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EBF1 Is Essential for B-Lineage Priming and Establishment of a Transcription Factor Network in Common Lymphoid Progenitors

Sasan Zandi, Robert Manssson, Panagiotis Tsapogas, Jenny Zetterblad, David Bryder, and Mikael Sigvardsson

Development of B-lymphoid cells in the bone marrow is a process under strict control of a hierarchy of transcription factors. To understand the development of a B-lymphoid-restricted functional network of transcription factors, we have investigated the cell autonomous role of the transcription factor EBF1 in early B cell development. This revealed that even though transplanted EBF1-deficient fetal liver cells were able to generate common lymphoid progenitors (CLPs) as well as B220+CD43− AA4.1+ candidate precursor B cells, none of these populations showed signs of B lineage priming. The isolated CLPs were able to generate T lymphocytes in vitro supporting the idea that the phenotype of EBF1-deficient mice is restricted to the development of the B lineage. Furthermore, EBF deficient CLPs displayed a reduction in IgH chain recombination as compared with their wild-type counterpart and essentially lacked transcription of B-lineage-associated genes. Among the genes displaying reduced expression in the EBF1 deficient CLPs were the transcription factors Pax5, Pou2af1 (OcaB), and FoxO1 that all appear to be direct genetic targets for EBF1 because their promoters contained functional binding sites for this factor. This leads us to suggest that EBF1 regulates a transcription factor network crucial for B lineage commitment. The Journal of Immunology, 2008, 181: 3364–3372.

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R.M. and D.B. performed transplantation experiments and cell sorting while genomics and RNA analysis work was performed by S.Z. and R.M., S.Z., P.T., J.Z., and M.S. performed promoter analysis and transfection experiments. All authors contributed to the experimental design and were involved in the writing of the manuscript.

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4 Abbreviations used in this paper: KO, knockout; FL, fetal liver; PL, propidium iodide; BM, bone marrow; WT, wild type; CLP, common lymphoid progenitors; LMPP, lympho myeloid primed progenitors; Q-PCR, real-time quantitative PCR.

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pre-pro-B cells is cell autonomous (18), however the identification of EBF1 proteins in other bone marrow populations (22, 23) also opens for alternative and more or less complex explanations of the phenotype observed in the absence of EBF1.

We report in this study that EBF1-deficient hematopoietic cells display a cell autonomous block of B cell priming already at the common lymphoid progenitors (CLP) stage. Reduced expression of genes associated with B-lineage development was observed in EBF1-deficient CLPs both at the population and single cell level. Global gene expression analysis also revealed a reduced expression of the transcription factors Pou2af1 (OcaB) and Foxo1 in EBF1-deficient CLPs and analysis of the promoter elements flanking these genes support the idea of them being direct targets for EBF1. Thus, we conclude that EBF1 deficiency results in a prominent block of B cell development already at the CLP stage and that EBF is directly involved in the establishment of a B-lineage-restricted transcription factor network.

Materials and Methods

In vitro reconstitution assay

Animal procedures were performed with consent from the local ethics committee at Lund University (Lund, Sweden). To obtain E14.5 fetal livers (FLs), heterozygous EBF-KO mice (on C57BL/6 background, CD45.2) were mated early evening and females checked the following morning for the presence of a vaginal plug designated as embryonic day 0.5 (E0.5). Embryos were collected from pregnant mice at E14.5 and feta liver dissected. Recipient mice (C57SL/CD45.1) were lethally irradiated 2 h before transplantation of 2 × 10^6 unfractonated bone marrow (BM) support cells (C57SL/CD45.1). Transplanted mice were analyzed at least 16 wk after transplantation. BM B-cells, BM CLPs, and thymocytes from respective tissues were stained as follows: B cell staining: purified Ter119 (Ter119), Gr1 (RB6-8C5) and CD11b (M1/70) (visualized with GAR-QD605), subsequently followed by Fc-Block (CD16/CD32, 2.4G2) and CD45.1 (A20) FITC, CD43 (57) PE, CD49d (1H3) PE-Cy5, CD45.2 (108) PE-Cy5.5, B220 (RA3-6B2) PE-Cy7, CD19 (ID3) allophycocyanin, CD4 (RM4-5) allophycocyanin-Allexa750, NK1.1 (PK136) Pacific Blue, AA4.1 (AA4.1) biotin (visualized with Streptavidin QuantumDot (QD) 655), and propidium iodine (PI); CLP staining: Fc-Block (CD16/32, 2.4G2) followed by CD45.1 (A20) FITC, FLT3 (A2F10) PE, TER119 (Ter119) PE, CD43 (145-2e11) PE-Cy5, Gr1 (RB6-8C5) PE-Cy5, CD11b (M1/70) PE-Cy5, CD45.2 (108) PE-Cy5.5, B220 (RA3-6B2) PE-Cy7, CD19 (ID3) allophycocyanin, Kit (2B8) allophycocyanin-Cy7, SCA1 (D7) Pacific Blue, IL7r (A7R34) biotin (visualized with Streptavidin QuantumDot (QD) 655), and propidium iodine (PI); Thymocyte staining: CD45.2 also opens for alternative and more or less complex explanations of the phenotype observed in the absence of EBF1.

