Figure S5). This is consistent with previous observations (Lin et al., 2010). In contrast, sites in the *Thy1* locus that lose H3K4 monomethylation upon differentiation into B cells showed slightly higher levels of enhancer activity in the T cell lines (Figure S5). Taken together, these findings indicate that in multipotent progenitors (Id2-HPCs), H3K4 monomethylation patterns mark a wide spectrum of enhancers associated with lineage-restricted genes that become further activated or repressed in a lineage-restricted manner.

Establishment of Lineage-Specific Enhancer Repertoires

The data described above raise the question as to whether changes in the levels of H3K4 monomethylation in the B versus myeloid cell lineages correlate with the presence of distinct *cis*-regulatory codes. To address this question, we examined H3K4me1-associated sites for enriched DNA sequences. First, we created scatter plots comparing the abundance of H3K4me1 in multipotent (Id2-HPCs) and differentiated cells (Figure 6). Next, H3K4me1 regions specifically associated with multipotent progenitors and differentiated cells were examined for the presence of enriched *cis*-regulatory elements via a de novo motif finder algorithm, HOMER (Heinz et al., 2010). The ranking of such associated sequences was based on their enrichment as compared to background genomic DNA sequences.

Ebf, *Ets*, *Runx*, and *E2A* were ranked among the top-scoring motifs associated with putative enhancers in cells with at least

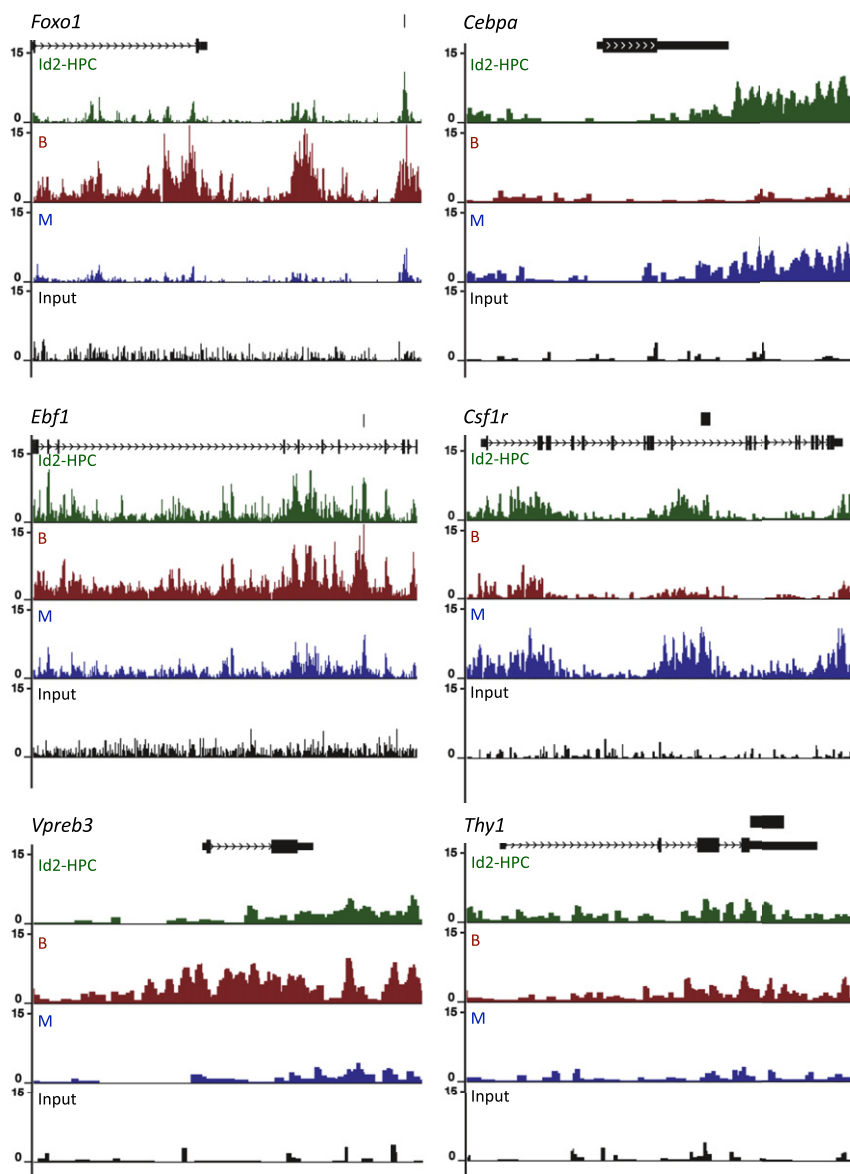


Figure 5. The Enhancer Repertoires of Multipotent Progenitors Are Primed for Activation

