BSAP/Pax5A Expression Blocks Survival and Expansion of Early Myeloid Cells Implicating Its Involvement in Maintaining Commitment to the B-Lymphocyte Lineage

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Early B lymphopoiesis is marked by plasticity between the myeloid and B lineages. An attractive model for B-lineage development is that commitment to this lineage is partly determined by the ordered expression of genes that prohibit switching to the myeloid lineage. In this regard, whereas the role of the B-cell-specific transcription factor BSAP/Pax5A in regulating B-lymphoid-restricted gene expression has been well-established, its role in maintaining B-lineage commitment is unclear. Thus, BSAP/Pax5A was constitutively expressed in the multipotent EML cell line, which can be directed toward the myeloid lineage by culture with interleukin-3 (IL-3) and retinoic acid. EML cells expressing BSAP/Pax5A successfully acquired the myeloid lineage markers CD11b and F4/80 in response to IL-3 and retinoic acid, indicating differentiation to the myeloid lineage. However, these early myeloid cells failed to expand in culture with granulocyte-macrophage colony-stimulating factor and were directed instead toward an apoptotic pathway. In parallel, primary bone marrow stem cells transduced with retrovirus constitutively expressing BSAP/Pax5A began myeloid cell differentiation, but like the transformed EML model failed to expand in response to myeloid growth factors. These studies identify a role for BSAP/Pax5A in suppressing the response to myeloid growth factors, which may be a component of the regulatory processes that limit plasticity of early B-lymphoid progenitors.

A FUNDAMENTAL QUESTION in hematopoiesis is how cells once directed to a particular lineage stay committed to that lineage. Lineage-determinant genes such as mafB/ets-1, GATA-1, E2A, and PU.1 are thought to play a role in these processes. These genes encode transcriptional regulators that direct or enhance expression of lineage-specific genes. In addition, these transcriptional regulators also repress expression of genes associated with closely related lineages. For example, whereas ectopic PU.1 expression in a chicken thromboblast progenitor was shown to activate genes associated with the myeloid lineage, enabling these cells to respond to myeloid growth factors, PU.1 also repressed expression of GATA-1, which is a determinant gene for erythroblast, thromboblast, and eosinophil commitment.

This question of how cells maintain lineage commitment is particularly important to the B-cell lineage as many transformed B cells fail to expand in culture with myeloid growth factors. Recent studies implicating E12, a product of the E2A gene, as a B-lineage–determinant protein, because it upregulates B-cell–specific genes and represses myeloid-associated genes. In these studies, ectopic expression of E12 in the macrophage cell line 70Z/3 caused a loss of macrophage morphology, downregulation of the myeloid cell genes CD11b and c-fms, inability to adhere to plastic, activation of the K light chain in response to lipopolysaccharide (LPS), and upregulation of the B-cell genes IL-7Ra, RAG-1, A5, BSAP/Pax5A, and EBF. Thus, E12 stripped a cell of its myeloid characteristics and reprogrammed it with B-cell characteristics, which led to the hypothesis that...
the endogenous expression of E12 in B cells normally prohibits them from switching to the myeloid lineage in vivo.\(^5\)

Target genes for E12 include the genes that encode the B-cell–restricted transcription factors EBF and BSAP/Pax5A.\(^3,5,18\)

The role of these molecules in B-lineage determination is only partially defined. Ectopic expression of EBF in the 70Z/3 macrophage cell line resulted in a limited expression of B-cell–associated genes, including upregulation of BSAP/Pax5A and \(\lambda_5\) expression and the activation of \(\kappa\) chain expression in response to LPS.\(^5\) However, the expression of the myeloid-lineage–associated genes covered in this study was unaffected by ectopic EBF expression.\(^5\) Thus, only a subset of the E12-linked processes that determine B-cell gene expression and none of the E12-linked myeloid-suppressing processes can be attributed to EBF or BSAP/Pax5A by extension, because it was upregulated by EBF.\(^5\) These data do not necessarily exclude a role for EBF or BSAP/Pax5A in the suppression of myeloid gene expression, because this study did not cover several myeloid genes.\(^5\)