In vitro reconstitution assay

RNA was extracted from purified adult BM subsets as described for Q-RT-PCR. RNA was labeled and amplified according to the Affymetrix GeneChip Expression Analysis Technical Manual. Chips were scanned on a GeneChip scanner 3000 and scanned to a median intensity of 100. RNA from cells sorted on separate occasions was separately hybridized for each investigated population. Probe level expression values were calculated using RNA and further analysis was done using dChip (http://biosoul.harvard.edu/complab/dchip/). Array data will be deposited in GEO upon acceptance of the report for publication.

Principal component analyses were performed using the online NIA Microarray analysis tools provided by national institute for aging (http://labs.niehs.nih.gov/ANOVA/) (24).

Ig recombination PCR analysis

Genomic DNA was prepared using TRizol (Life Technologies) according to the manufacturer’s instructions. IgD-JH rearrangements were amplified by 38 cycles (94°C, 30 s; 60°C, 45 s; and 72°C, 1 min) using the DH and JH primers at a μM. The PCR mix contained 1 μl of DNA, 1 μl of Expand Long Template DNA polymerase (Roche), 0.2 μl of each primer, and 10 g/L, BSA 10 g/L), 6 μL L-methionine, 1 mL NAc) supplemented with 0.1% SDS for 15 min and 0.1 × SSC with 0.1% SDS for 10 min. The hybridized membrane was subjected to autoradiography.

The following oligonucleotides were used for PCR: DH: 5′-GGAAATT CAGA/GAT/C/TTTGGTG/C/AAAGGGTGACTCTAGTTG-3′, J3: 5′-GTC TAGATTTCTCAACAAGAAGTCCGATAGACCTCG-3′. The following oligonucleotide was used for hybridization: JH3: 5′-AGACAGTGACCAGCACCTCCGCC-3′. The OPN promoter or a part of the HPRT gene was amplified as control for the samples contained amplifiable material.

Gene expression analysis of single cells by multiplex RT-PCR

Multiplex single-cell RT-PCR analysis was performed as previously described (25, 26). In brief, single cells were deposited into 96-well PCR plates containing 4 μl lysis buffer and frozen at −80°C. Cell lysates were reversed-transcribed using gene-specific reverse primers and 50 U MMLV-RT (Invitrogen) in a final volume of 10 μl. First-round PCR was performed by the addition of 40 μl PCR mix containing 1.25 U Taq polymerase (TaKaRa Bio) and gene-specific forward primers to the RT reaction and the mix subsequently subjected to 35 cycles of PCR. One-microliter PCR products were visualized by ethidium bromide staining. Primers sequences as follows: Hprt; 1) 5′GGGGGCTATAAGTTCTTTGC; 2) 5′GGGGGCTATAAGTTCTTTGC; 3) 5′GGGGGCTGCTGAAGGGATCTACTACTGTG-3′, J3: 5′-GTC TAGATTTCTCAACAAGAAGTCCGATAGACCTCG-3′. The following oligonucleotide was used for hybridization: JH3: 5′-AGACAGTGACCAGCACCTCCGCC-3′.