Multipotent progenitors (Id2-HPC) and differentiated progeny (B and M) were analyzed for active enhancer repertoires. Distributions of H3K4me1 in multipotent progenitors as well as differentiated progeny are shown across *Foxo1* (chr3: 52,356,303-52,540,000), *Ebf1* (chr11: 44,460,539-44,849,952), *Vpreb3* (chr10: 75,390,000-75,394,700), *Cebpa* (chr7: 34,825,439-34,833,310), *Csf1r* (chr18: 61,229,941-61,257,506), and *Thy1* (chr9: 43,793,835-43,800,231) genomic regions. Patterns were normalized against 10,000,000 tags. The transcript on top of each graph is shown as in the UCSC Browser. Numbers indicate the number of normalized mapped reads (tags) observed. Black bars on top of transcripts indicate regions cloned for luciferase studies.

Evolving Global Enhancer Repertoires in Developing B Cells

The development of a cell line that gives rise to differentiated progeny allows for the analysis of intermediate steps, not just the endpoints. We therefore used the Id2-HPCs cells to analyze intermediate changes in transcript levels from the multipotent HPC to the committed pro-B stage. To this end, Id2-HPCs were differentiated into CD19⁺ pro-B cells at different time points. We then analyzed patterns of gene expression by microarray analysis and performed hierarchical clustering of genes that had a 2-fold or greater change in expression at any given time point. From this analysis we observed three main patterns: transcript levels that were increased (cluster I), transcript levels that were transiently elevated (cluster II), and transcript levels that declined (cluster III) upon developmental progression (Figure 7A). Genes

a 2-fold increase in H3K4me1-mapped reads in B cells as compared to multipotent progenitors (Id2-HPCs) (Figure 6A). In multipotent progenitors (Id2-HPCs) compared to B cells, H3K4me1-marked regions were enriched for Runx, Mef2, and AP-1 consensus binding sites (Figure 6A). When comparing myeloid cells to Id2-HPCs and B cells, H3K4me1 peaks associated with the myeloid lineage were significantly enriched for the Pu.1, Runx, and AP-1 consensus binding sites (Figures 6B and 6C). Alternatively, Mef2, Stat, and Ets consensus binding sites were primarily enriched in Id2-HPCs compared to myeloid cells, whereas B cell-specific H3K4me1 sites were strongly enriched for Ebf, Ets, and E2A consensus binding sites (Figure 6C). Thus, lineage-specific enhancer repertoires, as characterized by H3K4me1, are established upon developing from multipotent progenitors into committed differentiated B and myeloid cells.

associated with elevated transcript abundance include *Ebf1*, *Foxo1*, and *Rag1*. Genes linked with a decline in transcript levels include those involved with alternate cell lineage programs of gene expression, such as *Hbb-b1*, *Tox*, and *Tcra*, as well as loci potentially involved in the maintenance of the HSC phenotype, including members of the *Hoxa* family (Figure 7A; Table S1). Interestingly, a group of genes associated with cell growth, including *Jun*, *Fos*, and *Xbp1*, showed a transient pattern of expression, possibly reflecting a stage in B cell development linked to cellular expansion (Figure 7A). Taken together, these data identify clusters of genes whose expression patterns are coordinately activated or silenced during developmental progression.

