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Transcription Elongation Factor S-II Is Required for Definitive Hematopoiesis

Takahiro Ito,[1]† Nagisa Arimitsu,[1]† Masaki Takeuchi,[2]† Nobuyuki Kawamura,[1] Makiko Nagata,[1] Kayoko Saso,[1] Nobuyoshi Akimitsu,[1] Hiroshi Hamamoto,[1] Shunji Natori,[1] Atsushi Miyajima,[2] and Kazuhisa Sekimizu[1]*

Division of Developmental Biochemistry, Graduate School of Pharmaceutical Sciences,[1] and Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences,[2] University of Tokyo, Bunkyo, Tokyo 113-0033, Japan

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Transcription elongation factor S-II/TFIIS promotes readthrough of transcriptional blocks by stimulating nascent RNA cleavage activity of RNA polymerase II in vitro. The biologic significance of S-II function in higher eukaryotes, however, remains unclear. To determine its role in mammalian development, we generated S-II-deficient mice through targeted gene disruption. Homozygous null mutants died at midgestation with marked pallor, suggesting severe anemia. *S-II*$^{-/-}$ embryos had a decreased number of definitive erythrocytes in the peripheral blood and disturbed erythroblast differentiation in fetal liver. There was a dramatic increase in apoptotic cells in *S-II*$^{-/-}$ fetal liver, which was consistent with a reduction in *Bcl-x*$_L$ gene expression. The presence of phenotypically defined hematopoietic stem cells and in vitro colony-forming hematopoietic progenitors in *S-II*$^{-/-}$ fetal liver indicates that S-II is dispensable for the generation and differentiation of hematopoietic stem cells. S-II-deficient fetal liver cells, however, exhibited a loss of long-term repopulating potential when transplanted into lethally irradiated adult mice, indicating that S-II deficiency causes an intrinsic defect in the self-renewal of hematopoietic stem cells. Thus, S-II has critical and nonredundant roles in definitive hematopoiesis.

Precise control of gene transcription is essential for the regulation of cell differentiation, proliferation, and survival. Transcriptional regulation involves the concerted actions of upstream activators, coactivators, basal transcription factors, and RNA polymerase II (RNAPII) at the gene promoter (34, 42). In addition, recent studies suggest that the factors regulating transcription elongation have critical roles in the activation of gene expression (46).

S-II, also designated TFIIS, is a transcription elongation factor widely found in eukaryotes (59). S-II helps RNAPII pass through transcriptional blocks on template DNA (9). Transcriptional blocks cause persistent catalytic inactivation (transcription arrest) of the RNAPII elongation complex. S-II promotes RNAPII-mediated endonucleolytic cleavage of the nascent RNA, which restores the catalytic activity of the polymerase, leading to the resumption of transcript elongation (18). This mode of action contrasts with that of other elongation factors such as ELL, DSIF (DRB sensitivity-inducing factor), and elongin, which function by increasing the overall rate of transcription elongation catalyzed by RNAPII (4, 16, 47, 57). It is also suggested that S-II has a role in overcoming the arrest induced by nucleosomes, thereby promoting chromatin transcription in vitro (21). Consistent with its role in transcription through nucleosomes, deletion of *DST1*, a gene encoding S-II in budding yeast, causes strong synthetic phenotypes in combination with mutations of genes involved in chromatin modification, such as *SNF2*, *SPT16*, *SET2*, and *SWR1* (5, 24, 25, 35).

Although *DST1* is not essential for viability in yeast, null mutation renders the yeast cells sensitive to oxidative stress and to drugs affecting nucleotide metabolism, such as 6-azauracil (6-AU) and mycophenolic acid (23, 29, 45). Structure-function relationship analyses established the importance of the C-terminal region of S-II for its in vivo function in *Saccharomyces cerevisiae* (30, 48, 49). For example, a mutant S-II gene encoding a truncated protein lacking C-terminal amino acid residues 260 to 309 (Δ260-309) does not suppress the 6-AU sensitivity of a Δ*dst1* mutant, and the mutant protein Δ266-309 does not stimulate transcription by RNAPII in vitro. On the other hand, a Δ2-141 mutation does suppress 6-AU sensitivity in vivo, and this mutant protein stimulates transcription by RNAPII in vitro. These results suggest that the 6-AU-sensitive phenotype of the yeast *S-II* deletion mutant is caused by the loss of function of S-II as a transcription factor.