The OPN promoter or a part of the HPRT gene was amplified as control for the samples contained amplifiable material.
ROLE OF EBF1 IN B-LYMPHOPOIESIS

GGAACATGC; 2) 5′CTACTCTCCGTATCAAGGC; 3) 5′TGTAAGCACTTCGCTTGGC; 4) 5′TAAAGATTTTCTCTTCTGGG; 5′GAACATGTACATGTTTTCG; ATTTGA. GTCTAA; Rag-E antisense: TTAGACAGAACCCGAGGGCTTAGC GAAGTCGGTG; Rag-E sense: ACAATGCTAAGCCCTCGGGTTCT GAGAGCTGCGCGA; Foxo1 EBF1 antisense: TCGCGCAGCTCTCGGT TCCCTCGGGAGGCGGCA; Foxo1 EBF2 sense: CACCGACTTCCC GACTGACTCTGAGG 3) 5′ATGTGCCCTCCTCCTGACTGACT 4) 5′TTAGGAAGAGAAGAAGGGC CGTGG; Sterile IgH: 1) 5′GCTAGCTCGGAAAGATTACC; 2) 5′TGAGTT TCTGAGCTTGG 3) 5′AGTACGACATCTGCTTGGC; 4) 5′AAG GACTGACTCCTGAGG

Tissue culture conditions and cell lines

BaF3 cells and transduced Ba/F3 lines (12) as well as HeLa cells were grown at 37°C and 5% CO2 in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM pyruvate, 50 μM 2-ME, and 50 μg/ml gentamicin (all purchased from Life Technologies). The medium for the BaF3 cells was supplemented with 10% of conditioned medium from confluent WEHI3 cells as a source of IL-3. OI9/OP9A1 cells were grown in OPTIMEM supplemented with 10% (v/v) FCS, 50 mM 2-ME, and 50 μg/ml gentamicin (Invitrogen) at 37°C and 5% CO2.

Protein extracts and EMSA

Nuclear extracts were prepared according to Schreiber et al. (27) DNA probes were labeled with γ [32P]ATP by incubation with T4 polynucleotide kinase (Roche) annealed with the complementary strand and purified on a microspin column (Roche). Five to ten micromolar of nuclear extract was incubated with the labeled probe (20,000 cpm, 3 fmol) for 30 min at room temperature in binding buffer (10 mM HEPES (pH 7.9); 70 mM KCl; 1 mM DTT; 1 mM EDTA; 2.5 mM MgCl2; 5% glycerol, 1 mM ZnCl2) with 0.75 μg Poly d(dC) (Amersham Biosciences). DNA competitors were added 10 min before addition of the labeled probe. The samples were separated on a 6% acrylamide TBE gel, which was dried and subjected to autoradiography. Competitors based on synthetic oligo-nucleotides were added at molar excesses indicated in the respective figure legends. Super shifts were performed under the same conditions but with additional presence of 2 μl polyclonal anti-EBF (SC-15333) or anti-actin (SC-1616) (Santa Cruz Biotechnology) as a negative control.

Oligo-nucleotides used for EMSAs were:

CD79α sense: AGGCCACCTC TCAAGGGGATAATGTTG; CD79α antisense: CCACAATTTTCTCAGAGG TTGCCT; mutated CD79α sense: AGGCCACCTC TCAAGGGGATAATGTTG; mutated CD79α antisense: CCACAACACCGTGAAGGTGGCTCCT; POU2af1 antisense: ATAGTGACCCCTGGAATCCTCGT; POU2af1 sense: ATAGTGACCCCTGGAATCCTCGT; CTCGTCGCTCGAGGAGGAGCAGGC; Foxo1 EBF1 antisense: GCTGAGTT TTCTCCCTGGAGGCGCA; Foxo1 EBF2 antisense: CACCGACTTCCC GAGAGCTGCGCGA; Foxo1 EBF1 antisense: TCGCCGACGTCTCCG GAAGTCTGGT; Rag-E antisense: ACAATCTAAGACCTCGGGTCTT GCTCTGA; Rag-E antisense: TTAGAACAAACCCGGAGGGTTACG ATTTGA.

Plasmids and cloning of promoter elements

The EBF-1 expression plasmid has been described before (11). The reporter plasmids were based on the luciferase reporter plasmid pGL-3 (Promega). The Foxo1 and POU2af1 promoters reporter plasmids were generated by blunt end cloning of PCR amplified promoter elements into the Smal site of pGL-3. All constructs were confirmed by sequencing. The PCR products were generated using the following primers: Pou2af1 – 5′-650sense: TCAGCCGGTGAGCCGTTGTTAAAAATGG; TCGAGCTGTGGAGACACCGGCTTTACCTCAGG; Foxo1 – 5′-499sense: TCGAGCCGCTCCACCCGACTCCTCCGGAGCTGC; Foxo1 antisense: AGCTCTCGAGACTGAACTGACCCTGGAGGCG.