The data described above show that the very early stages of B cell development are associated with a dynamic pattern of gene expression signatures. Specifically, we identified

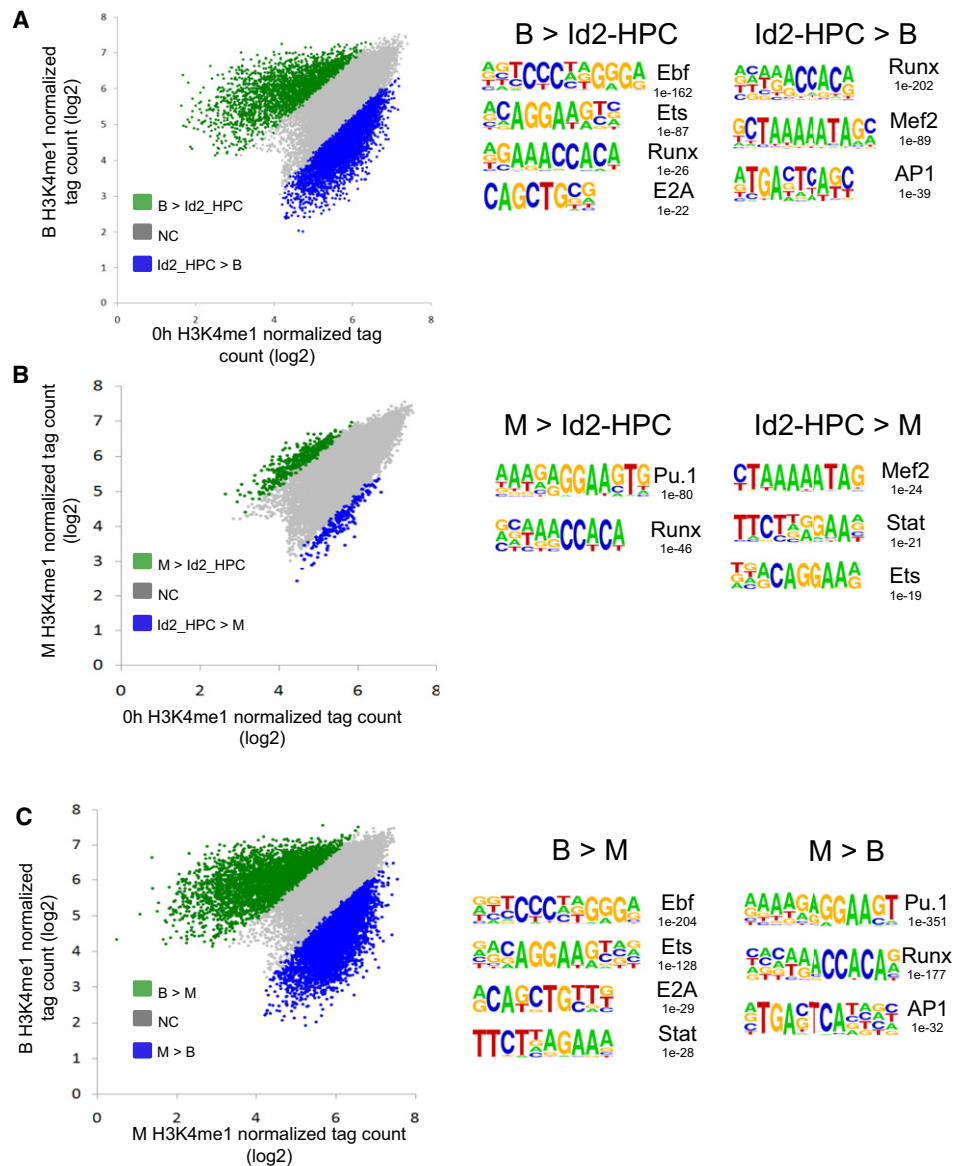


Figure 6. Establishment of Lineage-Specific Enhancer Repertoires

(A) Analysis of changes in H3K4me1-mapped reads (tags) between hematopoietic multipotent progenitors (Id2-HPCs) and CD19⁺ B cells. B cells were derived from Id2-HPCs upon in vitro differentiation. Scatter plot displays the number of H3K4me1-mapped reads across H3K4me1-marked regions in Id2-HPCs versus CD19⁺ B cells. Distributions of H3K4me1-mapped reads showing a 2-fold or greater increase in mapped reads in Id2-HPCs versus differentiated CD19⁺ B cells are shown in blue, and peaks with a 2-fold or greater increase in mapped reads in CD19⁺ B cells are indicated in green.