BSAP/Pax5A is the major alternatively spliced isoform of Pax5, a member of the Pax family of transcription factors.\(^19,20\) Pax5 is expressed in B cells developing neural tissue and testis and is abnormally expressed in certain B-cell lymphomas and medulloblastoma.\(^21,22\) Within the B-lineage, BSAP/Pax5A is first expressed immediately after commitment to the B-lineage in the bone marrow\(^17,23\) and continues to be expressed throughout B-cell development, except in plasma cells.\(^37\) Besides its regulation by E12 and EBF, other evidence suggests that BSAP/Pax5A may have B-cell–determinant properties. Similar to E2A\(-/-\) mice, Pax5\(-/-\) mice completely lack B220\(^+\) cells in the fetal liver, suggesting that BSAP/Pax5A is required for B-cell commitment.\(^24,25\) However, in the bone marrow, Pax5\(-/-\) mice display a complete block later in B-cell development.\(^26\) In this case B-cell development is blocked at the pro-B–cell stage after immunoglobulin D to J rearrangement, but before immunoglobulin V to DJ rearrangement of the heavy chain locus.\(^23\) Like E12, BSAP/Pax5A is also thought to upregulate several B-cell–specific genes that are first expressed during the early pro-B stage. These genes, which include \(V_{\text{preB}},\ \text{blk},\ \text{mb-1}\) (Igκ), and \(CD19\), encode both markers of early B-lineage commitment as well as proteins that are necessary for the transition through the initial stages of B-cell development in both the bone marrow and the fetal liver.\(^27,28\)

The combined evidence that BSAP/Pax5A plays a role in B-cell commitment, developmental progression, and B-cell marker expression throughout most of B-cell development strongly suggests that BSAP/Pax5A, like E12, may have its own B-cell–determinant properties. To test this possibility, BSAP/Pax5A was ectopically expressed in bone marrow stem cells. Because EBF/BSAP-expressing 70Z/3 cells failed to suppress the myeloid phenotype,\(^5\) it was expected that the B-cell–determinant properties of BSAP/Pax5A would be limited to upregulation of B-cell gene expression. Surprisingly, contrary to these expectations, whereas BSAP/Pax5A lacked the ability to alone mediate B-lineage differentiation, it nonetheless was found to limit myeloid-lineage potential by suppressing expansion and survival of early myeloid cells. By doing so, these results argue that one of the functions of BSAP/Pax5A in B-lymphoid commitment is to limit the ability of lineage-divergent cells to proceed through development.

**MATERIALS AND METHODS**

**Plasmid construction.** pGEM7(KI1)SalI contains the neomycin resistance gene downstream of the phosphoglycerokinase (PGK-1) gene.\(^32\) The PGK-1 promoter was removed from pGEM7(KI1)SalI after digestion with Xho I and Pst I and was ligated into pBluescript II SK (Stratagene, La Jolla, CA) digested with Xho I and Pst I to form pBSPGK. The BSAP/Pax5A cDNA and SV40 poly A signal was removed from pmBSAP-2\(^3\) (a kind gift from Dr Meinrad Busslinger, Research Institute of Molecular Pathology, Vienna, Austria) after digestion with \(\text{Cla I}\) and \(\text{Apa I}\) and was ligated into pBSPGK digested with \(\text{Cla I}\) and \(\text{Apa I}\) to form pPGK Pax5A. The Mig R1 plasmid, when transiently transfected into the Bosc23 packaging cell line, produces the Mig R1 retrovirus that expresses the green fluorescence protein (GFP) marker at an internal ribosomal entry site.\(^24\) BSAP/Pax5A cDNA was removed from pmBSAP-2 with \(\text{Cla I}\) and \(\text{HindIII}\), blunted-ended at the \(\text{HindIII}\) site, and ligated into the \(\text{Cla I}\) and \(\text{EcoRV}\) sites of pBluescriptII SK to form pBSBSAP. BSAP/Pax5A cDNA was then removed from pBSBSAP with \(\text{EcoRI}\) digestion and then ligated into the \(\text{EcoRI}\) site of Mig R1 to produce Mig R1BSAP.

**Hematopoietic growth factors.** Conditioned supernatant from the J558-IL-7 cell line (a kind gift from Dr Fritz Melchers, Basel Institute for Immunology, Basel, Switzerland) was used as a source of murine interleukin-7 (IL-7). Human flt3L and murine granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from Genzyme Diagnostics (Cambridge, MA). Conditioned supernatant from the WEHI-3B cell line was used as a source of IL-3. Erythropoietin was obtained from Amgen (Thousand Oaks, CA). Murine IL-6, stem cell factor (SCF), and IL-3 were purchased from R&D Systems (Minneapolis, MN).

