

# Single-cell analysis of early B-lymphocyte development suggests independent regulation of lineage specification and commitment in vivo

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Edited by George Klein, Karolinska Institutet, Stockholm, Sweden, and approved August 13, 2012 (received for review June 15, 2012)

To better understand the process of B-lymphocyte lineage restriction, we have investigated molecular and functional properties in early B-lineage cells from *Pax-5*-deficient animals crossed to a B-lineage-restricted reporter mouse, allowing us to identify B-lineage-specified progenitors independently of conventional surface markers. *Pax-5* deficiency resulted in a dramatic increase in the frequency of specified progenitor B-cells marked by expression of a  $\lambda 5$  (*Igll1*) promoter-controlled reporter gene. Gene expression analysis of ex vivo isolated progenitor cells revealed that *Pax-5* deficiency has a minor impact on B-cell specification. However, single-cell in vitro differentiation analysis of ex vivo isolated cells revealed that specified B-lineage progenitors still displayed a high degree of plasticity for development into NK or T lineage cells. In contrast, we were unable to detect any major changes in myeloid lineage potential in specified *Pax-5*-deficient cells. By comparison of gene expression patterns in ex vivo isolated *Pax-5*- and *Ebf-1*-deficient progenitors, it was possible to identify a set of B-cell-restricted genes dependent on *Ebf-1* but not *Pax-5*, supporting the idea that B-cell specification and commitment is controlled by distinct regulatory networks.

transcription | Notch-1 | Deltex

The development of B-lymphoid cells from multipotent progenitor cells in the bone marrow (BM) involves a gradual loss of alternative lineage potentials and sequential activation of the B-lineage-restricted genes. This process is dependent on the sequential action of transcription factor networks acting to specify lineage potentials at defined stages of development. Whereas the earliest stages of lymphoid specification depend on the transcription factors Pu.1 (1), Ikaros (2, 3), and E12/E47 (4, 5), activation of B-lymphoid-restricted genetic programs and commitment to B-lymphoid development are regulated by *Ebf-1* (6–8) and *Pax-5* (9–11). In addition to their ability to activate lymphoid-restricted genes, Pu.1 (12) and E2A (13) appear to be directly involved in the remodeling of chromatin to create a proper epigenetic landscape for downstream acting factors such as *Ebf-1* and *Pax-5*, to access lineage-specific target promoters. Deletion of *Pax-5* in B-lineage cells results in loss of lineage identity and opens a possibility for the cells to develop into functional T lymphocytes (14). Furthermore, cultured B-cell progenitors lacking *Pax-5* display plasticity for development into other hematopoietic lineages as well as osteoblasts, suggesting a crucial role for this transcription factor in achieving stable B-lineage commitment (10, 11). However, ectopic expression of the transcription factor *Ebf-1* has been reported sufficient to restrict lineage options for *Pax-5*-deficient cells (6), suggesting that the crucial role of *Pax-5* in B-cell commitment is mediated through activation of the *Ebf-1* gene (15). *Ebf-1* has also been shown to target the *Pax-5* gene, creating a feed forward loop that will result in stable B-lineage commitment (16, 17). However, it is becoming increasingly clear that the activation of this regulatory network is preceded by the activation of B-lineage genes in what can be called B-lineage priming or specification. High-resolution analysis of what was classically defined as the common lymphoid progenitor (CLP) compartment has allowed for the identification of additional reg-

ulatory networks that appear to cause activation of the B-lineage program before the expression of CD19 (18–20). This is critically dependent on *Ebf-1* that becomes transcriptionally activated in association with the up-regulation of the surface marker Ly6D on IL-7R<sup>+</sup>Flt-3<sup>+</sup>CD19<sup>+</sup>B220<sup>+</sup> progenitors (18, 20). In the absence of *Ebf-1*, the IL-7R<sup>+</sup>Flt-3<sup>+</sup>CD19<sup>+</sup>B220<sup>+</sup> cells lack expression of B-lineage-restricted genes (8) and they also display an abnormal lineage plasticity because they retain the NK-cell potential normally lost in association with surface expression of Ly6D on early lymphoid-restricted progenitors (21). The Ly6D-expressing cells form approximately half of the classical CLP compartment (18) but even though single-cell analysis suggests that all of the Ly6D<sup>+</sup> cells express *Ebf-1* message, only a small fraction of these express classical B-lineage genes such as  $\lambda 5$  and *Pax-5* (20). Using a  $\lambda 5$  reporter transgenic mouse, it was possible to prospectively isolate these cells and show that they represented CD19<sup>+</sup>B220<sup>+</sup> B-cell-committed progenitors (19).