The functional importance of the N-terminal region is inferred through the identification of S-II interaction partners. Through its N-terminal region (amino acid residues 1 to 132), yeast S-II associates with transcription factors Med13 and Spt8, subunits of the Mediator and SAGA coactivator complexes, respectively (58). The N-terminal region (residues 1 to 103) of human S-II interacts with human RNAPII holoenzyme (38). Mouse S-II interacts with transcriptional activators via its N-terminal half (31, 44). These results suggest that the N-terminal region of S-II acts as an interaction surface for several transcriptional regulators.

In spite of the detailed biochemical and structural analyses described above, the biologic significance of S-II function in higher eukaryotes remains unclear. To gain insight into the im-

* Corresponding author. Mailing address: Division of Developmental Biochemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, Hongo 7-3-1, Bunkyo, Tokyo 113-0033. Phone: 81-3-5841-4820. Fax: 81-3-5684-2973. E-mail: sekimizu@mol.f.u-tokyo.ac.jp.
† These authors contributed equally to this work.

portance of the transcription factor, we examined the function of S-II through targeted gene disruption in mice and revealed that S-II has essential roles in definitive hematopoiesis.

MATERIALS AND METHODS

Generation of S-II-deficient mice. Genomic DNA clones encoding the murine S-II gene (*Tcea1*) were described previously (17). We used a 6-kb SpeI-SacI genomic DNA fragment encompassing intron 4 through exon 8 of the mouse S-II gene as the 3′ homology arm in the targeting vector. For the 5′ homology arm, a 0.8-kb genomic DNA fragment was amplified by PCR with primer pair 5′-ATGCA GAGAGTAGATGAACA-3′ and 5′-GTCTTTATCAGTTGATGGTCC-3′. To create the targeting vector, these two homology arms were cloned in the pNeoDTA vector containing a phosphoglycerate kinase gene promoter-driven neomycin resistance cassette and a phosphoglycerate kinase gene promoter-diphtheria toxin A subunit gene (a gift from M. M. Taketo, Department of Pharmacology, Graduate School of Medicine, Kyoto University) (36). Electroporation of the targeting vector into E14TG2aIV embryonic stem (ES) cells, selection of neomycin-resistant clones, and injection of the correctly targeted ES cells into blastocysts were performed as described previously (2, 10).

Male chimeric mice were bred with C57BL/6J female mice (CLEA Japan Inc., Tokyo, Japan) to obtain F_1 mice heterozygous for the mutation. Heterozygotes were backcrossed with C57BL/6J mice. Heterozygous mice in the N_3 to N_{10} backcross generations were intercrossed to obtain homozygous mutant embryos for analyses. For genotyping mouse embryos and adult mice, genomic DNAs isolated from the yolk sac, forelimb, or tail were analyzed by Southern blotting using the probe depicted in Fig. 1A and/or PCR assays described below. For genotyping by PCR, the reaction mixture contained 0.5 U of ExTaq DNA polymerase (Takara Bio, Shiga, Japan), 1× ExTaq buffer, 0.2 mM deoxynucleoside triphosphates, genomic DNA, and 0.5 μM of each primer. The nucleotide sequences of the primers were as follows: S, 5′-GCTGCTCTGTTGACA GATCA-3′; AS, 5′-CTTGTCCTGGGATATGTACC-3′; and PGKR, 5′-CTA AAGCGCATGCTCCAGACT-3′. In this three-primer multiplex PCR, the wild-type allele gave a 730-bp fragment and the mutated allele gave a 440-bp product. PCR conditions were as follows: 4 min at 94°C, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. All animal experiments were approved by the institutional committee on animal experimentation and performed in compliance with the corresponding animal welfare laws.

Histology and cytology. Embryos were fixed in 4% paraformaldehyde and embedded in paraffin, and 5-μm sections were mounted on silane-coated glass slides according to standard procedures (39). Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining was performed using an in situ apoptosis detection kit (Takara Bio) according to the manufacturer's instructions. Nuclei were counterstained with methyl green. Cytospin preparations of peripheral blood cells collected from the cord vessels were stained with Wright-Giemsa. For diaminobenzidine (DAB) staining, deparaffinized sections were incubated in DAB solution (0.05% DAB tetrahydrochloride and 0.006% hydrogen peroxide in phosphate-buffered saline) for 30 min at room temperature.