**Antibodies.** To identify primitive stem cells, monoclonal antibodies RA3-6B2 (B220), RM4-5 (CD4), 53-6.7 (CD8a), RB6-8C5 (gr-1), M170 (CD11b/Mac-1), and Ter119 (erythroid lineage marker) were used coupled to phycoerythrin (PE) as lineage markers. Stem cell markers include 2B8 (c-kit) coupled to allophycocyanin (APC) and E13-161.7 coupled to biotin (sca-1) and streptavidin red-670 as a secondary reagent. These antibodies were handled according to the instructions of the supplier (PharMingen, San Diego, CA). The PE-coupled F4/80 antibody was handled according to the instructions of the supplier (Caltag, Burlingame, CA). Polyclonal antibodies directed against the paired domain of Pax5A were a kind gift from Dr Meinrad Busslinger, who generated them as previously described.\(^33\) All flow cytometry was performed using a Becton Dickinson (Franklin Lakes, NJ) FACScan at the University of Pennsylvania Flow Cytometry Facility (Philadelphia, PA) and Cellquest software.

**Cell lines.** EML cells\(^33\) (a kind gift from Dr Schickwann Tsai, Mount Sinai School of Medicine, New York, NY) were maintained in Iscove’s Modified Dulbecco’s Media (IMDM), supplemented with 20% heat-inactivated horse serum, and 12% to 15% BHK/MKL (a kind gift from Dr Fritz Melchers, Basel Institute for Immunology, Basel, Switzerland). EML cells were expanded and frozen in 0.1 M sucrose solution and stored at \(-80^\circ\)C.

**Electromobility shift analysis (EMSA).** Nuclear extracts were prepared as described elsewhere.\(^37\) The CD19Ains probe\(^37,38\) consisted of the following 2 oligonucleotides that were annealed to form a double-stranded oligonucleotide: 5’CTGGGAATGGGGGACTGAGGGGT-GACCCACGGCT3’ and 5’AGGCCGTTGTCACGGCTGACC-TCCATTCGCCAG3’ (Nucleic Acid Facility, University of Pennsylvania Cancer Center). Fifty nanograms of probe was end-labeled with 10 µCi [\(^32\)P]ATP using T4 kinase and purified over a G-25 spin column. Binding reactions consisted of 20,000 DPM probe, 2 µg poly dI-dC, 10
mmol/L HEPES, pH 7.9, 100 mmol/L NaCl, 10% glycerol, 0.5 mmol/L MgCl₂, and 1 mmol/L dithiothreitol for 15 minutes at room temperature. Nuclear extracts were then added to the probe mixture for an additional 15 minutes at room temperature. The reaction was then subjected to electrophoresis on a native 4% polyacrylamide gel in 1× Tris-borate/EDTA (TBE) buffer to separate protein-DNA complexes. Quantitation was performed with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

*In vitro generation of myeloid precursors from EML cells.* EML cells were induced to form myeloid cells, as previously described. After 3 days of induction in media A (IMDM supplemented with 10% WEHI-3B conditioned supernatant, 10 µmol/L all-trans retinoic acid (ATRA), and 20% heat-inactivated horse serum), the cells were harvested, washed, and replated at 1.5 × 10⁵ cells per 3.5-cm bacterial plate in media B (IMDM supplemented with 250 U/mL murine GM-CSF [muGM-CSF] and 20% heat-inactivated horse serum) and 1.0% methylcellulose. Colonies were counted after 7 days of growth. The entire plate of cells was harvested after 9 days, and the number of CD11b⁺/Gr-1⁻ cells was determined by flow cytometry.

*EML proliferation assays.* Two hundred microliters of undifferentiated EML cells at 2.5 × 10⁵ cells/mL were plated in triplicate in 96 flat well plates. Twenty-four hours later, 0.5 µCi of [³H]TdR was added to each well, and 17 hours later, the cells were harvested. Cells were then washed and 0.01% sodium azide overnight at 4°C. The percentage of cells with a signal greater than twice the background was determined by a scintillation counter (1290 Rackbeta; EG&G Wallace, Gaithersburg, MD).

