The SWI/SNF-like BAF Complex Is Essential for Early B Cell Development

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The SWI/SNF-like BAF Complex Is Essential for Early B Cell Development

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During the process of B cell development, transcription factors, such as E2A and Ebf1, have been known to play key roles. Although transcription factors and chromatin regulators work in concert to direct the expression of B lineage-specific genes, little is known about the involvement of regulators for chromatin structure during B lymphopoiesis. In this article, we show that deletion of Srg3/mBaf155, a scaffold subunit of the SWI/SNF-like BAF complex, in the hematopoietic lineage causes defects at both the common lymphoid progenitor stage and the transition from pre–pro-B to early pro-B cells due to failures in the expression of B lineage-specific genes, such as Ebf1 and Il7ra, and their downstream target genes. Moreover, mice that were deficient in the expression of Brg1, a subunit of the complex with ATPase activity, also showed defects in early B cell development. We also found that the expression of Ebf1 and Il7ra is directly regulated by the SWI/SNF-like BAF complex. Thus, our results suggest that the SWI/SNF-like BAF complex facilitates early B cell development by regulating the expression of B lineage-specific genes. The Journal of Immunology, 2012, 188: 000–000.

B

lood cells that constitute the three major hematopoietic lineages—erythroid, myeloid, and lymphoid—originate from pluripotent hematopoietic stem cells (HSCs) in the fetal liver and the bone marrow after birth (1, 2). HSCs differentiate into multipotent progenitors that have lost the capacity for self-renewal but retain the potential for multilineage differentiation; these cells, in turn, give rise to lymphoid-primed multipotent progenitors (LMPPs) (3). LMPP populations express high levels of the tyrosine kinase receptor Flt3 (Flk2) and have little potential for erythro-megakaryocyte differentiation (4). LMPPs are followed by common lymphoid progenitors (CLPs), which are the precursors of B lymphocytes, NK cells, dendritic cells, and T lymphocytes (5). After the CLP stage, B lineage differentiation progresses to pre–pro-B (Fr. A) cells by the upregulation of CD45R/B220 expression on the surface, but not yet of CD19, CD24, and BP-1 (6, 7). Pre–pro-B cells give rise to early pro-B (Fr. B) cells, which express CD19 and undergo a complete D_{HJ} rearrangement at the Ig H chain locus. The cells with the rearranged IgH locus are considered committed B lineage cells. Late pro-B (Fr. C) cells expressing pre-BCRs on the surface, which results in expression of the BCR (9).

B cell specification is controlled by cell-intrinsic transcription factors, such as E2A, paired box protein 5 (Pax5), and early B cell factor, as well as various cytokines produced from the local microenvironment, including IL-7. B cell generation is compromised in Il7ra-deficient mice, which show a decrease in CLP numbers and impairment in differentiation into the early pro-B cell stage, even though it is not clear whether IL-7 signaling is absolutely required for the generation of these cells (10, 11). E2A and Ebf1 act together as critical transcription factors to generate early pro-B cells from CLPs during B cell differentiation. E2A^{−/−} mice display significantly fewer LMPPs and CLPs and show defects in the transition from pre–pro-B (Fr. A) to early pro-B cells (Fr. B) (12, 13). B cell development in Ebf1-deficient mice is largely arrested at the pre–pro-B stage, similar to E2A^{−/−} mice. These mice fail to express B lineage-specific genes, including Rag1, Rag2, Ig-α (Cd79a, mb-1), Ig-β (Cd79b, B29), α5 (Igl1), and VpreB1 (VpreB1), and they do not initiate IgH recombination (14, 15). E2A and Ebf1 also induce Pax5, which is a transcriptional regulator that is required for B cell commitment and maintenance but not for B lineage specification. In addition, the ectopic expression of Ebf1 in HSCs from E2A^{−/−} mice and pre–pro-B cells from Il7ra^{−/−} mice rescues B cell development in vitro (10, 11, 14, 16).

Epigenetic modification and dynamic reorganization of the local chromatin structure are essential to poised lineage-specific genes for expression or repression. The key event in this process is the recruitment of chromatin-remodeling complexes, which mediate the active orchestration of transcriptional activation or silencing. Thus, the spatiotemporal regulation of chromatin reorganization can have significant impacts on the development and differentiation of the hematopoietic process. The mammalian SWI/SNF-like BAF
complex mobilizes nucleosomes by using the energy from ATP hydrolysis and is required for the expression of a number of genes (17, 18). The SWI/SNF-like BAF complex is composed of multiple subunits that are conserved through evolution. Brg1 with ATPase catalytic activity, Baf155 (also known as Srg3), Baf170, and Baf47 (also known as Snf5) are sufficient to induce remodeling activity in vitro; therefore, these subunits are considered the core subunits of the complex (17). Srg3 is a murine homolog of Swi3 in yeast and Baf155 in humans (19). Srg3/mBAf155 controls the stability of the SWI/SNF-like BAF complex and, thereby, serves as a scaffold protein (20, 21). It was also shown that the chromatin-remodeling activity is enhanced in transgenic mice overexpressing Srg3/mBAf155 (22).

It is still unknown how the process of hematopoiesis, particularly that of early lymphoid lineage differentiation, is epigenetically controlled, although there are a few studies suggesting its role during myeloid lineage differentiation (23, 24) and T cell development (25–27). In this study, we show that the SWI/SNF-like BAF complex plays a critical role in the process of early B cell lineage commitment by facilitating the expression of Ebf-1 and Il7ra.

Materials and Methods

Mice

Targeting constructs and strategy for the generation of conditional Srg3/mBAf155 knockout mice are described in Fig. 1D. Mice with loxP sites flanking exon 4 of Srg3 (Fig. 3) were backcrossed onto C57BL/6 mice for at least eight generations and were crossed with Mx1-Cre mice for deletion of Srg3 at various stages of the hematopoietic lineage. Mx1-Cre mice were purchased from The Jackson Laboratory. All mice were bred and maintained in specific pathogen-free barrier facilities at Seoul National University and were used following protocols approved by Institutional Animal Care and Use Committees of Seoul National University.