In vitro hematopoietic colony formation assays. Individual fetal livers (FL) from embryonic day 12.5 (E12.5) embryos were dissected in cold phosphate-buffered saline, disaggregated, and passed through a 74-μm nylon mesh (Corning) to obtain single-cell suspensions. Cells were counted using a hemacytometer and plated in triplicate in Iscove's modified medium-based methylcellulose medium supplemented with erythropoietin, stem cell factor, interleukin-3 (IL-3), IL-6, insulin, and transferrin (MethoCult M3434; Stem Cell Technologies, Vancouver, British Columbia, Canada). Erythroid (CFU-E and BFU-E) hematopoietic progenitors were scored by morphological criteria at day 3 and 5, respectively. Myeloid (CFU-GM, -G, and -M) and multilineage (CFU-Mix) hematopoietic progenitors were scored at day 8. Statistical significance was determined using nonparametric Kruskal-Wallis tests and Scheffe's multiple-comparison tests.

Flow cytometry. Flow cytometric analyses of FL cells were performed using a FACSCalibur (Becton Dickinson, San Jose, CA) as described previously (43, 54). Fluorochrome-conjugated anti-CD3ε, CD45, CD71, TER119, Mac-1, Gr-1, B220, c-Kit, and Sca-1 antibodies were obtained from BD Pharmingen (San Diego, CA). For KSL cell population analysis, FL cells were labeled with a lineage antibody cocktail (anti-TER119, Gr-1, B220, and CD3ε antibodies), anti-Sca-1, and anti-c-Kit antibodies for flow cytometry.

Western blot analysis of S-II. Embryonic tissues were homogenized in P buffer containing 25 mM HEPES (pH 7.8), 0.5 M potassium chloride, 0.1% Nonidet P-40, 5 mM magnesium chloride, 1 mM dithiothreitol, and protease inhibitors (Complete Mini; Roche Diagnostics KK, Tokyo, Japan) and centrifuged at 12,000 × *g* to remove debris. Protein extracts (40 μg) were resolved by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). After blocking with 5% skim milk, the blot was probed with anti-mouse S-II chicken immunoglobulin Y antibody (a generous gift from Toshiyuki Nakanishi, Daiichi Pharmaceutical Co., Ltd), followed by horseradish peroxidase-conjugated anti-chicken immunoglobulin Y (Promega KK, Tokyo, Japan). For α-tubulin detection, we used rabbit anti-α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-rabbit immunoglobulin G horseradish peroxidase-linked whole antibody (Amersham Biosciences, Piscataway, NJ). The horseradish peroxidase-labeled antibodies were detected using Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA).

Analysis of erythropoietin-dependent STAT5 phosphorylation. FL cells prepared from E13.5 embryos were cultured for 4 h in prestimulation medium (alpha minimal essential medium containing 0.5% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, 100 U/ml penicillin G, and 100 μg/ml streptomycin) and then stimulated with recombinant human erythropoietin (a generous gift from Kirin Brewery Co., Ltd.) at a final concentration of 50 U/ml. Cell lysis, immunoprecipitation of STAT5 protein, and Western blotting were performed as reported previously (55). The anti-STAT5 antibody and anti-phosphorylated STAT5 antibody were obtained from Santa Cruz Biotechnology and Cell Signaling Technology (Beverly, MA), respectively.

Real-time RT-PCR assays. FLs from E13.5 *S-II*$^{+/+}$ and *S-II*$^{-/-}$ embryos (n = 5 for each genotype) were dissected in cold phosphate-buffered saline, and total cellular RNAs were isolated with an RNeasy Mini kit (QIAGEN KK, Tokyo, Japan). Total cellular RNA (1 μg) was reverse-transcribed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Real-time PCR was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems) using TaqMan Universal PCR master mix and TaqMan gene expression assays (Applied Biosystems; Bcl-x catalog number, Mm00437783_m1; erythropoietin catalog number, Mm00433126_m1; erythropoietin receptor catalog number, Mm00438760_m1; and hypoxanthine guanine phosphoribosyltransferase catalog number, Mm00446968_m1). Real-time PCRs were performed in triplicate for each reverse-transcribed cDNA. After normalization by the hypoxanthine guanine phosphoribosyltransferase expression levels, relative expression values of the *S-II*$^{-/-}$ embryos were calculated by defining the mean value for *S-II*$^{+/+}$ embryos as 100%.

Preparation of *S-II*$^{-/-}$ EF cells and luciferase reporter assays. Embryonic fibroblast (EF) cells were harvested from *S-II*$^{-/-}$ embryos at E13.5 according to standard procedures. In brief, after removal of the extraembryonic and visceral tissues, embryos were minced with forceps and incubated in 0.25% trypsin–EDTA solution at room temperature for 15 min. Dispersed cells were suspended in EF medium (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin), and plated on gelatin-coated tissue culture dishes (Asahi Techno Glass, Tokyo, Japan). Adherent EF cells were maintained by passaging them once in 2 days.