*EML apoptosis assays.* EML cells were induced to differentiate in media A as described above were replated at 5 × 10⁵ cells/mL in 400 µL media containing 250 U/mL muGM-CSF. Two days later, the cells were washed with fluorescence-activated cell sorting (FACS) buffer (1× phosphate-buffered saline [PBS], 0.2% bovine serum albumin [BSA], and 0.01% sodium azide) and incubated with ice-cold 70% ethanol for several hours. Cells were then washed with FACS buffer and then stained with 1× PBS/50 µg/mL RNase/10 µg/mL propidium iodide/0.01% sodium azide overnight at 4°C. The percentage of cells with a subdiploid DNA content was determined using flow cytometry.

*Retroviral transduction of bone marrow stem cells.* Retroviral supernatants were prepared by transiently transfecting the M1R1 or M1RBSAP plasmids into the Bosc23 packaging cell line. For infection of stem cells, 8- to 12-week-old female BALB/C mice were injected with 200 µL of 25 mg/mL 5-fluorouracil. Four days later, bone marrow was harvested and then cultured at 2.5 × 10⁶ cells/mL in 1 mL Dulbecco’s modified Eagle’s medium (DMEM) in the presence of 15% heat-inactivated fetal calf serum, 5% WEHI-3B conditioned supernatant, 40 ng/mL flt3 ligand, 200 ng/mL SCF, 12 ng/mL IL-3, and 20 ng/mL IL-6. Forty-eight hours later, the cells were cultured with 1 mL of MIGR or MIGRPax5A retroviral supernatant as described.

Twenty-four hours later, the cells were plated on γ-irradiated OP9 stromal cells in IMDM plus 10% heat-inactivated fetal calf serum and 50 µmol/L β-mercaptoethanol. Fifty days later, the cells were washed and recultured in media A. Three days later, the cells were washed and recultured in media B. Three days later, the cells were harvested by gentle pipetting and then analyzed by flow cytometry.

**RESULTS**

*Establishment of EML cells that ectopically express BSAP/Pax5A.* To test whether BSAP/Pax5A stably transduced into EML cell line, a bone marrow-derived multipotent progenitor that expresses some markers associated with the B-lymphoid lineage as well as a dominant-negative retinoic acid receptor that blocks spontaneous differentiation to the myeloid lineage at low levels of ATRA. In the presence of IL-3 and high levels of ATRA, these cells undergo myeloid lineage differentiation, which can be followed by the de novo acquisition of the myeloid-restricted markers CD11b and F4/80 (previous studies and M. Chiang and J. Monroe, unpublished observations). The addition of GM-CSF causes further maturation to CD11b⁺/Gr-1⁻ granulocytes (M. Chiang and J. Monroe, unpublished observations). Three EML cell clones (EML/Pax5A-2, EML/Pax5A-3, and EML/Pax5A-5) were subcloned and shown to express BSAP/Pax5A by EMA (Fig 1A and B). BSAP/Pax5A was observed as a single band in the stable transfectants but not in the parental EML cells.

Furthermore, this specific complex was disrupted by preincubation of the nuclear extracts with an antibody directed against the paired domain of murine BSAP/Pax5A and was expressed at approximately one fifth to one fourth the level in pre-B–cell lines (M. Chiang and J. Monroe, unpublished observations). Parental EML cells and 4 EML cells stably transduced with only the hygromycin resistance plasmid (EML/Hyg-1, EML/Hyg-2, EML/Hyg-3, and EML/Hyg-4) were used as negative controls to study the effects of BSAP/Pax5A on lineage potential and differentiation.

*Enforced expression of BSAP/Pax5A does not suppress myeloid cell differentiation of EML cells.* To test whether BSAP/Pax5A stably transduced into EML cell line, a bone marrow-derived multipotent progenitor that expresses some markers associated with the B-lymphoid lineage as well as a dominant-negative retinoic acid receptor that blocks spontaneous differentiation to the myeloid lineage at low levels of ATRA. In the presence of IL-3 and high levels of ATRA, these cells undergo myeloid lineage differentiation, which can be followed by the de novo acquisition of the myeloid-restricted markers CD11b and F4/80 (previous studies and M. Chiang and J. Monroe, unpublished observations). The addition of GM-CSF causes further maturation to CD11b⁺/Gr-1⁻ granulocytes (M. Chiang and J. Monroe, unpublished observations). Three EML cell clones (EML/Pax5A-2, EML/Pax5A-3, and EML/Pax5A-5) were subcloned and shown to express BSAP/Pax5A by EMA (Fig 1A and B). BSAP/Pax5A was observed as a single band in the stable transfectants but not in the parental EML cells. Furthermore, this specific complex was disrupted by preincubation of the nuclear extracts with an antibody directed against the paired domain of murine BSAP/Pax5A and was expressed at approximately one fifth to one fourth the level in pre-B–cell lines (M. Chiang and J. Monroe, unpublished observations). Parental EML cells and 4 EML cells stably transduced with only the hygromycin resistance plasmid (EML/Hyg-1, EML/Hyg-2, EML/Hyg-3, and EML/Hyg-4) were used as negative controls to study the effects of BSAP/Pax5A on lineage potential and differentiation.