(B) Analysis of changes in H3K4me1-mapped reads (tags) between hematopoietic multipotent progenitors (Id2-HPC) and myeloid cells. Myeloid cells were derived from Id2-HPCs upon in vitro differentiation. Scatter plot displays the number of H3K4me1-mapped reads across H3K4me1-marked regions in Id2-HPCs versus CD11b⁺ myeloid cells. Distributions of H3K4me1-mapped reads showing a 2-fold or greater increase in mapped reads in Id2-HPCs versus differentiated CD11b⁺ myeloid cells are shown in blue, and peaks with a 2-fold or greater increase in mapped reads in CD11b⁺ myeloid cells are indicated in green.

(C) Analysis of changes in H3K4me1-mapped reads (tags) between myeloid and CD19⁺ B cells. Myeloid and B cells were derived from Id2-HPCs upon in vitro differentiation. Scatter plot displays the number of H3K4me1-mapped reads across H3K4me1-marked regions in CD11b⁺ myeloid versus CD19⁺ B cells. Distributions of H3K4me1-mapped reads showing a 2-fold or greater increase in mapped reads in CD11b⁺ myeloid cells versus differentiated CD19⁺ B cells are shown in blue, and peaks with a 2-fold or greater increase in mapped reads in CD19⁺ B cells are indicated in green.

Numbers reflect p values. *cis*-regulatory elements associated with H3K4me1 regions were identified by computational analysis (HOMER).

enhancers by the presence of H3K4me1 at 0 hr, 48 hr, and 120 hr postdifferentiation (Figures S3, S4, and S6). The data were obtained from two independent experiments generated for all three time points. To accomplish this, the reads from the ChIP-Seq analyses were merged. Next, H3K4me1 islands

with FDR (false discovery rate) less than 0.001 were selected from the merged files and then were analyzed for enriched transcription factor binding motifs within the three clusters at 0 hr, 48 hr, and 120 hr postdifferentiation via HOMER (Figure 7B).