Transfections and luciferase reporter assays were performed essentially as described previously (31). In brief, 2×10^4 EF cells were seeded in 24-well plates and cultured for 24 h. Transfections were performed according to the manufacturer's instructions using LipofectAmine 2000 (Invitrogen Japan KK, Tokyo, Japan) with *Bcl-x*$_L$ reporter plasmid pGL2-3.2 (a generous gift from N. Komatsu, Division of Hematology, Jichi Medical School) (22), the pMX-STAT5A1*6 expression vector (a generous gift from T. Kitamura, Division of Hematopoietic Factors, Institute of Medical Science, University of Tokyo) (32), pRL-TK (internal control; Promega), and a plasmid encoding mouse S-II (cloned in the pcDNA 3.1 vector; Invitrogen) as indicated. Cells were lysed in passive lysis buffer (Promega) 24 h after transfection, and firefly and *Renilla* luciferase activities were assayed using the dual-luciferase reporter assay system (Promega). All firefly luciferase activity values were normalized to *Renilla* luciferase activity values. Transfections were performed in quadruplicate.

In vivo transplantation assays. In vivo transplantation assays were performed essentially as described previously (26, 54), except that FL cells were prepared from E13.5 embryos expressing enhanced green fluorescent protein (EGFP). To obtain these embryos, we first generated *S-II*$^{+/-}$ mice hemizygous for the EGFP transgene by crossing *S-II*$^{+/-}$ mice with transgenic C57BL/6-Tg(ACTbEGFP)1Osb/J (stock no. 003291, Jackson Laboratory, Bar Harbor, ME). *S-II*$^{+/-}$ male or female mice hemizygous for the EGFP transgene were then mated with *S-II*$^{+/-}$ female or male mice, respectively, to obtain *S-II*$^{+/+}$, *S-II*$^{+/-}$, and *S-II*$^{-/-}$ embryos expressing EGFP. For transplantation, the indicated numbers of FL cells, together with wild-type support/competitor bone marrow cells, were injected intravenously into the tail veins of lethally irradiated C57BL/6J recipients. Peripheral blood cells of the recipient mice were analyzed at 9 days and 8 and 16 weeks posttransplantation by flow cytometry of EGFP fluorescence.