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BSAP/Pax5A inhibits the expansion of EML-derived myeloid progenitors. To test whether BSAP/Pax5A affected further maturation/expansion of the EML cells subsequent to the initial stages of myeloid lineage differentiation, BSAP/Pax5A-expressing EML cells were induced with IL-3 and ATRA, washed, and recultured in methylcellulose media containing GM-CSF. GM-CSF supports the further maturation and expansion of myeloid precursors from progenitors that are dependent on IL-3. The colonies derived from BSAP/Pax5A-expressing EML cells appeared to be unusually small compared with those derived from EML/Hyg cells (M. Chiang and J. Monroe, unpublished observations). To quantitate this observation, the colonies were counted, aspirated, and analyzed by flow cytometry. BSAP/Pax5A-expressing colonies on average contained 60% to 95% fewer Gr-1+/CD11b+ cells than did the negative control colonies (Fig 4A). These data suggest that BSAP/Pax5A inhibits the ability of myeloid cells to expand in response to myeloid growth factors.

To confirm that BSAP/Pax5A was suppressing myeloid cell expansion, the BSAP/Pax5A-expressing EML cells were induced with IL-3 and ATRA, washed, recultured in media containing GM-CSF, and then subjected to proliferation and apoptosis assays. Proliferation of BSAP/Pax5A-expressing EML cells in response to GM-CSF was markedly reduced relative to non-BSAP/Pax5A-expressing controls (Fig 4B). This failure to expand is unlikely to result from a nonspecific suppression by BSAP/Pax5A on the expansion of EML cells, because undifferentiated BSAP/Pax5A-expressing EML cells have a slightly but significantly higher expansion rate than control cells in response to SCF (Fig 4C). Instead of expanding in response to GM-CSF, the BSAP/Pax5A-expressing EML cells underwent a 15-fold increase in the rate of apoptosis as compared with controls (Fig 4D). In combination with the colony size data, these data strongly suggest that BSAP/Pax5A inhibits the ability of myeloid cells to expand specifically in response to myeloid growth factors. This reduced proliferative capacity results in a marked reduction in the generation of more mature myeloid cells, an effect that is then exacerbated by the high apoptotic frequency in the BSAP/Pax5A-expressing population.

Although CD11b is an early appearing myeloid-restricted marker, it has been known to be expressed at a low level in other cell types, such as the fetal liver stem cells, activated/memory CD8+ T cells, and B1 B cells. Thus, we repeated the experiments shown in Fig 2A with the F4/80 antigen, a marker that appears later in myeloid differentiation than CD11b, but is more tightly restricted to the myeloid lineage (Fig 3). In a typical experiment, the EML/Hyg cells generated approximately 23% B220+/F4/80+ cells and 11% B220+/F4/80− cells, whereas EML/Pax5A cells generated 15% B220+/F4/80+ cells and 1% B220+/F4/80− cells. These data using the F4/80 marker confirm the experiments using the CD11b marker in Fig 2 and reinforce the point that BSAP/Pax5A does not inhibit myeloid differentiation, as evidenced by the successful de novo acquisition of the CD11b and F4/80 markers during differentiation of the EML/Pax5A cells. However, the negative effect of BSAP/Pax5A on myeloid cell maturation/expansion subsequent to the initiation of myeloid cell differentiation, as reflected in the diminished generation of B220+/CD11b+ cells and B220+/F4/80+ cells, was of particular interest.