Even though previous work has provided data suggesting that B-lineage specification and lineage commitment are independently regulated, this work has to a large degree been based on analysis of in vitro cultured cells (10, 11, 22–25). Furthermore, the increased understanding of the functional properties of early lymphoid-restricted compartments has opened new possibilities to more carefully investigate the interplay between different regulatory networks in early B-cell development. To understand these processes in more detail, we crossed  $\lambda 5$  (*Igll1*) reporter mice (26) to *Pax-5*-deficient animals (9), creating a possibility to investigate B-cell specification in vivo, independently of direct *Pax-5* targets such as CD19. This revealed that even though *Pax-5* deficiency results in dramatic accumulation of cells specified for development toward B lineage, single-cell analysis shows that these cells display a defective lineage commitment. Hence, B-lineage specification and commitment are controlled by distinct but intervened regulatory networks.

## Results

***Pax-5* Deficiency Results in an Expansion of B-Lineage-Specified Progenitors in Vivo.** To investigate the molecular regulation of B-lineage commitment and specification in vivo, we crossed *Pax-5*-

Author contributions: S.Z., J.Å., P.T., J.S., H.Q., and M.S. designed research; S.Z., J.Å., P.T., J.S., and H.Q. performed research; S.Z., J.Å., P.T., J.S., H.Q., and M.S. analyzed data; and S.Z., J.Å., P.T., J.S., H.Q., and M.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo) (accession no. GSE39554).