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Transient transfections and luciferase assays

HeLa cells were seeded in a 24-well tissue culture plate so that they reached 60–80% confluence for transfection. A total of 400 ng of DNA was used to transfect the cells in each well. In brief, 50 ng of each reporter construct was cotransfected with 600 ng expression vector and all transfections included 50 ng pRl-0 Renilla luciferase, received from Dr. Björn Olde (Lund University, Sweden), as internal control, and used for normalization of the luciferase activities. The DNA was first added to 50 μl of serum free medium, incubated for 30 min in room temperature, mixed with the diluted DNA, and then incubated for another 15 min at room temperature. Meanwhile, the complete RPMI medium was replaced with 300 μl of serum-free medium, and the DNA plus lipofectamin mixture was added to the cells that then were incubated at 37°C in 5% CO2 for 3 h, after which each well was supplemented with 1 ml complete RPMI. Cells were harvested 48 h after transfection and protein extracts were prepared directly in the 24-well plates by adding 100 μl cell lysis buffer.

Statistics

All statistical analyses have been done either in Microsoft Excel and GraphPad Prism or appropriate software for microarray analysis (dChip and NIA microarray analyses).

Results

Lack of EBF1 results in a cell autonomous and lineage-restricted block of B cell development

Expression of EBF1 is within the hematopoietic system closely related to B lineage development (3). However, when comparing the changes in gene expression patterns between multipotent lymphoid myeloid primed progenitors (LMPs) (28) and the more developmentally restricted CLPs (29) by microarray analysis, we noted a striking difference in the expression levels of Ebf1 (53). These experiments suggested that the multipotent CLP expressed ~20% of the mRNA level observed in Cd19+B220+ CD43+ pro-B cells, opening the possibility that EBF1 may be involved in the development of CLPs. Due to a high early lethality of EBF1 deficient mice, and due to the reported function and expression of EBF1 proteins in bone marrow stroma cells (22), we investigated the ability of EBF1-deficient day 14.5 FL hematopoietic progenitor cells to generate hematopoietic cells after transplantation (Fig. 1, A–D). In mice analyzed 16 wk or more post-transplantation, the overall reconstitution of CD45.2+ cells out of the total CD45+ BM cells in mice receiving wild-type (WT) FL cells was >95% while in those transplanted with the EBF1 deficient FL cells, it was between 75 and 95% (Fig. 1A). Among the CD45.2+ cells, the fraction of early progenitors including the Lin−Scal+Kit+ (LSK) and the CLPs (Fig. 1B) was comparable as was the distribution of the major T cell subpopulations in the thymus (Fig. 1C), suggesting that EBF is not crucial for early lymphoid development or thymopoiesis. Investigations of early bone marrow EBF1-deficient cell, however, revealed a complete lack of pre- or pro-B cells derived from EBF1 deficient mice. In contrast, we detected slightly increased numbers of B220+CD43+ AA4.1+ cells, reported to represent the pre-pro-B cell compartment (Fig. 1D), (30, 31) supporting the previously raised idea that deficiency in EBF1 results in a lineage restricted early block in B cell differentiation. To verify the apparently normal ability of EBF1-deficient fetal liver cells to generate CLPs with lymphoid potential, we cultured CLPs (lineage-negative, I/B7R+FLT3+ SCA1low KIThigh, sorted from the FL transplanted mice) on either OP9 or OP9Δ1 stroma cells (see Materials and Methods). This revealed that T cell development of EBF1-deficient CLPs progressed normally in vitro while development of CD19+ cells was completely abrogated (Fig. 1F). These data suggest that EBF1-deficient hematopoietic progenitor cells have a lineage-specific cell autonomous defect and suggest that a CLP compartment forms independently of EBF1.