BSAP/Pax5A was introduced retrovirally into primary bone marrow stem cells to verify the BSAP/Pax5A-mediated suppression of myeloid cell expansion in a nontransformed cell. Stem cells transduced with BSAP/Pax5A-expressing retrovirus were tested for the ability to differentiate to the myeloid lineage and expand during 5 days of culture on OP9 stromal cells, followed by 3 days of IL-3/ATRA and 3 days of GM-CSF (Fig 5A). The last 6 days of culture were designed to replicate the conditions used in the experiments described in Fig 4, which demonstrated that EML-derived myeloid precursors failed to expand in response to GM-CSF. OP9 stromal cells were derived from the op/op mouse, which lacks functional macrophage colony-stimulating factor (M-CSF). Because of this mutation, hematopoietic cultures are not artificially skewed predominantly toward macrophages, as has been seen with other stromal cell...
Rather, normal bone marrow hematopoiesis is more closely approximated, including the efficient generation of early CFU-GM myeloid precursors, erythroid cells, nonmacrophage lineage myeloid cells such as granulocytes, B lymphocytes, and mature macrophages. Thus, the OP9 system offers the unique advantage of a more physiologically relevant in vitro bone marrow culture system and more efficient generation of GM-CSF/IL-3 responsive precursors and nonmacrophage myeloid cells.

Retrovirus containing BSAP/Pax5A cDNA was called MIGRPax5A and was derived from the parental retrovirus MIGR. To differentiate transduced cells from nontransduced cells, these retroviruses express the GFP marker from an internal ribosomal entry site. Thus, cells that express GFP also coexpress BSAP/Pax5A as a bicistronic message. To correct for differences in the retroviral transduction efficiencies of hematopoietic stem cells from experiment to experiment, the GFP+ myeloid cells were normalized to the number of GFP+ c-Kit+/Sca-1+/Lin− cells 72 hours after transduction (which allows time for GFP to be fully expressed). The c-Kit+/Sca-1+/Lin− bone marrow subset is highly enriched for the most primitive hematopoietic stem cells, which have long-term multilineage reconstitution potential. Normalization to this population ensures that any reduction in the number of GFP+ myeloid cells generated is not due to a lower number of stem cells that were successfully transduced but is rather due to the myelosuppressive effect of BSAP/Pax5A.

In all experiments, the level of GFP expression in MIGRPax5A-transduced hematopoietic stem cells (identified by sca-1+/c-kit+/lin−) was generally lower than that in MIGR-transduced hematopoietic stem cells. In a typical experiment, the mean fluorescence intensity of GFP+ hematopoietic stem cells transduced with MIGRPax5A was 41.37 ± 3.83, whereas the mean fluorescence intensity of GFP+ hematopoietic stem cells transduced with MIGR was 141.68 ± 0.02. This difference unlikely reflects uneven promoter activity between the 2 populations, but rather is likely the result of the integration of a...
large cDNA insert upstream of the GFP marker in the MIGR Pax5A retrovirus but not in the MIGR retrovirus (Chiang et al, unpublished observations).

Because the expression levels of GFP were different in the control and experimental cells, we determined the effect of GFP and retroviral transduction on myeloid differentiation. MIGR-transduced stem cells were cocultured with nontransduced stem cells and found to generate approximately 50% of the Gr-1<sup>+</sup> cells and 50% of the F4/80<sup>+</sup> cells present in the cultures on day 8 and on day 14 (Fig 5B). Thus, the frequency of transduced myeloid cells did not change over time, verifying that neither expression of GFP nor retroviral transduction had any measurable effects on myelopoiesis.

BSAP/Pax5A does not inhibit myeloid differentiation of primary stem cells but does inhibit the expansion of myeloid progenitors. Primary bone marrow stem cells were transduced with either MIGR or MIGR Pax5A retrovirus and then cultured in vitro according to the scheme in Fig 5A to generate myeloid cells. When cultured without myeloid growth factors during the first 8 days, MIGR Pax5A-transduced stem cells and control MIGR-transduced stem cells generated approximately equal numbers of Gr-1<sup>+</sup> and F4/80<sup>+</sup> cells (days 6 and 8 of culture, Fig 6A and B). However, upon addition of IL-3 and ATRA for the next 3 days, followed by GM-CSF for another 3 days, which simulated the experimental conditions used to differentiate EML cells in Fig 4, MIGR Pax5A-transduced stem cells generated 60% to 85% fewer Gr-1<sup>+</sup> cells (Fig 6A) and 60% to 75% fewer F4/80<sup>+</sup> cells (Fig 6B) than did MIGR-transduced controls when analyzed on day 14 of culture. This suppression of myeloid cell generation was not observed with stem cells that were cultured without growth factors for the entire 2 weeks of culture (Fig 6A and B and M. Chiang and J. Monroe, unpublished observations), but rather was observed only when the stem cells were cultured in the presence of myeloid growth factors. These data suggest that BSAP/Pax5A does not completely suppress myeloid cell differentiation, but does suppress myeloid cell expansion to myeloid growth factors, which is consistent with our interpretation of the EML studies.