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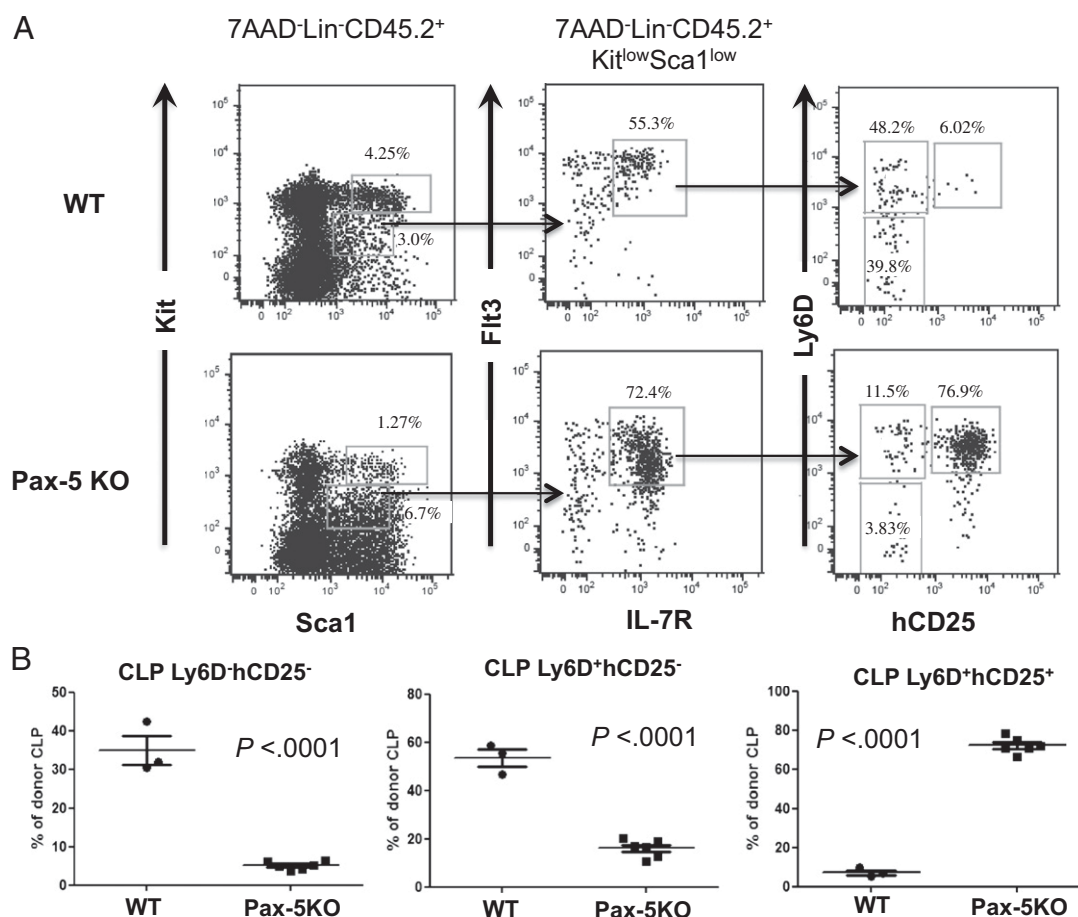
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deficient mice (9) to  $\lambda 5$  reporter ( $\lambda 5$ -hCD25) animals (26). The expression of this reporter is restricted to B-lymphoid-committed cells making it possible to identify B-lineage progenitors independently of B220 or CD19 (19). Because inbred C57B6 (CD45.2) Pax-5-deficient mice display a high embryonal lethality, we transplanted day 14.5 fetal liver cells into irradiated CD45 congenic mice (CD45.1) and analyzed the presence of CD45.2<sup>+</sup> cells in the BM 4–6 wk after transplantation. Analysis of lineage negative (Lin<sup>−</sup>) (CD19<sup>−</sup>B220<sup>−</sup>CD3<sup>−</sup>Ter119<sup>−</sup>NK1.1<sup>−</sup>Gr1<sup>−</sup>Mac1<sup>−</sup>CD11c<sup>−</sup>) cells revealed that the relative frequency of Sca<sup>Low</sup>Kit<sup>Low</sup> cells, including the CLPs (27), was slightly increased in mice transplanted with Pax-5-deficient cells compared with those transplanted with WT fetal liver cells (Fig. 1A). The majority of these cells expressed Flt-3 and IL-7R (Fig. 1A) in line with the fact that they represent progenitors in the classically defined CLP compartment (27, 28). Because this compartment has been reported to harbor several progenitor populations with different developmental potentials, we investigated the expression of Ly6D and the  $\lambda 5$  promoter-driven hCD25 reporter gene (Fig. 1A and B) (18, 20) on these cells. Whereas the CLP compartment generated from WT fetal liver contained ~40% Ly6D<sup>−</sup> cells, the relative frequency of this population was reduced 10-fold when fetal livers from Pax-5-deficient mice were used for transplantation (Fig. 1B). The frequency of Ly6D<sup>+</sup> cells not expressing the  $\lambda 5$  reporter transgene was reduced 4.2-fold from about half

to one-fifth of the classically defined CLP population (27, 28). Although the  $\lambda 5$  reporter-positive population composed  $\sim 7\%$  of the classical CLP population after transplantation of WT cells, using Pax-5-deficient fetal liver for transplantation, this population composed  $\sim 70\%$  of the CLP population (Fig. 1*A* and *B*). Hence, lack of Pax-5 results in a dramatic increase in the frequency of B220 $^+$  $\lambda 5^+$  cells in vivo.

The activation of the  $\lambda 5$  reporter gene has been shown to overlap with that of the endogenous  $\lambda 5$  gene and to the activation of a set of B-lymphoid-restricted genes including *Mb-1* and *Oca-B* but also *Pax-5* (19). To investigate whether Pax-5 is important for the coordinated activation of early B-lineage genes in the earliest B220<sup>+</sup> $\lambda 5$  reporter-positive B-cell progenitors, we sorted single  $\lambda 5$  reporter-positive B220<sup>+</sup>CD19<sup>+</sup>Sca<sup>Low</sup>C-Kit<sup>Low</sup>IL-7R<sup>+</sup>Flt-3<sup>+</sup> cells from mice transplanted with either WT or Pax-5<sup>-/-</sup> fetal liver to perform multiplex single-cell PCR analysis (Fig. S1). This revealed that the Pax-5-deficient reporter-positive cells expressed detectable levels of *Ebf-1* (100%), *Rag-1* (100%), *Oca-B* (81%), *Mb-1* (72%), and  $\lambda 5$  (68%), similar to that of WT cells (100, 82, 74, 66, and 63%, respectively) (Fig. S1). These data show that primary  $\lambda 5$  reporter-expressing progenitors generated in the absence of Pax-5 display a coordinated activation of B-lineage genes suggesting specification can be achieved in the absence of Pax-5 in vivo.