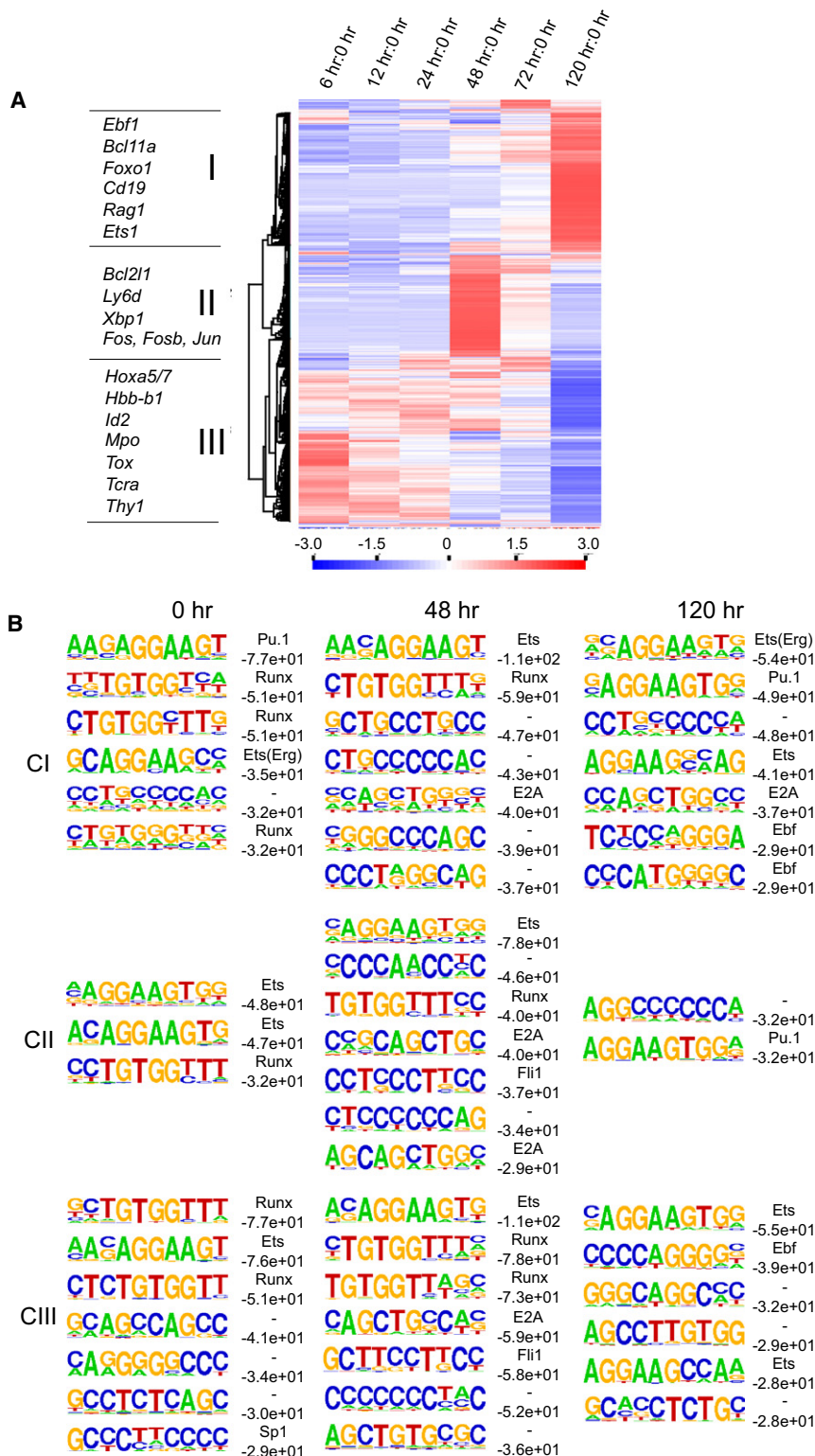


Figure 7. Evolving Enhancer Repertoires in Developing Multipotent Progenitors

(A) Multipotent progenitors (Id2-HPCs) were differentiated into pro-B cells over a 5-day time course, and RNA was taken at 7 time points for microarray analysis. Expression was normalized to day 0. Cluster I refers to genes activated 120 hr postdifferentiation. Cluster II refers to genes activated 48 hr postdifferentiation. Cluster III refers to genes repressed 120 hr postdifferentiation.

(B) Enriched regulatory motifs at distal H3K4me1 sites during early B cell differentiation. ChIP-Seq was performed on multipotent progenitors (Id2-HPC) at 0, 48, and 120 hr after induction of B cell differentiation. De novo motif finding was performed to determine transcription factor binding motifs associated with active enhancers of genes that are upregulated (CI), intermediately upregulated (CII), and downregulated (CIII). The known motif names are indicated to the left of the de novo motifs. Log p values of the de novo motifs are indicated below of the names of the motifs. Hyphen refers to unknown motif.

sion of cluster I loci frequently contain binding motifs for Pu.1, Runx, and a potential participant named Erg. Upon developmental progression to the committed B cell stage, initially E2A and ultimately Ebf binding sites were frequently associated with active enhancers, consistent with the induction of a B cell lineage program of gene expression (Figure 7B, cluster I at 120 hr).

Cluster II represents loci whose transcript levels are transiently elevated but decline prior to the commitment cell stage (Figure 7, cluster II). The cis-tromic elements associated with this subset of genes were distinct from that of cluster I. Analysis of this cluster showed a substantial enrichment for Runx and Ets binding sites (Figure 7B, CII). Two days after induction of differentiation, enhancer repertoires were identified that included E2A and a potential cis-regulatory element, Fli-1 (Figure 7B, CII at 48 hr). Five days postdifferentiation, the enhancer repertoire again was altered as characterized by the singular presence of Pu.1 binding sites (Figure 7B, CII at 120 hr).