DISCUSSION

During hematopoiesis, developing cells increasingly become more restricted with regard to their potential to develop along multiple lineages. Lineage-determinant transcription factors are thought to drive this commitment process by upregulating the genes of a specific lineage and downregulating genes specific to other lineages. For example, the transcription factor E12 is thought to prevent B cells from acquiring myeloid characteristics, because ectopic expression of E12 in a macrophage cell line upregulated B-cell genes such as A<sub>5</sub>, BSAP/Pax5A, EBF, and IL-7R<sub>a</sub> and downregulated myeloid-specific genes CD11b and c-fms. Because BSAP/Pax5A is upregulated by E12, it itself a transcription factor that is thought to upregulate B-cell–associated genes, and is required for B-cell commitment, as determined by gene deletion studies, it was of particular interest to determine if BSAP/Pax5A like E12 had B-cell–determinant properties.

To test whether BSAP/Pax5A had B-cell–determinant properties, BSAP/Pax5A was constitutively expressed in the EML cell. Surprisingly, BSAP/Pax5A-expressing EML cells did not
upregulate the B-cell–specific genes CD19 or mb-1 (M. Chiang and J. Monroe, unpublished observations). This finding suggested that BSAP/Pax5A, although required for the expression of CD19 and mb-1 in pre-B cells, as demonstrated by loss of function analysis, is not sufficient for their expression, presumably because of the absence of requisite cofactors or presence of suppressive factors in the EML cell line. This finding is consistent with the E12 study discussed earlier, which found that, although BSAP/Pax5A was upregulated in EBF-expressing 70Z/3 macrophages, only one B-cell gene, λ5, was also upregulated. Furthermore, BSAP/Pax5A is unlikely to be responsible for this λ5 upregulation, because gain-of-function/
loss-of-function genetic studies have shown that λ5 is a target gene of EBF, but not of BSAP/Pax5A.24,31,62 Therefore, in EML cells and 70Z/3 macrophages, BSAP/Pax5A is not sufficient to upregulate the B-cell genes that were examined. BSAP/Pax5A-expressing EML cells differentiated to the myeloid lineage in response to IL-3 by successfully acquiring de novo the myeloid-restricted markers CD11b and F4/80. However, these early myeloid cells then failed to expand in response to GM-CSF. To confirm these results in a nontransformed cell, BSAP/Pax5A was retrovirally transduced into primary bone marrow stem cells. When cultured in vitro, BSAP/Pax5A-transduced stem cells successfully differentiated to the myeloid lineage. However, the addition of myeloid growth factors to these cultures showed a defect in cell expansion. Taken together, the EML cell studies and primary bone marrow studies share consistent results and thus provide strong evidence that BSAP/Pax5A, although unable to completely suppress myeloid lineage differentiation, does suppress the expansion response to myeloid growth factors, a characteristic myeloid trait acquired shortly after the initiation of myeloid commitment.

In this study, BSAP/Pax5A was ectopically expressed in stem cells to demonstrate that BSAP/Pax5A can suppress myeloid cell expansion. However, in vivo, BSAP/Pax5A is not expressed in multipotential stem cells, but later in the fraction A1 stage of B-cell development after B-cell commitment has already occurred.15,17,23 It is formally possible that BSAP/Pax5A is expressed in rare multipotential progenitors at low levels undetectable by reverse transcriptase-polymerase chain reaction (RT-PCR) and that a higher level of BSAP/Pax5A expression in these cells would prevent them from responding to myeloid growth factors. However, because this possibility...
seems remote due to the high sensitivity of PCR, the finding of BSAP/Pax5A-mediated suppression of myeloid expansion from stem cells more likely supports a role for BSAP/Pax5A in preventing B cells from responding to myeloid growth factors. This effect may in part maintain commitment to the B lineage as some myeloid growth factors, notably IL-3 and GM-CSF, have been shown to convert certain pre-B cell lines to the myeloid lineage.