Administration of polyinosinic-polycytidlic acid

For the deletion of Srg3, 6–8-wk-old control (Srg3<sup>afl0</sup> or Srg3<sup>afl/afl</sup>) and Srg3<sup>mice</sup> mice crossed with Mx1-Cre mice were injected with 250 μg polyinosinic-polycytidlic acid (pdpC; GE Healthcare) three times every other day i.p. Deletion efficiency was determined by PCR or Western blot analysis.

DNA constructs

For sequence-specific knockdown of Srg3 and Brg1, target sequences (Srg3, 5′-CATCCTTTGGTATTAAAGG-3′ and Brg1, 5′-CGCTCAAAGTGTTAGATCAATA-3′) were cloned into MDH1–PGK–GFP2-0 (kindly provided by Dr. Chang-Zhen Cheng, Stanford University) or MCV-TL (Invitrogen) vectors (Invitrogen) using the Gateway cloning system (Invitrogen). Myc-tagged Baf57ΔN mutant and Myc-tagged Srg3 were cloned into pMIG vectors (kindly provided by Dr. Warren S. Pear, University of Pennsylvania).

Flow cytometry and cell sorting

Single-cell suspensions were prepared from the bone marrow (femur and tibia), spleen, lymph node, and thymus by passing through a cell strainer to remove cell debris. To deplete the RBCs, an equal volume of ACK RBC lysis buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, and 0.1 mM EDTA) was added to the cells and incubated on ice for 5 min. Then up to 10 ml PBS was added to the cells and incubated for 10 min with rocking at 37°C, followed by washing twice with ice-cold PBS. Then, cells were lysed for 10 min with SDS lysis buffer. The samples were sonicated to an average length of 100–500 bp, and the sonicated cell supernatants were diluted 10-fold in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1], and Protease Inhibitor mixture [Roche]). Then, lysates were precleared for 1 h with salmon sperm DNA/protein-A agarose (50% slurry), followed by incubation overnight at 4°C with isotype-control anti-rabbit IgG (Upstate), anti-Brg1, anti-Srg3, or anti-H3K9<sup>ac</sup> (Upstate). The beads were washed sequently once with a low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM SDS, 2 mM Tris-HCl [pH 8.1], 150 mM NaCl), a high-salt buffer (0.1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]) and twice with 10 mM Tris (pH 8) plus 1 mM EDTA. Cross-linked chromatin–histone complexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). After washing and elution steps, cross-linking was reversed by heating at 65°C for 4 h, followed by proteinase K treatment for an additional 1 h. DNA was purified with a QiAquick spin kit (QiAGEN, Seoul, Korea), and DNA was analyzed by PCR with primers within the Ebf1 or Il7ra promoter regions. The sequences of the primer pairs are as follows: Ebf1 promoter regions, P1, 5′-TCGACTGTCGATCACAGTCCCTTGA-3′, P2, 5′-ATCGATGAGTGGCCTTGTCC-3′, P3, 5′-CTACGACACCGACGTCTCC-3′, and P4, 5′-GGGCTGAGTTCTTCTTGGTG; Il7ra promoter regions, P1, 5′-CACATTCGCTAAGAATGACG-3′ and P2, 5′-GGAAGCCCTGCTTGTACGTG-3′; and Gapdh intron regions, sense, 5′-TTGCTTCTCCGCCCCATG-3′ and antisense, 5′-GGGTTTATCTCTTCCTTCT-3′.

Semiquantitative and quantitative RT-PCR

Total RNA was purified from cells with TRIzol reagent, following the manufacturer’s instructions (Invitrogen). Equivalent quantities of total RNA were reverse transcribed with SuperScript III (Invitrogen), and diluted cDNAs were analyzed by semiquantitative PCR. PCR products were separated on agarose gels and visualized by ethidium bromide. SYBR Green PCR mix (Applied Biosystems) or TaqMan Master mix (Applied Biosystems) was used for quantitative real-time PCR analysis (primer sequences are available upon request), and the results were quantified with StepOnePlus (Applied Biosystems).

Western blot analysis

Whole-cell lysates were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Blotted proteins were detected using Abs to SRG3, BRG1, FLAG epitope (AC-15; Sigma), or FLA3 (epitope (M2; Sigma) in TBST (150 mM NaCl, 10 mM Tris-Cl [pH 8], 0.5% Tween-20) containing 5% low-fat milk. Antiserum against SRG3 and BRG1 were raised in rabbits in our laboratory.

Southern blot analysis

Genomic DNA preparation and Southern blotting were done mainly as described (28). Briefly, DNA was purified from B220<sup>-</sup>CD4<sup>+</sup> Ig<sup+m</sup> pro-B cells obtained from pIpC-treated control and Mx1-Srg3<sup>afl</sup> mice by phenol-chloroform extraction and resuspended in 10 mM Tris (pH 8) and 1 mM EDTA. PCR analyses of Ig genes were performed with published primers. The PCR product of HSS3 was used as a loading control. DNA products amplified by PCR were separated on 1% agarose gels by electrophoresis and transferred to Hybond-N membranes (Amersham). Southern blotting was probed with 32P-labeled oligonucleotides.