To investigate the impact of Pax-5 deficiency on the classically defined pro-B-cell compartments, we investigated the expression



**Fig. 1.** Pax-5-deficiency results in an increase of  $\lambda 5$ -expressing cells in early B220<sup>+</sup> progenitors. (A) Representative FACS plots showing the gating strategies used to identify CLPs among lineage low/negative progenitors hCD25( $\lambda 5$ )<sup>+</sup> cells among the CD45.2<sup>+</sup> cells generated by transplantation of WT or Pax-5-deficient fetal liver cells to congenic CD45.1 mice. Percentages in the gates are of the gated populations in one experiment. Lineage (Lin) mixture contains antibodies against CD11b, GR1, TER119, CD3, CD11c, NK1.1, CD19, and CD45R/B220. (B) Relative frequencies of Ly6D<sup>+</sup> as well as hCD25( $\lambda 5$ )<sup>+</sup> cells collected from two independent experiments. Each dot represents one transplanted mouse and the horizontal line, the mean in each group. Unpaired t test was used to compare the difference between WT and Pax-5<sup>-/-</sup> cells.

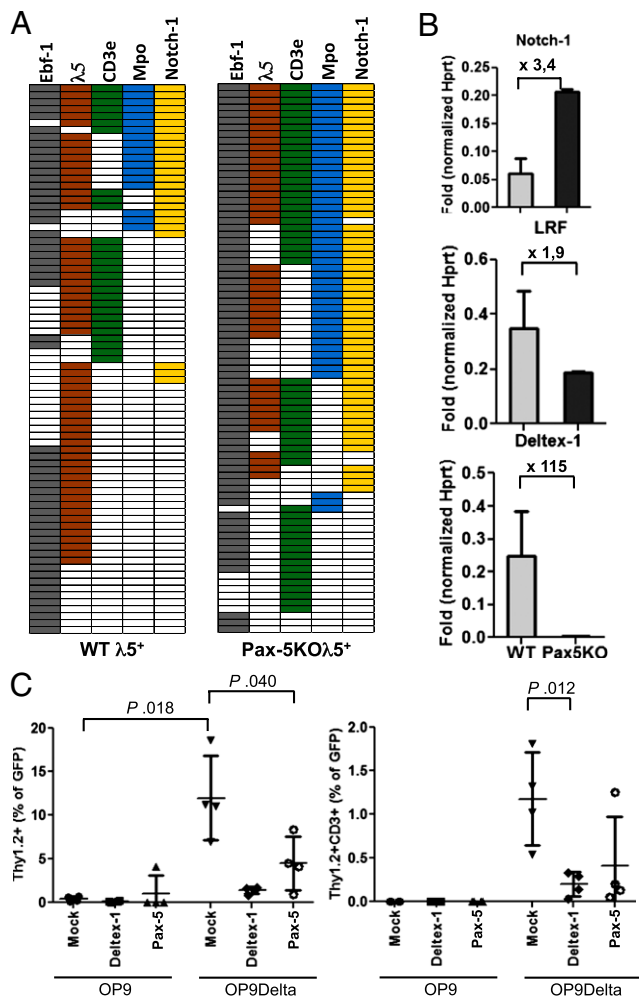


genitors express higher levels of Notch-1 (32) (Fig. 3*A* and *B* and Table S2), the microarray analysis suggested that the expression of the Notch target gene *Deltex-1* was lower in the freshly isolated Pax-5-deficient cells (Table S2). To verify this, we analyzed the expression of *Deltex-1* and the Notch antagonist *LRF* (33) in freshly isolated B220<sup>+</sup>λ5<sup>+</sup> cells by qPCR (Fig. 3*B*). Whereas the expression level of *LRF* was comparable in WT and Pax-5-deficient cells, the expression of *Deltex-1* was reduced in cells generated from Pax-5-deficient progenitors. *Deltex-1* has been reported to repress Notch signaling (34), opening for the possibility that the plasticity toward T-lineage development observed in Pax-5-deficient pro-B cells exposed to high levels of Notch ligand