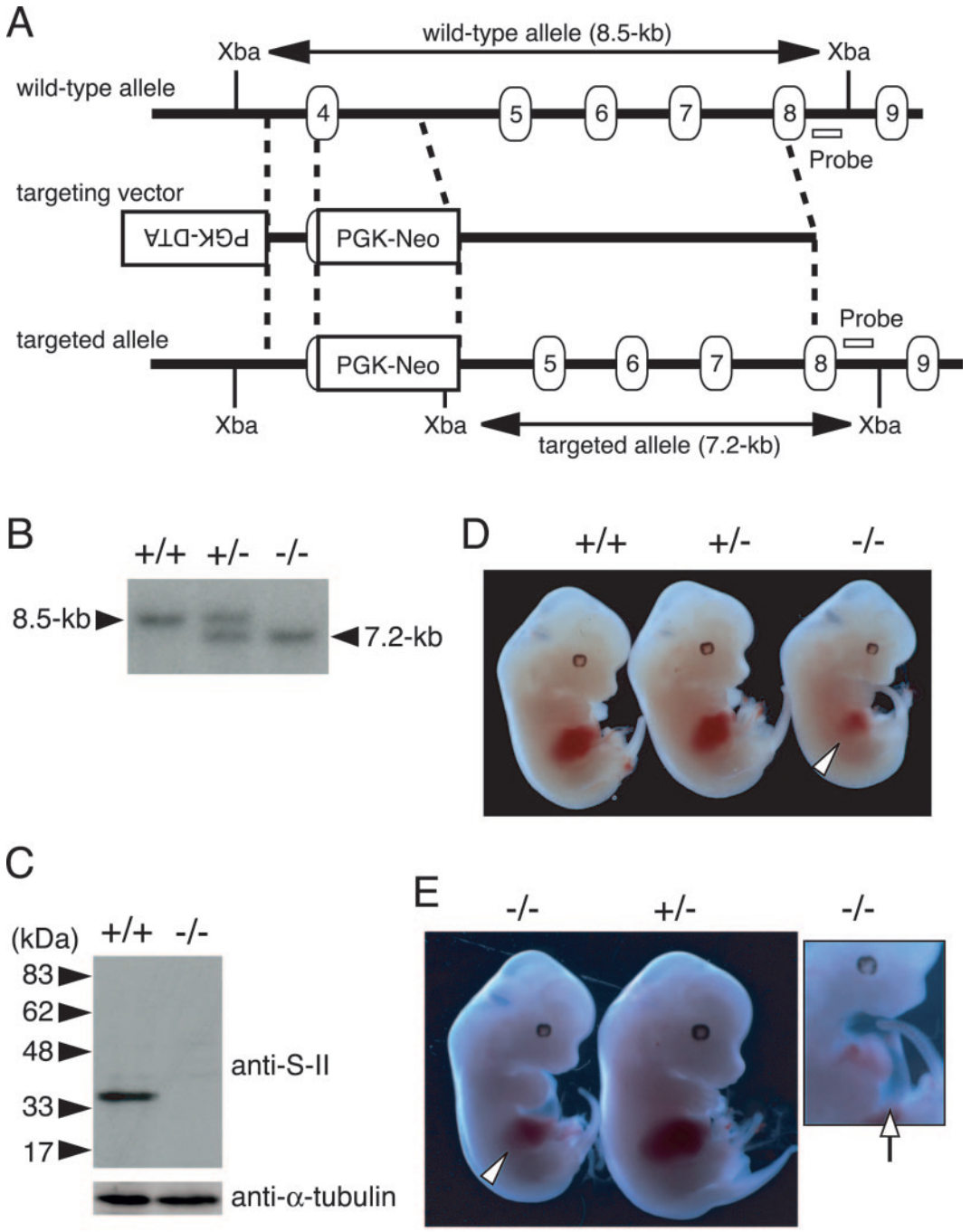

FIG. 1. Targeted disruption of the *S-II* gene. (A) Diagrams of the wild-type allele of the murine *S-II* gene, the targeting vector, and the targeted allele. Numbered boxes indicate exons. Broken lines indicate the regions of homology used for homologous recombination. Wild-type (8.5-kb) and targeted (7.2-kb) alleles were distinguished by Southern blot analysis of XbaI-digested genomic DNA hybridized with the indicated probe (open box). (B) Southern blot analysis of E12.5 embryos from the heterozygote intercross. +/+, wild type; +/−, heterozygous mutant; −/−, homozygous mutant. (C) Western blot analysis of E13.5 embryos probed with anti-S-II antibody (top). Equal protein loading was assessed by probing the blot with anti-α-tubulin antibody (bottom). (D and E) Gross morphology of control and *S-II*$^{-/-}$ embryos at (D) E12.5 and (E) E13.5. Arrowheads indicate liver hypoplasia, and the arrow indicates pericardial edema in *S-II*$^{-/-}$ embryos. The mutant embryo was similar to the control in body size at E12.5 but became smaller at E13.5.

RESULTS

Homozygous null mutation of the *S-II* gene resulted in embryonic lethality. To disrupt the *S-II* gene, we constructed a targeting vector to replace most of the fourth exon encoding amino acids 78 to 106 of the S-II protein with a neomycin resistance cassette (Fig. 1A) and obtained two embryonic stem cell clones that contained a correctly targeted allele (data not shown). These ES cell clones were injected into C57BL/6J blastocysts to generate male chimeric mice, which were then bred with C57BL/6J females.

TABLE 1. Genotype distribution of progeny from *S-II*$^{+/-}$ intercrosses

Stage	Total	No. of embryos with indicated genotype)[a]		
		+/+	+/−	−/−
Postnatal	103	42	61	0
E18.5	20	6	14	0
E16.5	25	9	14	0 (2)
E14.5	53	14	23	4 (12)
E13.5	366	84	204	59 (19)
E12.5	117	31	60	26
E11.5	38	10	17	11

[a] Number in parentheses are number of dead embryos. Live embryos were defined as those with beating hearts at the time of dissection.

Heterozygous mutant (*S-II*$^{+/-}$) mice appeared normal and were viable and fertile.

S-II$^{+/-}$ mice were then intercrossed to determine whether mice homozygous for the *S-II* deletion (*S-II*$^{-/-}$) were viable. There were no *S-II*$^{-/-}$ mice among 103 pups examined at 4 weeks of age, suggesting embryonic lethality of the homozygous mutants (Table 1, postnatal). Examination of embryos from timed pregnancies demonstrated that all *S-II*$^{-/-}$ embryos were alive until embryonic day 12.5 (Table 1 and Fig. 1B). The proportion of viable *S-II*$^{-/-}$ embryos gradually decreased after E13.5, and all were dead by E16.5. Western blot analysis of proteins from E13.5 embryos demonstrated a loss of S-II protein in *S-II*$^{-/-}$ embryos (Fig. 1C). These results indicated that homozygous null mutation of the *S-II* gene resulted in embryonic lethality between E13.5 and 16.5.