Chromatin immunoprecipitation assay

For the chromatin immunoprecipitation (ChIP) assay, a solution of 37% (v/v) formaldehyde was added to the culture medium of PD36 cell line or primary pro-B cells to a final concentration of 1%, and cells were incubated for 10 min with rocking at 37°C, followed by washing twice with ice-cold PBS. Then, cells were lysed for 10 min with SDS lysis buffer. The samples were sonicated to an average length of 100–500 bp, and the sonicated cell supernatants were diluted 10-fold in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1], and Protease Inhibitor mixture [Roche]). Then, lysates were precleared for 1 h with salmon sperm DNA/protein-A agarose (50% slurry), followed by incubation overnight at 4°C with isotype-control anti-rabbit IgG (Upstate), anti-Brg1, anti-Srg3, or anti-H3K9<sup>ac</sup> (Upstate). The beads were washed sequently once with a low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM SDS, 2 mM Tris-HCl [pH 8.1], 150 mM NaCl), a high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), and LiCl washing buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]) and twice with 10 mM Tris (pH 8) plus 1 mM EDTA. Cross-linked chromatin–histone complexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO<sub>3</sub>). After washing and elution steps, cross-linking was reversed by heating at 65°C for 4 h, followed by proteinase K treatment for an additional 1 h. DNA was purified with a QiAquick spin kit (QiAGEN, Seoul, Korea), and DNA was analyzed by PCR with primers within the Ebf1 or Il7ra promoter regions. The sequences of the primer pairs are as follows: Ebf1 promoter regions, P1, 5′-TCGACTGTCGATCACAGTCCCTTGA-3′, P2, 5′-ATCGATGAGTGGCCTTGTCC-3′, P3, 5′-CTACGACACCGACGTCTCC-3′, and P4, 5′-GGGCTGAGTTCTTCTTGGTG; Il7ra promoter regions, P1, 5′-CACATTCGCTAAGAATGACG-3′ and P2, 5′-GGAAGCCCTGCTTGTACGTG-3′; and Gapdh intron regions, sense, 5′-TTGCTTCTCCGCCCCATG-3′ and antisense, 5′-GGGTTTATCTCTTCCTTCT-3′.
In vitro B cell-differentiation assay pro-B cell culture

The murine OP9 stromal cells were maintained in α-MEM supplemented with 5% FBS. For in vitro culture, HSCs or pro-B cells were isolated from plpC-treated control and Mx1-Srg3<sup>fl/fl</sup> mice by FACS (The Journal of Immunology II (2012) B Cell Biologies) at day 5 or 10 after the final induction and cocultured (BD Biosciences) with gamma-irradiated (30 Gy) OP9 cells in RPMI 1640 supplemented with 10% FBS and cytokines. Stem cell factor (25 ng/ml), Flk2/Fli3 ligand (10 ng/ml), and IL-7 (20 ng/ml; all from PeproTech) were used for the culture of hematopoietic progenitor cells, and IL-7 (20 ng/ml; PeproTech) only was used for the culture of pro-B cells. After 3–6 d of culture, both suspended and adherent cells were harvested, stained with lineage markers, including Gr-1, B220, and CD19, and analyzed using a FACS Canto II (BD Biosciences).

Retroviral infection and bone marrow reconstitution

Bone marrow cells were flushed out of femurs and tibias after i.p. injection with 5-fluorouracil at 150 μg/kg body weight for 4 d. RBCs were removed using ACK lysis buffer (0.15 M NH₄Cl, 10 mM KHO₂C, and 0.1 mM EDTA). Then, the cells were cultured overnight in complete RPMI 1640 (10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 50 μg/ml 2-ME, 1% Glutamax [Life Technologies], 1% nonessential amino acid [Life Technologies], and 1% sodium pyruvate [Life Technologies]) containing 5 ng/ml IL-3, 10 ng/ml IL-6, 50 ng/ml stem cell factor, and 50 ng/ml Flt3 ligand (all from PeproTech) before the initial retroviral infection. The packaging Phoenix-ecco cells were transfected with a control empty vector and Brst1-short hairpin RNA (shRNA) using a standard calcium phosphate protocol to generate retroviruses. After 48 h, the viral supernatants were harvested and used to spin-infect HSC-enriched cells for 1.5 h at 2500 rpm and 30°C in the presence of 10 μg/ml polybrene. This procedure was repeated three times, followed by the injection of 2–3 × 10<sup>6</sup> infected cells via the tail vein into each recipient that was lethally gamma-irradiated with 1100 rad at 2-h intervals (137Cs); GC 3000 Elan; MDS Nordion.

Statistical tests

Two-tailed Student t tests were used to calculate p values where indicated; p values < 0.05 were considered statistically significant.

Results

The SWI/SNF-like BAF complex is required for normal B cell development

We first examined the expression pattern of the core components of the SWI/SNF-like BAF complex in different lineage cells. Hematopoietic progenitor-enriched populations (Lin<sup>-</sup>) and B cells expressed Srg3<sub>3bnq155</sub> (hereafter referred to as Srg3) protein highly expressed with erythroid and myeloid lineage cells (Fig. 1A). We also analyzed Srg3 transcript levels throughout the lymphoid lineage differentiation. Srg3 was highly expressed in HSCs (LSKs), CLPs, and pre-pro-B (Fr. A) cells (Fig. 1B). Srg3 expression was gradually increased during B cell maturation from early pro-B cells to pre-B cells. Brst1, a core subunit of the SWI/SNF-like BAF complex with ATPase activity, also showed a similar expression pattern (Fig. 1C).