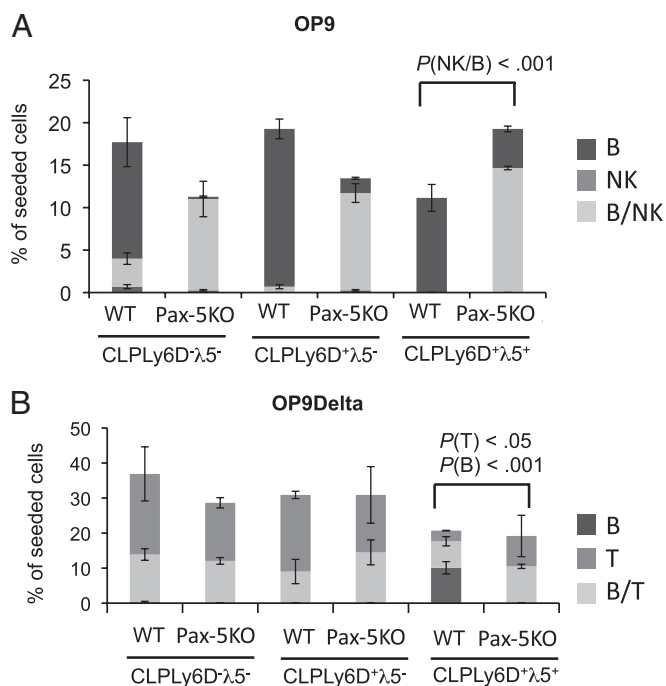
is regulated in a more complex manner than simply direct deregulation of Notch-1 expression. To investigate whether *Deltex-1* is capable of modulating the ability of Pax-5-deficient pro-B cells to adopt alternative cell fates, we transduced cultured Pax-5-deficient λ5<sup>+</sup> pro-B cells with a retrovirus expressing *Deltex-1* and GFP, and cultured the cells on either OP9 or Notch ligand-expressing OP9Δ stroma cells. After 8 d of incubation of the pro-B cells on OP9Δ, a fraction of the GFP virus transduced Pax-5-deficient progenitors expressed Thy1.2 on the cell surface, indicating that they are changing their primary fate and initiate progression into T lineage (Fig. 3*C*). The generation of these cells was significantly reduced when the progenitors were transduced with virus encoding either *Deltex-1* or Pax-5 (Fig. 3*C*). After 14 d of culture, a fraction of the control virus-transduced cells had converted into CD3<sup>+</sup> cells, a process dramatically reduced by ectopic expression of *Deltex-1* (Fig. 3*C*). Hence, expression of *Deltex-1* is sufficient to reduce the lineage plasticity of Pax-5-deficient pro-B cells, suggesting that the molecular adoption of T-cell fate in the absence of Pax-5 extends beyond direct repression of Notch-1 expression.