Cluster III represents loci whose transcript levels decline upon developmental maturation from the multipotent HPC to the committed B cell (Figure 7B, CIII). They include genes whose expression is

As aforementioned, cluster I represents loci whose expression is activated during the developmental progression from the multipotent (0 hr) to the committed (120 hr) B cell stage. At the multipotent HPC stage, putative enhancers modulating the expres-

associated with alternate lineages and HSC cell identity, including *Hoxa5*, *Hoxa7*, *Hbb-b1*, *Ptcr*, and *Thy1*. Interestingly, upon developing into committed B cells, H3K4me1 islands in these loci were substantially enriched for E2A and Fli binding

sites (Figure 7B, CIII at 120 hr). Five days postinduction, the enhancer repertoires were changed as demonstrated by the lack of E2A and Fli1 binding sites and the presence of Ebf consensus *cis*-regulatory elements (Figure 7B, CIII at 120 hr). Thus, it appears that these combinations of regulatory elements suppress rather than activate the expression of genes associated with alternate cell lineages in committed B cells. In sum, we describe the differentiation of multipotent HPCs into committed B lineage cells in terms of evolving enhancer repertoires that involve both known regulators such as E2A, EBF, and PU.1 as well as potential participants such as Erg and Fli1.

DISCUSSION

Recent genome-wide studies have provided insight into how transcriptional regulators act in concert to specify a developmental stage (Ghisletti et al., 2010; Heinz et al., 2010; Treiber et al., 2010; Lin et al., 2010; Natoli, 2010). We are now faced with the fundamental question as to how such networks are established during the developmental progression from the multipotent HPC to a committed lymphoid or myeloid cell stage. Here we describe experimental and computational strategies to address this question.

Previous studies have demonstrated that long-term cultures of E2A-deficient progenitors remain pluripotent both in vitro and in vivo (Ikawa et al., 2004). A limitation of these cells is that E2A-deficient cells cannot be differentiated into committed B cells because the E2A proteins are required for the initiation of the B cell pathway. Additionally, forced E47 expression has, until now, not permitted rescue of the developmental block observed in E2A-deficient pre-pro-B cells. Here we have developed a strategy that overcomes this obstacle. In brief, we have generated a long-term culture of HPCs by enforcing the expression of the E-protein inhibitor Id2 (Rivera and Murre, 2001). Id2-HPCs are multipotent both in vitro and in vivo (Ikawa et al., 2004). However, unlike E2A-deficient progenitors, Id2-HPCs have the ability to differentiate into the B cell lineage upon downregulation of Id2. These studies indicate that it is the activity of E2A that plays a causative role in B cell specification. It is the accumulation of E2A activity in multipotent progenitors that permits B cells to begin their developmental journey.

A tight correlation between enhancers associated with transcriptionally active genes and H3K4 monomethylation has recently been established (Heintzman et al., 2007). We demonstrate that multipotent HPC lineage-restricted enhancer elements are already primed as demonstrated by the presence of H3K4 methylation prior to differentiation. As expected, upon commitment to the B or myeloid cell lineage, we observed an increase in H3K4 mono- and trimethylation across genomic regions associated with a B cell or myeloid cell lineage programs of gene expression, respectively. In addition, the degree of H3K4 methylation declined across genomic regions linked with the alternate programs of gene expression. Taken together, these data confirmed the previous notion that H3K4me1 regions are associated with transcriptionally poised or active regions. These findings are in line with the concept of multilineage priming, previously proposed to be involved in the maintenance of multipotency in HSCs (Hu et al., 1997).

The simplest explanation of multilineage priming is that a low level of expression of several lineage-determining transcription

factors at the precursor stage “primes” genomic regions characteristic of different hematopoietic lineages. Therefore, it is likely that, in progenitor cells, these premarked enhancers are already associated, possibly at low levels, with the very same transcription factors that will bind these regions in fully differentiated cells. It is very well possible that prelineage priming involves E2A. E2A proteins are expressed in HSCs and act to maintain the HSC pool (Dias et al., 2008; Semerad et al., 2009; Yang et al., 2008). Thus, it may be that E2A functions in progenitor population to premark lymphoid enhancer repertoires. This raises the question as to why this premarking generates a low degree of H3K4 monomethylation in multipotent progenitors relative to lineage-committed cells. The level of E2A at these developmental stages is similar to that of pro-B cells. Thus, it does not seem likely that the expression of E2A in HSCs versus pro-B cells generates differences in the degree of H3K4 monomethylation. Although still to be proven, it is conceivable that E2A associates with different partners including SCL/TAL1 in HSCs. The SCL proteins are well known to heterodimerize with E2A. Thus, we suggest that the E2A proteins could function in HSCs to promote the premarking of a lymphoid-specific enhancer repertoire, whereas in committed B cells, they act as homodimers to elevate H3K4me1 abundance across poised enhancer regions and thus induce a B cell lineage program of gene expression.