Previous studies showed that the complete deletion of Srg3 resulted in embryonic lethality during the peri-implantation stage (29). To overcome this restriction and examine the function of Srg3 in hematopoietic lineage differentiation, we generated conditional Srg3<sup>-</sup> mice by crosses with Mx1-Cre transgenic mice, in which the deletion of Srg3 occurred in hematopoietic cells (30). After treatment with plpC, we confirmed the deletion of Srg3 alleles in genomic DNAs (Fig. 1G). We also confirmed a drastic reduction in the protein levels in B cells purified from the bone marrow and spleen of Mx1-Srg3<sup>-</sup> mice compared with control (Fig. 1H, I). We then analyzed the effect of Srg3 deficiency on the hematopoietic system, especially B cell development, by comparing Mx1-Srg3<sup>-</sup> mice with control littermates. Srg3-deficient mice showed a drastic decrease in the percentage of B220<sup>+</sup>CD19<sup>+</sup> B cells in the bone marrow (14.2± 1.21% compared with control (26.09 ± 1.456%)) (Fig. 2A). In accordance with the reduction in B cell frequency, the B cell numbers were also greatly reduced (40.94 ± 4.493 versus 17.51 ± 3.233 × 10<sup>5</sup>) (Fig. 2B). In the spleen, B cell generation was also defective in the absence of Srg3 (Fig. 2C). Moreover, Srg3-deficient mice showed a significant reduction in B cell numbers to half that of control mice (Fig. 2D). In addition, interestingly, the percentage of follicular (FO) B cells was significantly decreased, whereas the frequency of the marginal zone (MZ) B cells was increased in Mx1-Srg3<sup>-</sup> mice (Fig. 2E). The numbers of FO B cells were also reduced by as much as half (Fig. 2F). These results indicate that Srg3 is essential for proper B cell development in both the bone marrow and the periphery.

Impairment of B-lineage potential in Srg3-deficient lymphoid progenitors

Srg3 is expressed at the early stage of hematopoietic precursor cells. Thus, we investigated the function of Srg3 in HSC maintenance and the development of lymphoid progenitors. The frequency of the Lin<sup>-</sup>IL-7Rα<sup>+</sup>c-Kit<sup>+</sup>Sca-1<sup>-</sup> (LSK) subset was comparable to that of control mice. The subsets of long-term HSCs, short-term HSCs, and the LMPPs were not significantly altered in the absence of Srg3, although a mild decrease in LMPPs was observed (data not shown). The frequency and cell numbers of traditional Lin<sup>-</sup>IL-7Rα<sup>+</sup>c-Kit<sup>+</sup>Sca-1<sup>-</sup> CLPs, which are the precursors of B and T cells, were not significantly altered by Srg3 deletion (Fig. 3A, 3B). The CLP population is heterogeneous, and genes involved in early B cell development, including Ebf1 and Rag1, are highly transcribed in B cell-restricted CLPs (31–34). Interestingly, CLPs highly expressing AA4.1 on the surface were significantly reduced in Mx1-Srg3<sup>-</sup> mice compared with control mice (Fig. 3C). AA4.1<sup>+</sup> CLPs are more potent in the generation of B lineage cells, but not myeloid lineage cells, than are traditional CLPs (35, 36). To assess whether the Srg3-deficient CLPs have normal lymphoid potential, we analyzed the expression of lineage-specific genes in these CLPs. Notably, CLPs purified from Srg3<sup>-</sup> mice showed less Ebf1 expression compared with those from control mice, whereas the expression of jh3 remained unchanged (Fig. 3D). It was reported that Ebf1-deficient CLPs express very low levels of B lineage-specific genes (37). We also found that expression of B lineage-specific genes, such as Cd79a (mb-1), Cd79b (B29), Rag1, Rag2, and FoxO1, were significantly reduced in Srg3-deficient CLPs (Fig. 3E).

Pro-B cell development was defective in the absence of BAF complex

Although Srg3-deficient lymphoid progenitors showed defects in the expression of genes that are associated with the development of early B-lineage cells, a considerable number of CLPs was still detected in the absence of Srg3. Therefore, we assessed the cell specification processes in Mx1-Srg3<sup>-</sup> mice. Consistent with the lower numbers of B cells in Srg3-deficient mice, B cell development was blocked at the early stage of B-lineage cells in the bone marrow of Mx1-Srg3<sup>-</sup> mice. Analysis of Mx1-Srg3<sup>-</sup> mice showed that most of the B cells were arrested at the pro-B cell stage, resulting in a significant reduction in the frequency of pre-B cells and immature B cells compared with those from control mice (Fig. 4A, 4B). The number of pro-B cells was decreased in Mx1-Srg3<sup>-</sup> mice, even though the Srg3-deficient mice had a higher percentage of pro-B cells (Fig. 4C). To further define the defective stage in pro-B cell development in Srg3-deficient mice, we subcategorized the pro-B cells using the differential cell surface markers BP-1 and CD24 (HSA) or CD19. The proportion of CD19<sup>+</sup>...
cells was severely reduced in the pro-B cells of Mx1-Srg3 fl/fl mice (Fig. 4D). In addition, Mx1-Srg3 fl/fl mice had significantly lower frequencies of early pro-B cells (Fr. B) and late pro-B cells (Fr. C) but higher frequencies of pre–pro-B (Fr. A) cells than did the control mice (Fig. 4D, 4E). In fact, the numbers of early and late pro-B cells were also severely decreased, whereas the number of

FIGURE 1. Generation of Srg3 conditional knockout mice. (A) Western blot analysis of SRG3 expression levels from the indicated subsets in the bone marrow. Expression of β-actin was used as a loading control. Representative results of three independent experiments are shown. (B) Relative expression of Srg3 mRNA in sorted lymphoid lineage compartment in the bone marrow using TaqMan real-time PCR analysis. The expression of gapdh served as a loading control. Data are shown as mean ± SEM (n = 3). (C) Relative expression of Brg1 mRNA in sorted lymphoid lineage compartment in the bone marrow using TaqMan real-time PCR analysis. The expression of gapdh served as a loading control. Data are shown as mean ± SEM (n = 3). (D) Schematic representation of the targeting strategy. (E) Southern blot analysis of genomic DNA from wild-type (+/+) and targeted (flox/+) embryonic stem cells with a probe overlapping exon 3 reveals a different HindIII (7.5 versus 10.2 kb) fragment in the targeted cells. (F) PCR analysis of tail DNA from wild-type (+/+), heterozygous (flox/+) and homozygous (flox/flox) mice using primers in introns 3 and 4 [P1 and P2 or P3 and P4, as indicated by arrows in (D)]. (G) Representative PCR analysis of bone marrow cells after pIpC treatment of control and Mx1-Srg3 fl/fl mice. (H) Western blot analysis of SRG3 expression in B220+CD19+ B cells of bone marrow purified from pIpC-treated control, Mx1-Srg3 fl/fl, and Mx1-Srg3 homo mice. (I) Western blot analysis of SRG3 expression in splenic B220+CD19+ B cells purified from pIpC-treated control and Mx1-Srg3 homo mice. B220+, B lineage cells; B, BamHII; DT-A, diphtheria toxin A-chain gene; Gr-1+Mac-1+, myeloid lineage cell population; H, HindIII; Lin−, hematopoietic progenitor-enriched population; neo, neomycin-resistance gene; Ter-119−, erythroid lineage cells.
pre–pro-B cells remained largely unchanged (Fig. 4F). In previous studies, Fr. A population was shown to be heterogeneous and to contain AA4.1+ and AA4.12 cells. AA4.1+ Fr. A cells are subdivided into A1 and A2, and they are considered the earliest B-lineage population (7). Mx1-Srg3 fl/fl mice showed significantly fewer AA4.1+ Fr. A cells compared with the control mice (Fig. 4G). Overall, Srg3 deficiency leads to a defect in the transition from pre–pro-B to early pro-B cells.