Although early B cells in vivo seem to acquire some myeloid traits, such as low levels of CD11b, they are prevented from expanding in response to myeloid growth factors and further maturation, perhaps in part due to BSAP/Pax5A expression. Because BSAP/Pax5A is not sufficient to completely prevent maturation down the myeloid lineage, especially in the absence of growth factors, there probably exists additional lineage-determinant genes that act singly or together with BSAP/Pax5A to suppress myeloid differentiation. These genes are likely to be target genes of E12 in view of previous studies already discussed in which ectopic expression of E12 in a macrophage cell line completely suppressed all myeloid characteristics that were reported.

Whether BSAP/Pax5A is sufficient to suppress myeloid expansion in vivo is currently being studied by generating mice transgenic for BSAP/Pax5A. This question is particularly interesting, because studies involving myeloid growth factor–deficient mice have indicated the existence of multiple redundant mechanisms that can drive myelopoiesis. These mice display either normal or reduced (but never abrogated) myelopoiesis. For example, IL-3/GM-CSF/IL-5R–deficient mice, IL-3–deficient mice, and GM-CSF–deficient mice all display normal steady-state myelopoiesis. Granulocyte colony-stimulating factor (G-CSF)–deficient mice display neutropenia, which is somewhat exacerbated when the GM-CSF gene is additionally deleted. M-CSF–deficient mice have fewer macrophages and osteoclasts, but these cells accumulate to near normal levels in older mice. Thus, the number of growth factor responses that can be suppressed by BSAP/Pax5A will dictate the degree to which enforced expression of BSAP/Pax5A will suppress myelopoiesis in vivo. We predict, based on our studies reported here, that IL-3–mediated responses may remain largely unaffected in the presence of BSAP/Pax5A expression.

How BSAP/Pax5A suppresses myelopoiesis has proven elusive in part because the effect of BSAP/Pax5A on the function and expression of the downstream mediators of myeloid growth factor signal transduction cascade is unknown. Because the paired domain of BSAP/Pax5A can bind the transcription factor Ets-1, which is itself involved in the regulation of many myeloid genes, it was postulated that BSAP/Pax5A could suppress Ets-1–mediated transactivation. However, transient transfection of BSAP/Pax5A failed to inhibit Ets-1–mediated transactivation of the CD18 promoter. Currently, the effect of BSAP/Pax5A on the expression of the various myeloid growth factor receptors, such as IL-3Rα, β-common, IL-3Rβ, and GM-CSFRα, is being studied.

These studies expand on the results of the earlier E12 study by using cells that are growth-factor dependent, early in development, and nontransformed. Using growth-factor–dependent cells instead of the growth factor–independent macrophage cell line 70Z/33 has shown that BSAP/Pax5A affects the growth response of cells to myeloid growth factors. Using progenitor stem cells instead of mature macrophages has shown...
that BSAP/Pax5A can act in a model of early development to prevent the acquisition of the myeloid growth factor response, one of the earliest appearing myeloid-specific characteristics after myeloid commitment. This aspect of our studies is relevant, because anecdotal reports of lineage switching to myeloid cells in vitro have almost always involved cells early in development when plasticity is thought to be greatest.6-8 In this regard, our studies provide additional evidence that suppressive factors, which prohibit the acquisition of lineage-specific traits, may be integral to maintaining B-lineage commitment as developing cells chose between the B-cell and myeloid fates.

ACKNOWLEDGMENT

The authors thank Dr M. Busslinger for providing the pmBSAP-2 plasmid and the anti-paired domain antisera, Dr S. Tsai for providing the EML and BKH/MKL cell lines, Dr F. Melchers and A. Groenewegen for providing the J558-IL-7 cell line, Dr W. Pear for providing the MigeR1 plasmid and Bosc23 cell line, P. Sandel and Dr M. Atchison for critical reading of the manuscript, and Dr L. King and Dr L. Xu for assistance in designing the experimental protocols.

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BSAP/Pax5A Expression Blocks Survival and Expansion of Early Myeloid Cells Implicating Its Involvement in Maintaining Commitment to the B-Lymphocyte Lineage

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