### Pax-5 Is Essential for Preserved Lineage Stability of ex Vivo Isolated Cells.

It is well established that cultured Pax-5-deficient progenitor cells can adopt alternative cell fates (10, 11, 25, 30, 31); however, our experiments using short-term cultured Pax-5-deficient pro-B cells suggested that only a limited fraction of the cultured cells initiated the expression of T-cell genes. A low level of conversion would not be fully compatible with a key role for Pax-5 in the preservation of B-lineage identity. Hence, to directly investigate the frequency of cells that display plasticity ex vivo, we sorted single Ly6D<sup>+</sup>, Ly6D<sup>+</sup>, or λ5<sup>+</sup>Sca-1<sup>low</sup>Kit<sup>low</sup>Flt-3<sup>+</sup>IL-7R<sup>+</sup>B220<sup>+</sup>CD19<sup>+</sup> progenitors and performed in vitro differentiation experiments under conditions stimulating either myeloid-, B/NK-, or T-lineage development. Culturing lymphoid primed multipotent progenitors (LMPPs) (35) under conditions stimulating the development of myeloid cells for 7 d resulted in an overall cloning frequency of around 90%, where a majority of the clones contained more than 100 cells (Fig. S2*A*). λ5 reporter-expressing Lin-Sca-1<sup>low</sup>Kit<sup>low</sup>Flt-3<sup>+</sup>IL-7R<sup>+</sup>B220<sup>+</sup>CD19<sup>+</sup> progenitors from WT mice were dramatically reduced in their ability to generate myeloid cells compared with LMPPs under these conditions and only 16% of the wells contained small clones with less than 10 cells (Fig. S2*B*). Using Pax-5-deficient progenitors, the frequency of clones was increased to 34%; however, all of the clones were small, containing less than 10 cells (Fig. S2*B*). Culturing single WT or Pax-5-deficient cells under conditions stimulating the development of B and NK cells, the majority of the seeded cells from WT Ly6D<sup>+</sup> progenitors generated colonies composed of mainly B220<sup>+</sup>λ5<sup>+</sup> cells without the development of NK1.1 expressing cells (NK cells) (Fig. 4*A*). Using Pax-5-deficient progenitors, we were unable to obtain clones lacking NK1.1 cells and rather all of the generated clones contained a mixture of B and NK lineage cells. A further reduction of NK cell development was observed using Ly6D<sup>+</sup>λ5(hCD25)<sup>+</sup> CLPs from WT mice, in line with the idea that the expression of Ly6D is associated with a reduced NK cell potential (18, 20). The corresponding population of Pax-5-deficient cells generated mainly mixed NK/B colonies. Similar data were obtained when we investigated the lineage potential of Ly6D<sup>+</sup>hCD25(λ5)<sup>+</sup> progenitors because, whereas no NK1.1<sup>+</sup> cells were detected using WT cells, the absolute majority of the clones from Pax-5-deficient progenitors contained NK1.1<sup>+</sup> cells. Investigating the T-lineage potential of the progenitors by culturing single cells on OP9Δ cells (Fig. 4*B*) revealed that, whereas the lineage potential of the more immature compartments was comparable—the majority of the WT Ly6D<sup>+</sup>hCD25(λ5)<sup>+</sup> cells generated clones lacking Thy1.2 high cells—all of the clones from Pax-5-deficient mice containing either a mix of B- and T-lineage cells or only T cells. Hence, stable B-lineage restriction of normal progenitors is dependent on Pax-5, even in cells specified for B-lineage development.



**Fig. 3.** Ex vivo analysis of Pax-5-deficient pro-B cells suggests partial disruptions of B-lineage-specific transcription. (A) Color-coded display of data collected by single-cell multiplex RT-PCR analysis of hCD25(λ5)<sup>+</sup>Sca-1<sup>low</sup>Kit<sup>low</sup>Flt-3<sup>+</sup>IL-7R<sup>+</sup>CD19<sup>+</sup>B220<sup>+</sup> sorted from mice transplanted with normal and Pax-5-deficient fetal liver. Each horizontal line of boxes represents a single investigated cell. Colored box indicates that an RT-PCR product from a given gene could be detected on an ethidium bromide-stained agarose gel. (B) qPCR data generated from sorted B220<sup>+</sup>hCD25<sup>+</sup> cells from normal or Pax-5-deficient mice. The data were normalized to the expression of *Hprt* and collected from two independent sorting experiments analyzed by triplicate qPCR reactions. The data are presented as mean ± SD. (C) Diagrams displaying the frequency of in vitro expanded Pax-5-deficient λ5<sup>+</sup> cells transduced with either an empty GFP virus or virus coding for *Deltex-1* or Pax-5 that express T-lineage markers after in vitro culture. Left shows the frequency of Thy1.2<sup>+</sup> cells after 8 d of culture on OP9/OP9Δ cells as indicated. Right displays the frequency of Thy1.2<sup>+</sup>CD3<sup>+</sup> cells generated after 14 d of culture on OP9/OP9Δ cells. The data are collected from two transduction experiments containing two cultures. The statistical analysis is based on two-tailed t test.



**Fig. 4.** Pax-5 deficiency results in abnormal differentiation capacity. (A) Generation of B (hCD25(λ5)<sup>+</sup>) and NK (NK1.1<sup>+</sup>) cells when single WT or Pax-5-deficient Ly6D<sup>-</sup>, Ly6D<sup>+</sup>, or hCD25(λ5)<sup>+</sup> Lin<sup>-</sup> Sca<sup>Low</sup> Kit<sup>Low</sup> Flt-3<sup>+</sup> IL-7R<sup>+</sup> CD19<sup>-</sup> B220<sup>-</sup> progenitors were cultured on OP9 cells using conditions allowing for NK cell development. The data are presented as mean ± SEM from three experiments based on 96 seeded cells of each phenotype. (B) Generation of B (hCD25(λ5)<sup>+</sup>) or T (Thy1.2<sup>high</sup>) cells when WT or Pax-5-deficient progenitors were incubated on OP9Δ cells using conditions allowing for T-cell development. The data are presented as mean ± SEM from three experiments including 96 cells of each phenotype/experiment.