The deletion of Brg1 in HSCs also leads to defects in early pro-B cell development

Because the impairment of B cell development in Mx1-Srg3 fl/fl mice appeared to be due to a functional deficiency of the SWI/SNF-like BAF complex, it was likely that the inactivation of other subunits of the complex would also lead to similar defects in B cell development. To confirm this hypothesis, we generated Brg1-deficient chimeras using Brg1-shRNA (Fig. 5A, 5B). The deficiency in Brg1 also significantly decreased the frequency of B cells in the bone marrow (Fig. 5C). The frequencies of pre-B, immature B, and mature B cells were reduced in Brg1-deficient chimeras (Fig. 5D). Furthermore, most of the B cells were arrested at the stage of pre–pro-B cells, similarly to Srg3-deficient mice (Fig. 5E). These results indicate that proper activity of the SWI/SNF-like BAF complex is required for normal B cell development.

The SWI/SNF-like BAF complex is required for IL-7Rα expression and pro-B cell survival

Having shown that early pro-B cell development is blocked in the absence of Srg3/mBaf155, we speculated that pro-B cells of Mx1-Srg3 fl/fl mice would be defective in IL-7R signaling, which leads to more cell death and less proliferation (38, 39). In fact, we found that
pro-B cells from Mx1-Srg3fl/fl mice failed to upregulate IL-7Rα expression on the surface, especially at the early pro-B cell stage, whereas the control pro-B cells showed a high level of expression (Fig. 6A, 6B). In addition, we found that the Srg3-deficient pro-B cells showed a much higher frequency of Annexin V+ apoptotic cells compared with the control pro-B cells (Fig. 6C). These results indicate that pro-B cells lacking Srg3, especially early pro-B cells, are more susceptible to apoptosis, which may explain, at least in part, the severely reduced numbers of early pro-B cells in the bone marrow of Mx1-Srg3fl/fl mice. Notably, pro-B cells purified from Mx1-Srg3fl/fl mice showed higher levels of proapoptotic Bim expression and lower levels of antiapoptotic Bcl-xL expression (Fig. 6D).

To analyze the responsiveness of pro-B cells to IL-7 signaling, pro-B cells that were isolated from plpC-treated control or Mx1-Srg3fl/fl mice were cultured on OP9 stromal cells with IL-7. Srg3-deficient pro-B cells were severely reduced on day 3, and fewer pro-B cells were observed on day 6 after culturing compared with control mice (Fig. 7A). In addition, Srg3-deficient pro-B cells showed a much higher frequency of Annexin V+ cells on day 3 of in vitro culture (Fig. 7B). Srg3-deficient pro-B cells also showed a significant reduction in cell proliferation compared with the control pro-B cells, as measured by BrdU incorporation after 3 d of in vitro culture of the pro-B cells (Fig. 7C). These results indicate that Srg3-deficient pro-B cells were defective in survival as a result of the impairment in IL-7 signaling.
Impaired B lineage-specific gene expression and VDJ rearrangement in Srg3/mBaf155-deficient pro-B cells

We next examined the expression of genes that are critical for early B cell development in pro-B cells isolated from pIpC-treated control and Mx1-Srg3 fl/fl mice. Srg3-deficient pro-B cells expressed much less E47 (Tcfe2a) and Ebf1, as well as their downstream target genes, such as Pax5, Il7ra, Cd79a, Cd79b, Igll1, Vpreb1, Rag1, and Rag2, than did the control pro-B cells (Fig. 8A, 8B). However, it is possible that the diminished expression of B lineage-specific genes in Srg3-deficient pro-B cells is due to fewer early pro-B cells. To rule out this possibility, we analyzed gene expressions in PD36 cells ectopically transduced with control or Srg3-shRNAs. We found that the expression of Ebf1 and its target genes was decreased in the absence of Srg3 (Fig. 8C). Brg1-deficient B cells also showed a reduction in the expression of B lineage-specific genes (Fig. 8D), which strongly indicated that Srg3-deficient pro-B cells fail to express genes that are crucial for B cell development.

In accordance with the reduced expression of Rag1 and Rag2 in Srg3-deficient CLPs and pro-B cells, the expression of the intracellular μ-H chain in Srg3-deficient pro-B cells was much lower than in the control pro-B cells (Fig. 8E). To determine
whether the Srg3-deficient pro-B cells failed to undergo V(D)J and DJ rearrangement of the IgH (Igh) genes, we analyzed the genomic DNAs isolated from pro-B cells obtained from control and Mx1-Srg3 fl/fl mice by semiquantitative PCR, followed by Southern blotting. The recombination frequencies of the proximal VH locus, including VH7183 and VH52, as well as the more distant VH locus, including VH3609 and VHJ558, were reduced in Srg3-deficient pro-B cells compared with control pro-B cells. Furthermore, the incidence of rearrangement of DH-JH was also much lower in Srg3-deficient pro-B cells than control pro-B cells (Fig. 8F). Consistent with the genomic DNA analysis, the rearranged transcripts of the proximal VH7183 gene and the distal VJ558 gene were also decreased in Srg3-deficient pro-B cells compared with the control pro-B cells (Fig. 8G). These results indicate that the Srg3-deficient pro-B cells had defects in the V(D)J rearrangement of the Igh genes. This is consistent with impairment of IL-7R signaling, which is required for the regulation of VH gene rearrangement, in pro-B cells from Mx1-Srg3 fl/fl mice.