Severe reduction in the number of definitive erythrocytes in *S-II*$^{-/-}$ embryos. *S-II*$^{-/-}$ embryos at E12.5 were comparable in overall development to *S-II*$^{+/+}$ and *S-II*$^{+/-}$ littermates except for fetal liver hypoplasia and tail curling (Fig. 1D). At E13.5, the mutant embryos were smaller than their littermates and had pericardial edema (Fig. 1E). The FL of *S-II*$^{-/-}$ embryos was distinctly smaller and paler than that of *S-II*$^{+/+}$ littermates (Fig. 2A). Consistent with its appearance, there were significantly fewer nucleated cells in *S-II*$^{-/-}$ FL (Fig. 2B).

FL is the predominant site for hematopoiesis during the midgestational period. Hematopoiesis occurs in two waves during embryogenesis (37). Primitive erythropoiesis occurs first in blood islands of the yolk sac at E7.5 to produce large nucleated primitive erythrocytes and macrophages. The second wave, definitive hematopoiesis, takes place in FL during midgestation. Various blood cell types, mainly enucleated definitive erythrocytes, are generated from hematopoietic stem cells (HSCs) (33). Thus, we examined hemoglobin-producing cells by diaminobenzidine staining of the FL sections. The number of DAB-positive cells was severely reduced in *S-II*$^{-/-}$ FL (Fig. 2C). Consistently, the fraction of enucleated definitive eryth-