**Ebf-1 Is Crucial for B-Cell Specification.** The finding that genes indicative of B-lineage specification are expressed in the absence of Pax-5 prompted us to investigate whether we could identify a set of genes that could serve as markers for B-lineage specification compared with those associated with B-lineage commitment. To identify such genes, we performed global gene expression analysis to investigate intensity levels of genes expressed to an at least 15-fold higher level in WT B220<sup>+</sup>λ5<sup>+</sup> pro/pre-B cells compared with Ly6D<sup>-</sup> CLPs (Fig. S3 and Table S3). The relative expression levels of these genes were compared between WT and Ebf-1-deficient Ly6D<sup>+</sup> CLPs as well as Pax-5-deficient B220<sup>+</sup>λ5<sup>+</sup> cells. This comparison identified a limited set of genes (Table S3, light gray) including λ5 (*Igll1*), *Vpre-B1*, *CD79β* (*B29*), *FoxO1*, *Blk*, *Pde24*, and *Rag-1* that all appeared to be dependent on Ebf-1 and to be up-regulated in the Ly6D<sup>-</sup> to Ly6D<sup>+</sup> transition, whereas they appeared rather independent of Pax-5. A second group of genes (Table S3, dark gray), including *CD79α* (*mb-1*), appeared to be dependent on both Ebf-1 and Pax-5. These data suggest that B-cell specification is dependent on Ebf-1 and involves the activation of a defined set of genes not critically dependent on Pax-5 supporting the idea that B-cell specification and commitment are controlled by distinct regulatory networks.

## Discussion

Even though the CLP compartment has been proposed to contain a population of multipotent cells (27), accumulating evidence suggests that this population is highly heterogeneous and contain both multipotent cells and committed B-lymphocyte progenitors (18–20). Analyzing B-lymphocyte development based on this concept provides increased possibilities to obtain a high-resolution map of the molecular events that result in committed B-cell progenitors. In this report, we have used this model to investigate the molecular basis of B-cell specification and commitment in the

early lymphoid compartments. The dramatic enrichment of λ5 reporter-positive cells in the absence of Pax-5 could be a direct result of a developmental block causing an accumulation of cells; however, the higher levels of cyclin D1 and CDK-4 and -6 (Table S2) suggest that the Pax-5-deficient progenitor population is more actively dividing than their normal counterparts in vivo. The finding of coordinated B-lineage gene expression in λ5 reporter-positive cells even in the absence of Pax-5 (Fig. S3), strongly suggest that this protein is not crucial for B-lineage specification in vivo. However, the finding that almost all of the Pax-5-deficient progenitors could silence the expression of λ5 and enter other lymphoid lineage pathways (Fig. 4A and B) is in line with the idea that Pax-5 is crucial for stable B-lineage commitment. Previous work has suggested that Pax-5-deficient B-cell progenitors display a dramatic plasticity to both lymphoid and myeloid lineages (11, 25). A direct role for Pax-5 in lymphomyeloid-lineage restriction has also been suggested from the finding that combined deletion of Pax-5 and expression of cEBPα is sufficient to drive B-lineage cells into a myeloid cell fate (30). However, even though we were able to detect aberrant expression of myeloid genes in Pax-5-deficient pro-B cells ex vivo, we were unable to detect any robust myeloid-lineage potential in short-term differentiation assays (Fig. S2). Even though care always should be taken in interpretations of negative data, the finding that transplanted B cells carrying a conditional mutation of Pax-5 mainly generated T-lineage cells upon deletion of the gene, whereas the contribution to the myeloid compartment was limited and could not be detected in all of the recipient mice (14) are in line with the idea that the myeloid potential in Pax-5-deficient progenitors is low. Hence, even though the loss of Pax-5 function in B-lineage cells can open a possibility to differentiate into myeloid lineages, this may represent a rather rare event or result in cells with limited expansion potential. A minimal role for Pax-5 in lymphomyeloid-lineage restriction is also in line with the finding that Pax-5 is only expressed in a small fraction of the classical CLP compartment (19, 36) even though these cells display dramatically reduced myeloid potential (19, 27).