EBF1-deficient CLPs display defective transcription of B-lineage-associated genes

The apparent block of B cell development in fraction A, according to the Hardy nomenclature (30, 31) would suggest that EBF1 is involved in regulating critical genes in the pre-pro-B cell compartment. These data suggest that EBF1-deficient hematopoietic progenitor cells have a lineage-specific cell autonomous defect and suggest that a CLP compartment forms independently of EBF1.
microarray gene expression analyses on sorted CD45.2\(^+\) CLPs and B220\(^-\)CD43\(^+\)AA4.1\(^+\) cells from mice reconstituted with either WT or EBF1-KO FL cells (Fig. 2A). To place the gene expression patterns observed into a context, expression data from LMPPs as well as CD19\(^-\)CD43\(^{low}\)AA4.1\(^+\) pro B cells, B220\(^-\)CD43\(^-\)CD19\(^+\)IgM\(^-\) pre-B cells and IgM\(^+\)B220\(^+\) spleen B cells sorted from WT mice were included in the analysis. These experiments suggested that WT and EBF1 KO B220\(^-\)CD43\(^+\)AA4.1\(^+\) cells, generated in vivo in mice reconstituted with FL cells, expressed very low levels of B-lineage-associated transcripts, indicating that in this setting and from the gene expression point of view, this population of cells cannot be linked to progressive B lineage development. This was also supported by a global principal component analysis because CLPs displayed a higher overall similarity to pro-B cells than the B220\(^-\)CD43\(^+\)AA4.1\(^+\) population (data not shown). The principal component analysis also supported the idea that the EBF1-deficient cells displaying a CLP surface phenotype indeed represent a CLP population (data not shown). The microarray data suggested that there were no significant alterations in the expression of TCF3, Id, or Ikaros proteins in WT or EBF-deficient CLPs (Data not shown), however, we could detect reductions of gene expression of B-lineage-associated genes including Pou2af (OcaB), Pax5, Vpreb1, and Blnk, in the cells generated from EBF1-deficient fetal livers. To verify a reduced expression of B-lineage-associated genes in the EBF1-deficient CLPs from transplanted mice, we performed Q-PCR analysis that supported reductions in Cd79a, Pou2af, Pax5, and Ilgll1 expression (Fig. 2B). We could also detect reductions in the levels of Cd79b and Foxo1 message, suggesting that normal expression of these genes were dependent of functional EBF1. To investigate these alterations in gene expression patterns at a single cell level, WT and EBF1-KO CLPs (sorted from FL reconstituted mice), were analyzed for gene expression patterns with multiplex single cell PCR (Fig. 2C). This revealed that while a small subpopulation of the WT CLPs coordinately expressed Mb-1, Pax-5, and Pou2af (p < 0.03), these cells are absent in the CLPs from EBF1-deficient mice (Fishers exact test, p < 0.03). We were also able to detect transcripts from the WT as well as the mutated Ebf1 gene in a large number of the cells. These findings support the idea that in the absence of EBF1, B cell development is blocked already at the level of the CLP.

FIGURE 1. Lack of functional EBF1 results in a cell autonomous deficiency in B cell development in vivo. A–D, The panel shows data from individual mice (represented by dots) transplanted with 2.5 \times 10^6 E14.5 EBF1-KO or WT FL donor cells (from CD45.2\(^+\) mice) transplanted together with 2.5 \times 10^5 unfractionated BM support (from CD45.1\(^+\) mice) to lethally irradiated mice. The mean value in an experimental group (indicated by a horizontal line) as well as representative FACS plots from the generated BM cells. A, Graph containing the obtained overall reconstitution levels (percentage of CD45.2\(^+\) cells of total CD45\(^+\) cells in the BM). B, Percentage of LSKs and CLPs of total CD45.2\(^+\) cells and representative FACS plots from WT or EBF1-deficient mice as indicated. C, CD48 thymocytes subpopulations and D displays B cell progenitor populations. The diagrams show the percentage of CD45.2\(^+\) donor cells. E, Representative FACS plots of resulting cells when 50 CLPs (purified from transplanted mice of indicated genotype 16 wk posttransplantation) were cocultured for 14 days on OP9 or OP9\(\times\) stroma cells (see Materials and Methods). The data are representative of at least two independent sorting experiments (KO; Ebf1 KO mice, WT; wild-type C57BL/6 mice, HT; heterozygote Ebf1 KO mice, DN; double negative, DP; double positive, SP; single positive, n = 5).
Absence of EBF1 results in reduced IgH D-J recombination in CLPs