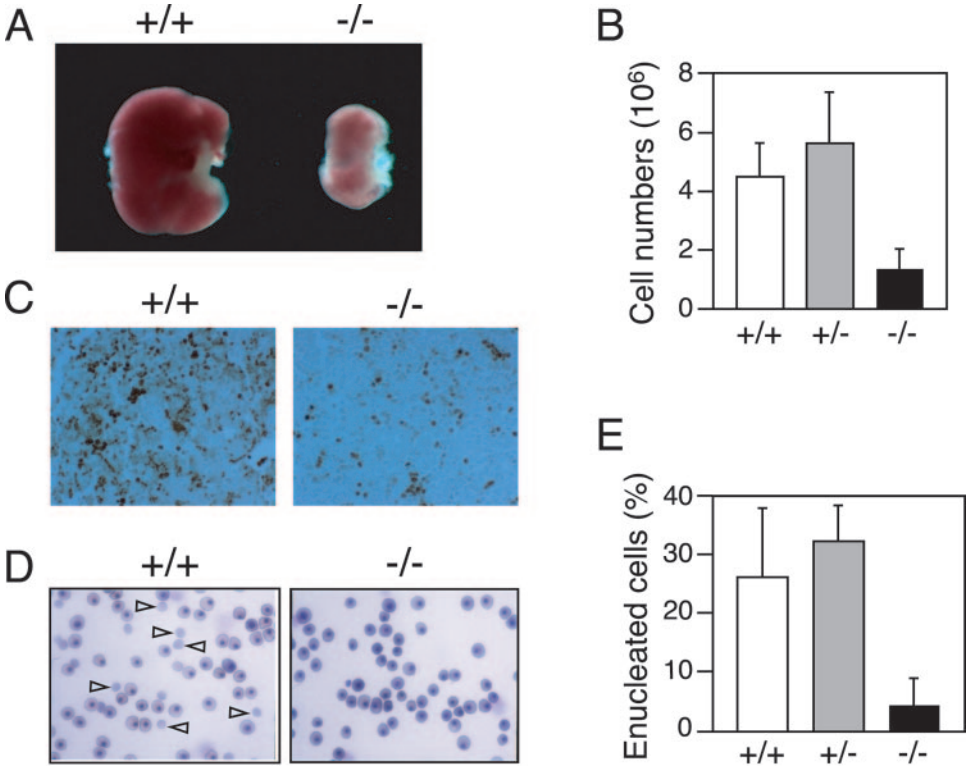

FIG. 2. Liver hypoplasia and impaired definitive erythrocyte production in *S-II*$^{-/-}$ embryos. (A) Liver morphology at E13.5. The *S-II*$^{-/-}$ FL is smaller and paler than that of the *S-II*$^{+/+}$ embryo. (B) Cellularity of the E13.5 FL. There was a more than fourfold reduction in the total number of nucleated cells in the *S-II*$^{-/-}$ FL. Results are means with standard deviation (+/+, $n = 4$; +/−, $n = 15$; −/−, $n = 6$). (C) DAB staining of the FL sections showing fewer DAB-positive erythrocytes in *S-II*$^{-/-}$ than in *S-II*$^{+/+}$ FL. (D) Cytocentrifuge preparations of peripheral blood from E13.5 stained by Wright-Giemsa. Blood from the *S-II*$^{+/+}$ embryo contained enucleated definitive erythrocytes (arrowheads), while that from the *S-II*$^{-/-}$ embryo contained no enucleated cells in this field. (E) Frequencies of enucleated red blood cells in the peripheral blood of E13.5 embryos. Data are means with standard deviation (+/+, $n = 8$; +/−, $n = 18$; −/−, $n = 5$).

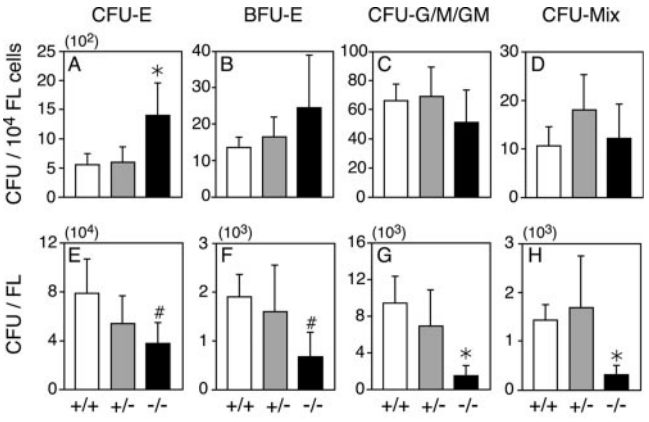

FIG. 3. Lineage-committed hematopoietic progenitors in FL. Single-cell suspensions of E12.5 FL cells from each genotype were plated in semisolid methylcellulose medium, which contained erythropoietin, stem cell factor, IL-3, IL-6, insulin, and transferrin. Hematopoietic colonies of the indicated types were scored per 10^4 FL cells (A to D) or per FL (E to H). Results are means with standard deviation (+/+, $n = 5$; +/−, $n = 7$; −/−, $n = 8$). *, $P < 0.03$ compared with either *S-II*$^{+/+}$ or *S-II*$^{+/-}$; #, $P < 0.03$ compared with *S-II*$^{+/+}$.

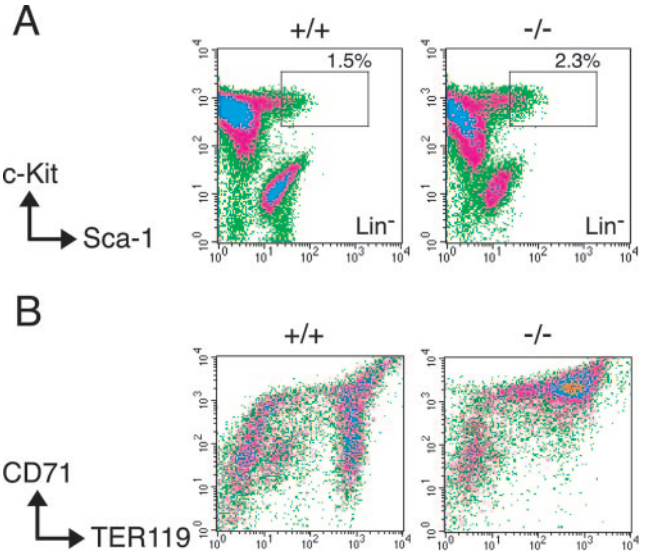

FIG. 4. Presence of phenotypically defined HSCs and impairment of erythroblast differentiation in *S-II*$^{-/-}$ FL at E13.5. (A) c-Kit/Sca-1 expression profile of the Lin$^-$ population. The *S-II*$^{-/-}$ FL contained the KSL population, and the frequency was comparable to that observed in *S-II*$^{+/+}$ FL. (B) TER119/CD71 expression profile. In *S-II*$^{-/-}$ FL, the TER119^{high} CD71^{high} population (early erythroblasts) was greatly increased, whereas the TER119^{high} CD71$^{medium\text{-}low}$ population (late erythroblasts and reticulocytes) was severely reduced compared with that of *S-II*$^{+/+}$ FL.

rocytes in the peripheral blood was lower in *S-II*$^{-/-}$ embryos than in *S-II*$^{+/+}$ and *S-II*$^{+/-}$ embryos (Fig. 2D and E). These findings indicated that definitive erythropoiesis was severely impaired in *S-II*$^{-/-}$ embryos.