The core components of the SWI/SNF-like BAF complex directly bind to the promoter of I7ra and Ebf1 Our results suggest that defects in the expression of B lineage-specific genes, including Ebf1 and IL-7Rα, on the surface of

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**FIGURE 5.** Impairment in pro-B cell development in the absence of Brg1. (A) Schematic representations of retroviral constructs for shRNA targeting Brg1. Empty control (MSCV–LTRmiR30–PIG vector) and Brg1-shRNA virus express GFP only or both GFP and shRNA targeted to Brg1 transcripts. (B) Reduction in Brg1 expression by shRNA-Brg1 retrovirus. NIH3T3 cells were transduced with control or shRNA-Brg1 viruses, and GFP– cells were sorted by flow cytometry. Whole-cell extracts were isolated from the transduced cells, and SRG3 and BRG1 proteins were quantified by immunoblotting. β-Actin served as a loading control. One of two independent experiments is shown. (C) Flow cytometric analysis of bone marrow B cells (B220+ CD19+) from control and Brg1-deficient chimeras. Numbers indicate the percentage of populations (left panel); summary of the percentage of B cells (right panel). Individual symbols represent one mouse; the horizontal lines show the mean percentage (n = 3). (D) Flow cytometry of B cell subsets of IgM+B220+, IgM+B220−, and IgM+B220low cells. Numbers indicate the percentage of B cell subsets (left panel). Summary of the percentage of subsets described above (right panel). The horizontal lines show the mean percentage (n = 3). (E) Expression of CD43 on gated CD20+IgM+B cells and of CD19 on gated CD20+IgM+CD43+ pro-B cells was analyzed to discriminate the pre-pro-B fractions. Numbers represent the percentage of each subset. Data are representative of three independent experiments (left panel). Summary of the percentage of subsets described above (right panel). The lines show the mean percentage (n = 3). *p < 0.05, **p < 0.001.
pro-B cells led to the impairment of pro-B cell development in Mx1-Srg3fl/fl mice. We confirmed reduction of the Ebf1 transcript in PD36 cells transfected with a small interfering RNA duplex targeting Srg3 or Brg1 transcripts (Fig. 9A). It is known that the expression of Ebf1 is controlled through two other promoters (Fig. 9B): a distal promoter that is regulated by E2A and a proximal promoter that is regulated by PU.1 and Pax5 (40). Previous studies showed that PU.1 or Pax5 can cooperate with the BAF complex to activate target genes in various conditions (41–44). These results led us to investigate whether SRG3 directly regulates the expression of Ebf1 at the proximal region. To test the potential binding of SRG3, we used ChIP analysis with cross-linked chromatin extracted from PD36 cells. We found that immunoprecipitation with SRG3-specific Abs, but not isotype-control Abs, was enriched in the proximal promoter regions (Fig. 9C). BRG1 was also recruited to the same region (Fig. 9C). In addition, ChIP analysis using primary pro-B cells showed the binding of SRG3 and BRG1 to the proximal promoter of the Ebf1 gene (Fig. 9D). The forced expression of Ebf1 in LSK cells purified from pIpC-treated Mx1-Srg3fl/fl mice partially rescued the generation of B cells in vitro, whereas the generation of B cells was absent in Srg3-deficient LSKs that were ectopically transduced with control vectors (0.3937 ± 0.2889% versus 3.117 ± 0.5429% for B220+CD19+ cells) (Fig. 9E). However, ∼30% of the LSKs obtained from control mice differentiated into B cells (Fig. 9E). Although ectopic expression of Ebf1 produced B cell progenitors, the recovery of the cells was very inefficient in the absence of Srg3 (Fig. 9F). These results indicate that Ebf1 is very inefficient in activating B lineage-specific genes in the absence of Srg3 and suggest that it requires the SWI/SNF-like BAF complex for its full functional activity. We also found that SRG3 and BRG1 bind to the proximal promoter regions of Il7ra in PD36 cells and primary pro-B cells (Fig. 9G, 9H). These results collectively indicate that the SWI/SNF-like BAF complex is required for the transcriptional activation of Ebf1 and Il7ra in pro-B cells.

Discussion

The development of B lineage cells in the bone marrow occurs in a stepwise manner with critical checkpoints. The rearrangement of Ig genes and the proper expression of B lineage-specific genes are required for B cell commitment, survival, and proliferation. Epigenetic changes in the chromatin structure are essential for controlling the transcriptional regulation of these genes. In addition, the chromatin-remodeling complexes cooperate with sequence-specific transcription factors to promote the expression of several genes in many cell-specification events (17, 18). In this study, we demonstrated that Srg3/mBaf155, a core subunit of the SWI/SNF-like BAF complex, is essential for normal B cell development. Our data indicate that the BAF complex plays a pivotal role at both the CLP stage and the transition from pre–pro-B to early pro-B cells by regulating the expression of the B lineage-specific genes, such as Ebf1 and Il7ra.