It has been reported that a fraction of the CLPs in the mouse BM has undertaken the first steps of the IgH recombination process by joining D segments to J segments (31). To investigate whether EBF1 has a direct role in recombination of the IgH genes, we extracted genomic DNA from WT as well as EBF1-deficient CLPs obtained by transplantation as above, and investigated the presence of IgH D-J recombination in these cells (Fig. 3A). Recombination events were easily detected in the WT CLPs, while they appeared to be dramatically reduced in CLPs generated from EBF1 deficient cells. To approximate the differences in recombination efficiency, we performed a semiquantitative PCR (Fig. 3A). This suggested that the difference was in the order of 2–4 cycles, or 12.5–25% of WT values. To further investigate whether D-J recombination occurs in the absence of EBF1, we sorted CD3+ cells generated by transplantation of WT or EBF1 KO fetal liver cells as above and investigated IgH recombination in this compartment (Fig. 3B). This indicated that IgH D-J recombination could be detected supporting the idea that IgH D-J recombination occur, at least in the developing T cell compartment, in the absence of EBF1. Thus, even though DJ recombination may occur in the absence of EBF1,
Figure 3. Reduction of IgH D-J rearrangement in CLP compartment in absence of EBF1. A, Autoradiograms of blotted PCR products from the D-J recombinated IgH locus on ethidium bromide stained agarose gels with a PCR product generated by the amplification of the osteopontin promoter (OPN) or the Hprt gene as a control for the presence of amplifiable DNA. Each lane in the left panel represents one independent sort from different transplanted mice (LMPPs was generated by sorting directly from WT mice and not from transplanted animals). The semiquantitative experiment in the right panel displays autoradiograms of PCR products generated by two cycle intervals as indicated. The data show one representative of two independent experiments from different sorts. B, D-J PCR analysis of sorted thymic CD3+ cells. OP9 represents OP9 stroma cells and serves as a negative control for recombination. C, Quantitative RT-PCR analysis of Rag1, Rag2, and sterile IgH expression in WT and EBF1 KO reconstituted CLP populations (data are collected from one representative WT and two EBF KO reconstituted mice, error bars indicate experimental SE). The left autoradiogram in D shows EMSAs using nuclear extract isolated from 230 to 238 pre-B cell line and the potential EBF1 binding site in the E-Rag enhancer. ACTIN or EBF1 Abs are included as indicated. Right panel, EMSAs using nuclear extracts from 230 to 238 and the EBF1 binding site from the Cd79a promoter. Complex formation is competed for by the addition of a 500-fold excess of unlabelled Cd79a or mutated CD79aM oligo or 150 and 500-fold excess of E-Rag enhancer EBF1 site. The gels have been cut to display only the EBF/DNA complex. (EMSAs; EMSA, Ab; Ab, Com; competitor, Cd79aM; mutated Cd79a prob).

This event is largely reduced even at the level of the CLP. The global gene expression analysis suggested that EBF1-deficient CLPs expressed a lower level of Rag1 essential for IgH recombination. To verify this finding and expand the analysis, we investigated the expression levels of Rag1, Rag2, and sterile IgH transcripts in EBF1-deficient CLPs (Fig. 3C). We found that the Rag1 expression was only ~25% of that in the WT cells while the expression of Rag2 and sterile IgH transcripts were only marginally reduced. Because the single cell PCR analysis suggested that a comparable number of CLPs express Rag1 either in the absence or presence of EBF1 (Fig. 2C), this differential level is likely to be reflected in a reduction of transcription rather than a loss of a given population. This could indicate that Rag1 would be a direct target for EBF1, but we were unable to identify any EBF binding sites in the published Rag1 promoter. To investigate whether an alternative promoter was used in the CLP, we performed 5′ RACE analysis using CLP mRNA. This did, however, result in identification of the identical promoter region arguing against this explanation. Another possibility would be a direct involvement of EBF in the regulation of the E-Rag enhancer (32). Targeted deletion of this element has a rather dramatic effect on the transcription of Rag1 while the effect on Rag2 transcription is comparably small (32). Investigation of the E-Rag enhancer sequence revealed a potential EBF binding site, and using EMSA we were able to show that this site had the ability to compete for binding of a CD79a promoter EBF site to EBF1 in nuclear extracts from a mouse pre-B cell line (Fig. 3D). Furthermore, upon incubation with nuclear extracts, this site was able to form a complex that could be supershifted with anti-EBF Ab (Fig. 3D). Therefore, we conclude that EBF1 is of large importance for IgH D-J recombination in the CLP and that lack of EBF1 is associated with a reduced expression of Rag1, possibly as a direct result of EBF activity at the E-rag enhancer or via reduced levels of Fox proteins acting at the same element (see below).