The establishment of committed lymphoid cells from multipotent HPCs involves multiple intermediate stages. How are such intermediate stages established? Developmental stages can be characterized in terms of gene expression signatures. Gene expression signatures are in turn primarily established by enhancer repertoires. Here, we have used genome-wide and computational approaches to describe the evolving enhancer repertoires that underpin early B cell development. These repertoires include E2A, Ebf, Ets, Runx, and Pu.1, as well as Erg and Fli1 consensus binding sites. Erg and Fli1 are particularly intriguing because they have previously been suggested, based on their expression patterns, to play critical roles in early B cell development (Rivera et al., 1993).

In sum, we have generated long-term cultures of HPCs that are multipotent and self-renew indefinitely in vitro. Remarkably, the distribution of motifs across enhancer repertoires does not change dramatically during developmental progression. However, we note that a fraction of the enhancer repertoires has changed upon becoming committed to the B cell lineage. This is particularly apparent by the increased frequency of Ebf motifs at 120 hr relative to 0 hr. Finally, by using directed differentiation, gene expression microarrays, and identification of enhancer repertoires by H3K4me1, we describe the early stages of B cell development in terms of evolving global enhancer and silencer repertoires. In principle, this analysis can be applied to any developmental setting including β -selection, positive selection, receptor editing, and beyond.

EXPERIMENTAL PROCEDURES

Viral Vectors and Virus Production

Human *Id2* was amplified from 293T cDNA with the addition of BamHI sites at both 3' and 5' ends and then cloned into pRRL.sin-18.PPT.PGK.MCS.IRES.GFP.pre, kindly provided by I. Weissman (Stanford University). The *hId2*_IRES-GFP expression cassette was excised with AgeI and BsrGI, replacing the eGFP gene in pBob_TA1_R2_corrected120803,

kindly provided by I. Verma (Salk Institute). hld2 was then cut out of the resulting vector to create an empty vector control. These vectors were named TetOff_hld2 and TetOff_Empty, respectively.

Transduction and Cell Culture

CD45.1 congenic animals (8–12 weeks of age) were injected with 250 mg/kg of 5-fluorouracil 4 days before bone marrow was harvested and lineage depleted by auto-MACS. Purified lineage-negative cells were cultured overnight in expansion media (DMEM + 15% FBS in the presence of 10 ng/mL IL-3, 10 ng/mL IL-6, 1:200 SCF, and 1:20 WEHI). After 12–18 hr in culture, cells were pelleted and resuspended in fresh expansion media plus TetOff_hld2 or TetOff_Empty lentivirus in the presence of 4 μ g/mL polybrene and the above cytokines. Cells were spin infected twice for 1.5 hr at 2500 rpm, 30°C, with a 12–18 hr rest in between spin infections. After the second spin, cells were resuspended in fresh expansion media and cultured for 4–5 weeks. Cells were then cultured in IMDM + 10% FCS, 2% PSG, and 2 μ l β -me on subconfluent S17 feeder cells in the presence of 1:100 IL-7, 1:100 Flt3L, and 1:200 SCF. S17 feeder cells were maintained in α -MEM + 10% FBS and 2% PSG. Animal studies were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

In Vitro Differentiation

TetOff_hld2 expanded cells were depleted of small (<1%–5%) numbers of CD19⁺, CD25⁺, and CD11b⁺ cells by auto-MACS. For myeloid differentiation, cells were cultured for up to 6 days in IMDM + 10% FCS and β -me in the presence of IL3, Flt3L, GM-CSF, and MCSF and in the presence or absence of 1 μ g/mL doxycycline. To promote B cell differentiation, cells were cultured for up to 10 days in IMDM + 10% FCS and β -me on S17 feeder cells in the presence of 1:100 IL-7 and 1:200 SCF in the presence or absence of 1 μ g/mL doxycycline, or alternatively, in α -MEM in the presence of cytokines and the Tst-4 stromal cell line (kind gift from T. Ikawa). Fresh doxycycline and cytokines were added every 2 days. *E2A*^{−/−} cells were cultured as previously described (Ikawa et al., 2004).