Ebf1-deficient CLPs showed defects in the expression of B lineage-specific genes, although the numbers of CLPs are comparable between WT and Ebf1−/− mice (37). E47-deficient mice showed severe reduction in AA4.1+ CLPs (36). Notably, the
OFFICIAL EXCLUDE THE POSSIBILITY THAT WITH EARLY B CELL DEVELOPMENT (31–34, 37). ALTHOUGH WE CANNOT EXPRESSION OF AA4.1, IT IS UNLIKELY BECAUSE THERE ARE STILL AA4.1+/− Cd79a Srg3−/− OF PRE–PRO-B CELLS WAS NORMAL IN IN VIVO AND IN VITRO (31). CONSISTENT WITH THE DEFECTS IN THE EX- GIVE RISE TO B AND T CELLS, BUT NOT MYELOID LINEAGE CELLS, BOTH 2 B CELLS COMPLETELY LACK AA4.1+ POPULATION, ALTHOUGH THE NUMBER OF IL-7. (A) Flow cytometry of residual B220+ pro-B cells purified from FlpC-treated control or Mx1-Srg3fl/fl mice 6 d after in vitro culture with OP9 stromal cells. (B) Expression of Annexin V on pro-B cells from control and Mx1-Srg3fl/fl mice 3 d after in vitro culture (left panel). Numbers represent the percentage of Annexin V+ cells. Summary of the percentage of Annexin V+ cells from control and Mx1-Srg3fl/fl mice (right panel). Each symbol represents one mouse; the horizontal lines show the mean percentage of Annexin V+ cells (n = 4 for control and n = 6 for Mx1- Srg3fl/fl mice). (C) Flow cytometry of the proliferation of pro-B cells, using BrdU staining (left panel), purified from FlpC-treated control and Mx1- Srg3fl/fl mice by flow cytometry and then cultured for 3 d on OP9 stromal cells with IL-7. NUMBERS INDICATE THE PERCENTAGE OF BRDU+ CELLS. ERROR BARS INDICATE SEM OF THREE INDEPENDENT EXPERIMENTS.

FIGURE 7. Srg3-deficient pro-B cells are defective in survival in the presence of IL-7. (A) Flow cytometry of residual B220+ pro-B cells purified from FlpC-treated control or Mx1-Srg3fl/fl mice 6 d after in vitro culture with OP9 stromal cells. (B) Expression of Annexin V on pro-B cells from control and Mx1-Srg3fl/fl mice 3 d after in vitro culture (left panel). Numbers represent the percentage of Annexin V+ cells. Summary of the percentage of Annexin V+ cells from control and Mx1-Srg3fl/fl mice (right panel). Each symbol represents one mouse; the horizontal lines show the mean percentage of Annexin V+ cells (n = 4 for control and n = 6 for Mx1-Srg3fl/fl mice). (C) Flow cytometry of the proliferation of pro-B cells, using BrdU staining (left panel), purified from FlpC-treated control and Mx1-Srg3fl/fl mice by flow cytometry and then cultured for 3 d on OP9 stromal cells with IL-7. NUMBERS INDICATE THE PERCENTAGE OF BRDU+ CELLS. ERROR BARS INDICATE SEM OF THREE INDEPENDENT EXPERIMENTS.

SURFACE EXPRESSION OF AA4.1 ON CLPS WAS SIGNIFICANTLY REDUCED IN THE ABSENCE OF Srg3, WHEREAS Flt3 EXPRESSION REMAINED LARGELY UNCHANGED. IT WAS NOTED THAT AA4.1+ Flt3+ CLPS HAVE THE ABILITY TO GIVE RISE TO B AND T CELLS, BUT NOT MYELOID LINEAGE CELLS, BOTH IN VIVO AND IN VITRO (31). CONSISTENT WITH THE DEFECTS IN THE EXPRESSION OF AA4.1 ON CLPS, Srg3-deficient CLPS DISPLAYED DECREASED EXPRESSION OF Ebf1 AND ITS DOWNSTREAM TARGET GENES, SUCH AS Cd79a, Cd79b, Rag1, Rag2, and FoxO1, WHICH ARE ASSOCIATED WITH EARLY B CELL DEVELOPMENT (31–34, 37). ALTHOUGH WE CANNOT OFFICIALLY EXCLUDE THE POSSIBILITY THAT Srg3 DIRECTLY REGULATES THE EXPRESSION OF AA4.1 (31), IT IS UNLIKELY BECAUSE THERE ARE STILL AA4.1+/−med subsets in CLPS in Srg3-deficient mice. AA4.1+ pre-pro-B cells, but not AA4.1− cells, could develop into B220+CD43+ pro-B cells (7). OUR DATA SHOWED THAT Srg3-deficient pre-pro-B cells completely lack AA4.1+ population, although the number of pre-pro-B cells was normal in Srg3-deficient mice. THEREFORE, THE pre-pro-B cells found in Srg3-deficient mice could not generate early pro-B cells, and these defects might be due to the impairment of the expression of B lineage-specific genes at the stage of CLPs in the absence of Srg3. TAKEN TOGETHER, THIS APPEARS TO RESULT IN THE SEVERE REDUCTION IN THE NUMBER OF EARLY PRO-B CELLS IN THESE MICE.

TWO MAJOR DEFECTS WERE OBSERVED IN PRO-B CELLS, CONSISTING OF PRE–PRO-B, EARLY PRO-B, AND LATE PRO-B CELLS, FROM Srg3-deficient mice. The first defect is the failure in cell survival and proliferation via IL-7 signaling. We have clearly shown that the Srg3-deficient pro-B cells, especially early pro-B stage cells, failed to express IL-7Rα on the surface, which resulted in more apoptosis and less proliferation of pro-B cells, even in the presence of IL-7. IN ADDITION, Srg3-deficient pro-B cells showed a higher expression of Bim and a significantly lower expression of Bcl-1-XL relative to the control mice. THESE RESULTS ARE CONSISTENT WITH THE DECREASED EXPRESSION OF IL-7Rα ON THE SURFACE, WHICH INDUCES Bcl-1-XL EXPRESSION AFTER THE ACTIVATION OF Stat5 by Jak-mediated phosphorylation (45). NOTABLY, THE DEVELOPMENT OF FO B CELLS WAS SEVERELY IMPAIRED BY Srg3 DELETION, WHEREAS MZ B CELLS CONSTITUTED A MUCH HIGHER PROPORTION IN THE SPLENIC B CELL POPULATION IN THE ABSENCE OF Srg3. THESE RESULTS ARE CONSISTENT WITH THE PHENOTYPE IN I77α−/− mice, in which the peripheral B cell defects are most apparent in the FO B cell compartment (46). Moreover, it was shown that the FO B cells increase in number, whereas the proportion of MZ B cells decreases in IL-7 transgenic mice (47).