EBF activates a network of B-lineage-associated transcription factors in the CLP

Knowing that the expression of Pou2af and Foxo1 is reduced in EBF-deficient CLPs, we wanted to examine a potential direct role of EBF1 in the regulation of these genes. Because expression of the EBF gene is not perfectly correlated to the expression of its target genes (Fig. 2C, 53), we sorted CLPs from mice carrying an Igfl promoter-controlled human CD25 gene (33). The Iglfl promoter is a direct target for EBF and these mice can therefore be used to identify early cells that have initiated the B lineage program (53). Q-PCR analysis suggested that the increased expression of Iglfl was correlated to that of Pax5, Pou2af1, Foxo1, and Ebf1 suggesting the idea that these genes are coordinately regulated in early B cell development (Fig. 4A). In addition, we were able to detect low but significant amounts of both Pou2af1 and Foxo1 transcripts in Ba/F3 progenitor cells after stable ectopic expression of the combination of EBF and E2A (12) (Fig. 4B). These data suggest that EBF1 might be directly involved in the regulation of both the Pou2af1 and Foxo1 genes. RACE analysis supported the idea that the published promoter sequences (Ref. 34, 35 and NCBI-Gene) were valid also in immature cells (data not shown) and inspection of the 5′ flanking regions revealed two potential EBF1 sites in the Foxo1 and one potential site in the Pou2af1 promoters (Fig. 4C). To investigate whether these sites were able to interact with EBF1, we performed EMSA experiments using nuclear extracts from a mouse pre-B cell line and competed for complex formation between EBF1 and the high affinity binding site from the Cd79a promoter (Fig. 4C). This revealed that both the Foxo1 and Pou2af1 promoters contained high affinity binding sites able to compete for EBF1 binding. To verify the ability of these binding sites to interact with EBF1 in a nuclear extract from a pre-B cell line, we performed a second round of EMSAs using labeled Foxo1 or Pou2af1 EBF binding sites. Both gave rise to one
major complex that was supershifted by the addition of anti-EBF Ab (Fig. 4C). To investigate the ability of EBF1 to directly activate the Foxo1 and Pou2af1 promoters, we cloned these regulatory elements in front of a luciferase reporter gene and transfected them either with empty or EBF1 encoding expression vector into epithelial HeLa cells lacking endogenous expression of EBF1. This revealed that both promoter elements responded to the ectopic expression of EBF1, supporting the idea that the Foxo1 and Pou2af1 genes are direct targets for EBF1.

Discussion

In this article, we present data suggesting that even though EBF1 expression is dramatically increased in the transition from the multipotent LMPP stage to the lymphoid-restricted CLP stage, the transcription factor is not critical for the development of cells with a general CLP phenotype. The presence of surface proteins and general gene expression patterns as well as preserved potential for development into T-lineage cells in vitro all support this conclusion. This appears to be in contrast to what has been reported for TCF3 because mice lacking this factor display a rather dramatic reduction in the number of CLPs (36). This would indicate that even though EBF1 and TCF3 act in synergy in the earliest stages of B cell development (9, 13), TCF3 might have an EBF1-independent function in the generation of CLPs. This would be in line with the finding that while TCF3 target genes can be detected in LMPPs, these cells lack transcription of EBF target genes (28). However, even though CLP-like cells were generated from EBF1-deficient fetal liver cells, they lacked the transcription of B-lineage associated genes normally found in the CLP compartment. Even though we were able to detect cells with surface expression of AA4.1, B220, and CD43, indicative of pre-pro-B cells, we were unable to obtain evidence that these cells would represent a homogeneous population of B-lineage cells and we conclude that EBF1-deficient progenitors are severely compromised in their ability to take on B lineage fate. A role for EBF1 in lineage commitment is also supported by the finding that the factor restricts the lineage choices of PAX5 deficient progenitor cells (37). The finding that EBF1-deficient CLPs lack expression of B-lineage genes found in normal CLPs supports the notion that EBF1 is a key coordinator of the transcriptional program that drives B-lymphocyte development already in the CLP. This activity could be explained by the apparent ability of EBF1 to pioneer transcription by mediation of epigenetic changes (18) but it could also be contributed to EBF1’s ability to directly activate a set of B-lineage associated transcription factors (Fig. 5). EBF1 has previously been reported to be involved in the activation of the Pax5 gene (13) coding for a key transcription regulator in early B cell development. PAX5 has in turn been suggested to regulate a large set of
FIGURE 5. Ebf1 plays a central role in transcription regulatory network of B-Cell development. The diagram shows some of the functional interactions of selected transcription factors with Ebf1 based on the current knowledge about transcription regulatory networks in B cell development. Solid arrowheads represent stimulatory and the open diamond end shows inhibitory effect. Dashed lines show the hypothetical or controversial interactions. The diagram designed in yEd-Java Graph Editor available at http://www.yworks.com/en/products_yed_about.html.