Microarray Profiling

All RNA was prepared with RNeasy Columns (QIAGEN). Gene expression profiling depicted in Figure 1 was performed by hybridization to the Affymetrix MOE430 2.0 gene expression array according to the manufacturer's instructions. Gene expression profiling depicted in Figures 1, 4, and 7 was performed by amplification and hybridization to the Illumina Mouse WG-6 v1.1 and v2 gene expression arrays according to the manufacturer's instructions. Differentially expressed genes were determined with the Limma package from BioConductor. A Benjamini-Hochberg-adjusted p value cut-off of 0.05 and a fold-change threshold of 2 were utilized. Hierarchical clustering was performed on this set of genes. The distance metric used was (1 – Pearson correlation coefficient), and the “average” (unweighted pair-group average method, UPGMA) method was used for agglomeration. Clusters were obtained by empirically cutting the tree.

ChIP-Sequencing

The chromatin immunoprecipitation (ChIP) protocol was essentially the same as previously described (Agata et al., 2007; Lin et al., 2010). Anti-H3K4me1 (ab8895) and anti-H3K4me3 (07-473) were purchased from Abcam and Millipore, respectively. Anti-H3K4me1 ChIP-Seq was performed in duplicate. Peaks from duplicate experiments were merged to identify putative peaks. Those peak positions and each of the individual experiments were used to score the peak and to ensure significance relative to the input. The R package “edgeR” was used to perform the significance calculations.

Data Analysis

Clusters of tags across the analyzed genomes were identified based on the significant enrichment of tags relative to background as well as local tag counts. Data were analyzed with HOMER software (<http://biowhat.ucsd.edu/homer/>). Bound sites were identified with HOMER. ChIP-Seq tag counts were normalized between experiments to a total of 10⁷ mapped tags. The peak region was required to show 4-fold more tags (as compared to total tag count) than controls. Additionally, DNA bound elements were required to exhibit at least 2-fold more tags within a 1 kb region versus flanking 10 kbp

DNA regions. The threshold for the number of tags generating an interacting site was determined for a false discovery rate of 0.001. Furthermore, peaks were required to exhibit at least 4-fold more tags (normalized versus total number) versus input control samples. To avoid identifying DNA elements that contain genomic duplications or nonlocalized occupancy, a 4-fold increase in the number of tags relative to tags located within immediate genomic proximity was chosen as a threshold. The detailed description of the heat map generation was found in <http://biowhat.ucsd.edu/homer/>. In brief, a peak file of the first biological replicate of the top 5000 high confidence peaks that had at least 4-fold more tags within a 1 kb region versus flanking 10 kbp DNA regions. Then, the sequences within 3000 bp upstream and downstream of the peak centers of each replicate were clustered by the average linkage method with Cluster 3.0. The heat map was visualized by Java Tree View.

Replicates for ChIP-Seq of H3K4me1 in Id2-HPCs, Id2-HPCs induced to differentiate toward the B cell lineage for 2 and 5 days, and myeloid cells were merged into one file each in a single “META”-experiment. EdgeR was used to calculate the false discovery rates to determine the difference between the merged files and input. Motifs associated with transcription factor occupancies were identified with HOMER software (C.B., unpublished data; Heinz et al., 2010). To analyze H3K4me1 islands for enriched motifs, DNA sequences (plus or minus 500 bp centered across H3K4me1 peaks) were compared to 50,000 randomly identified genomic fragments of similar size and matched for the proportion of GC content.

ACCESSION NUMBERS

The microarray and ChIP-Seq data are available in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/gds>) under the accession number GSE30859.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at doi:10.1016/j.immuni.2011.06.013.

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