The second defect in Srg3-deficient pro-B cells is the impaired expression of B lineage-specific genes, such as E47 (Tcf2a), Ebf1, and Pax5. OUR DATA ALSO DEMONSTRATED THAT Srg3-deficient pro-B cells expressed less Rag1 and Rag2, which led to the failure of V(D)J rearrangement of the IgH genes and the expression of the cytoplasmic μ-chain. Moreover, the reduced expression of the downstream target genes of E2A and Ebf1, such as I77α, Cd79a, Cd79b, Igll1, and Pax5, was observed in the absence of Srg3. Proper functioning of E2A or Ebf1 is required during B lineage differentiation. THE LOSS OF EITHER E2A OR Ebf1 RESULTS IN A DEVELOPMENTAL BLOCK AT THE EARLY STAGE OF B CELL DEVELOPMENT (12–14). THE RECOMBINATION OF Dγ-H1 SEGS, AS WELL AS THE EXPRESSION OF B LINEAGE-SPECIFIC GENES, IS DEFECTIVE IN THE ABSENCE OF EITHER E2A OR Ebf1. Unlike E2A-deficient mice, which show significantly lower numbers of CLPS and LMPPs (13), Ebf1-deficient mice have normal numbers of CLPS and pre-pro-B cell populations (14, 32). THE OVERT CHARACTERISTICS OF PRO-B CELL DEVELOPMENT BLOCKADE AND IMPAIRED EXPRESSION OF B LINEAGE-SPECIFIC GENES IN CLPS IN Srg3-deficient mice closely resemble those in Ebf1−/− mice. THEREFORE, A DECREASED EXPRESSION OF Ebf1 ITSELF AND/OR DOWNSTREAM TARGET GENES IS A PLausible EXPLANATION FOR THE COMPROMISED B LINEAGE DIFFERENTIATION POTENTIAL IN LYMPHOID PROGENITORS AND THE BLOCKADE OF EARLY B CELL DEVELOPMENT IN THE ABSENCE OF Srg3.

IN AGREEMENT WITH THE FINDING THAT THE EXPRESSION OF Ebf1 AND ITS DOWNSTREAM TARGET GENES WAS REDUCED IN CLPS AND PRO-B CELLS OBTAINED FROM Srg3-deficient mice, OUR RESULTS ALSO PROVIDED EVIDENCE THAT THE EXPRESSION OF Ebf1 WAS DIRECTLY MODIFIED BY THE SWI/SNF-LIKE BAF COMPLEX. Srg3 AND BrG1 WERE FOUND TO BIND TO THE PROXIMAL PROMOTER REGIONS OF THE Ebf1 GENE IN PRO-B CELLS. ALTHOUGH THE RELATIVE ACTIVITY OF THE DISTAL PROMOTER OF Ebf1 (Ebf1−α) IS HIGHER THAN THE ACTIVITY OF THE PROXIMAL PROMOTER (Ebf1−β) IN THE PD36 CELL LINE, Ebf1−β TRANSCRIPT IS PROMINENTLY EXPRESSED IN ALL B LINEAGE CELLS, ESPECIALLY IN CLP AND PRO-B CELLS (THE RELATIVE ABUNDANCE OF Ebf1−β mRNA IS >100-fold HIGHER THAN THAT OF Ebf1−α) (40). MOREOVER, IT WAS PREVIOUSLY REPORTED THAT RECRUITMENT OF SWI/SNF-LIKE BAF COMPLEX IS REQUIRED FOR THE FUNCTIONAL ACTIVITY OF Pax5, AND THE EXPRESSION OF Ebf1−β WAS REDUCED IN Pax5−/− pro-B cells, whereas the expression of Ebf1−α was comparable (40–43). Therefore, it appears that the SWI/SNF-like BAF complex may act cooperatively with Pax5 to activate the expression of Ebf1 through the proximal promoter. Thus, in the absence of Srg3, Ebf1−β expression, which is reported to be transcribed by the proximal promoter, would be
significantly decreased, and this will affect the early B cell development.

Enforced expression of Ebf1 in Srg3-deficient LSK cells rescued, although very inefficiently, the generation of B cells in vitro (GFP control: 0.3937 ± 0.2889% versus GFP + Ebf1: 3.177 ± 0.5429% for B220 + CD19+ cells and control LSK: 28.57 ± 1.489%). Therefore, enforced expression of Ebf1 drives, with very low efficiency (∼10% of control), the recovery of B cell progenitors that lack SWI/SNF-like BAF complex. This very low efficiency of the recovery appeared to be due to the absence of SWI/SNF activity in Srg3-deficient mice. Therefore, we believe that our results reflect the requirement of SWI/SNF for the functional activity of Ebf1, which is consistent with previous reports (41–43).

In summary, our results collectively establish the critical functions of the SWI/SNF-like BAF complex in the process of early priming of B lineage specification. The functional requirement for the BAF complex at both the CLP stage and the transition from pre-pro-B to early pro-B cells is correlated with defects in the expression of B lineage-specific genes, such as Ebf1, Il7ra, Tcfe2a,
and Pax5, and in the V(D)J rearrangement of the IgH locus in the absence of Srg3. Thus, our study suggests that proper control of epigenetic remodeling of chromatin is essential for the sequential steps of early B cell development.
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