genes including the expression of the transcription factor Lef1 (38) and Ebf1 itself (3). PAX5 has also been suggested to positively regulate expression of Cd19, Cd79a, and Rag2 (38–40) and to repress expression of Notch1(41). In addition, we report in this study that EBF1 is directly involved in the regulation of Pou2af1 transcription. This protein was originally identified as a regulator of OCT protein activity in B-lymphoid cells and even though the effect of targeted mutation of the Pou2af1 gene is most pronounced in late stages of B cell differentiation (42–44), reduced levels of Pou2af1 have been reported to result disturbances also in early B cell development (45). In addition, Pou2af1 has been reported to regulate the expression of the Ets transcription factor SpiB (46) as well as of other genes encoding proteins involved in signaling and cell cycle regulation (47). EBF1 also appear to be directly involved in the regulation of the Foxo1 gene that might be in a large network of redundant FOXO family transcription factors (48). Among other FOXO proteins, Foxp1 has been shown to be important for early B cell development (49), to some degree possibly via its ability to modulate Rag1 transcription though a direct interaction with the E-Rag enhancer. Several of the FOX family members also share binding sites, and it is possible that there exist a large redundancy among these proteins similar to what has been reported for basic-helix-loop-helix transcription factors (50, 51). Thus, it is possible that the link between EBF1 and the FOX-factor network is mediated via FOXO1. Using available information about target genes for this network of transcription factors allows for the creation of a scheme where several genes known to define the B lineage can be incorporated (Fig. 5).

It is also notable that with the possible exception of Ig H chain DJ recombination, the activation of EBF1 target genes is not directly linked to the transcription of the Ebf1 gene itself. A majority of the CLPs express Ebf1 message, while only a small percentage of the cells express known target genes. We have investigated this in more detail using mice transgenic for a Igfl1 promoter-controlled reporter gene, and it appears as if the activation of EBF1 target genes is linked to a 4-fold increase in Ebf1 message in a B cell committed subpopulation of the CLPs (53). We believe these data indicate that transcription of the Ebf1 gene is a precommitment process, crucial for but not directly coupled to activation of the B-lineage program. This could be a result of a direct dose-dependent function, translational inhibition by inhibitory RNA or posttranslational modifications, for instance due to active Notch signaling (52). Regardless, because we find expression of Ebf1 even in the absence of the expression of other B-lineage genes, this provides strong support to the idea of a defined precommitment stage of B cell development represented by the expression of Ebf1. Because Ebf1 expression is low in LMPPs (53), a cell type that contains Tdt, sterile Ig, and Ragl transcripts (28), this could indicate that the pathway to B cell commitment is achieved at least at three different levels. In the LMPP, lymphoid priming is established, represented by expression of genes common for B and T lymphoid cells. Upon transition into the CLP compartment, Ebf1 is up-regulated, making the cell competent to develop into B-lineage, but as the full function of EBF1 would be regulated at a posttranscriptional level, cells still remain uncommitted even though they undergo Ig DJ recombination, facilitated by a low EBF1 activity incapable of activating B cell specific target genes. The final step is then associated with the functional activation of EBF1 resulting in the transcription of a set of B lineage specific genes, including Pax5, Foxo1, and Pou2af1.

Thus, it may be so that there is no single crucial target gene for EBF1 but rather that EBF1 is the key regulator of the B-lineage transcriptional program and commitment through its ability to activate B-lineage genes as well as a set of transcription factors capable to strengthen the B cell identity, possibly already at the level of CLP.

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References