The in vitro differentiation analysis strongly suggested that every single clonogenic Pax-5-deficient progenitor was able to generate T-lineage progenitors in the presence of a strong Notch signals such as that provided by OP9Δ cells (37). One possible explanation to this has come from the finding that Pax-5 represses transcription of Notch-1 (24, 29, 32). Increased Notch signaling may serve to maintain T-cell potential in a Pax-5-deficient pro-B-cell by inhibiting the action of Ebf-1 (38) and E2A (39) and their activation of Pax-5. However, the reduction in *Deltex-1* expression in pro-B-cell generated from Pax-5-deficient fetal liver ex vivo (Fig. 3B) suggests that the microenvironment in the BM does not provide sufficient levels of Notch ligands to effectively induce Notch signaling in early B-cell progenitors. It is possible that Pax-5 activate *Deltex-1* expression as a mechanism to restrict cell fate; however, the differences in *Deltex-1* expression may also reflect a more progressed development of the λ5-expressing cells from the WT compared with the Pax-5-deficient cells because it has been shown that *Deltex-1* expression is up-regulated upon progressed development of B-lineage cells (34).

Single-cell PCR analysis revealed that Pax-5-deficient progenitors activated the B-lineage program in an apparently normal fashion, suggesting that the differences in relative expression in Pax-5-deficient progenitors is a result of reduced relative expression of genes rather than changes in the composition of the populations. This is well in line with the idea that Pax-5 is not involved in B-cell specification and activation of the B-lineage program and argues against the idea that Pax-5 should be directly crucial for the generation of the epigenetic landscape associated with B-cell-specific gene expression. Hence, we believe our data are consistent with a model where Ebf-1 activates a large set of B-lineage-restricted transcriptional targets (Table S3) in collaboration with FoxO1 and E2A (40, 41) resulting in B-lineage specification. Activation of Pax-5 would then be one of these target genes driving development further into the committed

B-lineage cell. Hence, even if specification and commitment are controlled by distinct regulatory circuits, these processes would not be functionally separated but represent a continuum of events resulting in B-lymphoid restriction in the BM.

## Materials and Methods

**Animal Models.** Animal procedures were performed with consent from the Institutional Animal Care and Use committee at Linköping University. To obtain E14.5 fetal livers, Pax-5<sup>+/−</sup> mice (9) were mated to Pax5<sup>+/−</sup>λ5<sup>+</sup> (hCD25 reporter) mice (26), both on C57BL/6 background (CD45.2) and unfractionated fetal liver was transplanted to CD45.1 recipients. For details see *SI Materials and Methods*.

**FACS Staining and Purification of Bone Marrow Cells.** FACS analysis and sorting of hematopoietic progenitor cells was conducted as described in ref. 21 and in *SI Materials and Methods*.

**In Vitro Evaluation of Lineage Potentials.** In vitro differentiation of single hematopoietic progenitor cells was conducted as described in ref. 21 and in *SI Materials and Methods*.

**Quantitative and Single-Cell RT-PCR.** qRT-PCR analysis of sorted cells was performed as previously described (42) and multiplex single-cell RT-PCR analysis was performed as previously described (19). For qPCR probes and primer sequences please see *SI Materials and Methods*.

**Affymetrix Gene Expression and Data Analysis.** RNA was extracted from purified adult BM subsets as described in ref. 19. RNA was labeled and amplified according to Affymetrix GeneChip Expression Analysis Technical Manual and hybridized against MOE430 2.0 Affymetrix gene expression arrays chip. Chips were scanned using a GeneChip Scanner 3000. Probe level expression values were calculated using RMAexpress (43) and further analysis was done using dChip (44) ([www.dchip.org](http://www.dchip.org)). The data have been deposited in the GEO database (accession no. GSE39554).

**ACKNOWLEDGMENTS.** We thank Drs. Pear, Busslinger, Grosschedl, Mårtensson, and Zuniga-Pflucker for help with transgenic mice and cell lines and Liselotte Lenner for advice and assistance. These studies were generously supported by grants from the Swedish Cancer Society, the Swedish Research Council, the Swedish Childhood Cancer Foundation, and the faculty of Medicine at Linköping University.

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