Hematopoietic progenitors and hematopoietic stem cells in *S-II*$^{-/-}$ FL. To further characterize the defects in hematopoiesis, we analyzed hematopoietic progenitor cells by in vitro colony formation assays. Cells from E12.5 FL were cultured in semisolid medium under conditions that allow the growth and differentiation of erythroid (CFU-E and BFU-E), myeloid (CFU-GM, -G, and -M), and multilineage (CFU-Mix) hematopoietic progenitors. Compared with *S-II*$^{+/+}$ and *S-II*$^{+/-}$ FL cells, there were no significant differences in the number of BFU-E, CFU-GM/G/M, and CFU-Mix colony-forming cells per 10^4 nucleated FL cells from *S-II*$^{-/-}$ embryos (Fig. 3B to D). The mean number of CFU-E per 10^4 cells was increased 2.5- and 2.3-fold over the *S-II*$^{+/+}$ and *S-II*$^{+/-}$ values, respectively (Fig. 3A). These findings indicated that S-II is not required for the generation of the lineage-committed hematopoietic progenitors. The total number of colony-forming progenitors per whole FL, however, was significantly lower in *S-II*$^{-/-}$ than in *S-II*$^{+/+}$ and *S-II*$^{+/-}$ littermates as a result of the hypocellularity in *S-II*$^{-/-}$ FL (Fig. 3E to H). These results suggest that expansion of the hematopoietic progenitors and/or HSCs was compromised in the absence of S-II.

We next analyzed the expression of lineage-specific cell surface antigens by flow cytometry. There were no significant differences in the expression of the myeloid-specific antigens Mac-1 and Gr-1 among the three genotypes (data not shown), suggesting that the maturation of myeloid-committed progenitors was not altered in the absence of S-II. In contrast, the population expressing the TER119 antigen, an erythroid-specific surface antigen, was decreased in *S-II*$^{-/-}$ FL (+/+ or +/−, 83% ± 2%, $n = 16$; −/−, 67% ± 6%, $n = 5$; $P = 0.003$), which is consistent with the reduction of definitive erythrocytes in *S-II*$^{-/-}$ (Fig. 2C to E).

Definitive HSCs expand vigorously in FL during E11.5 to E16.5 (8). To investigate whether *S-II*$^{-/-}$ cells had defective HSC expansion, we analyzed the abundance of HSCs in E13.5 FL by measuring the frequencies of c-Kit$^+$ Sca-1$^+$ lineage-marker-negative (Lin$^-$) cells (KSL cells), in which HSCs are enriched. There was no apparent reduction in the frequency of KSL cells in *S-II*$^{-/-}$ FL (Fig. 4A). The absolute numbers of KSL cells per whole FL, however, were significantly different between *S-II*$^{+/+}$ and *S-II*$^{-/-}$ (+/+ or +/−, $14 \times 10^4 \pm 3.8 \times 10^4$, $n = 15$; −/−, $5.8 \times 10^4 \pm 1.8 \times 10^4$, $n = 2$; $P = 0.008$). Taken together, these results suggested that S-II is not required for the generation of phenotypically defined HSCs but is essential for their expansion in FL.

Impaired erythroblast differentiation in *S-II*$^{-/-}$ embryos. Although there was a severe reduction in the number of definitive erythrocytes in FL and peripheral blood in *S-II*$^{-/-}$ mice (Fig. 2C to E), the relative frequency of the late erythroid progenitor CFU-E in FL was significantly increased over that in *S-II*$^{+/+}$ or *S-II*$^{+/-}$ embryos (Fig. 3A). These results suggested that erythropoiesis in the mutant embryos was disturbed during erythroblast differentiation. To examine this possibility, we used a flow cytometry assay developed by Socolovsky et al. that allows the assessment of the maturation stage of differentiating erythroblasts by expression of the TER119 and CD71 antigens (53). The erythroid-specific antigen TER119 is first expressed at the proerythroblast stage, and all subsequent erythroid precursors express TER119 at high levels (20). In contrast, CD71 (transferrin receptor) is expressed at high levels by immature erythroid precursors (proerythroblasts and early erythroblasts), and its expression decreases as the precursors mature (late erythroblasts) (63).

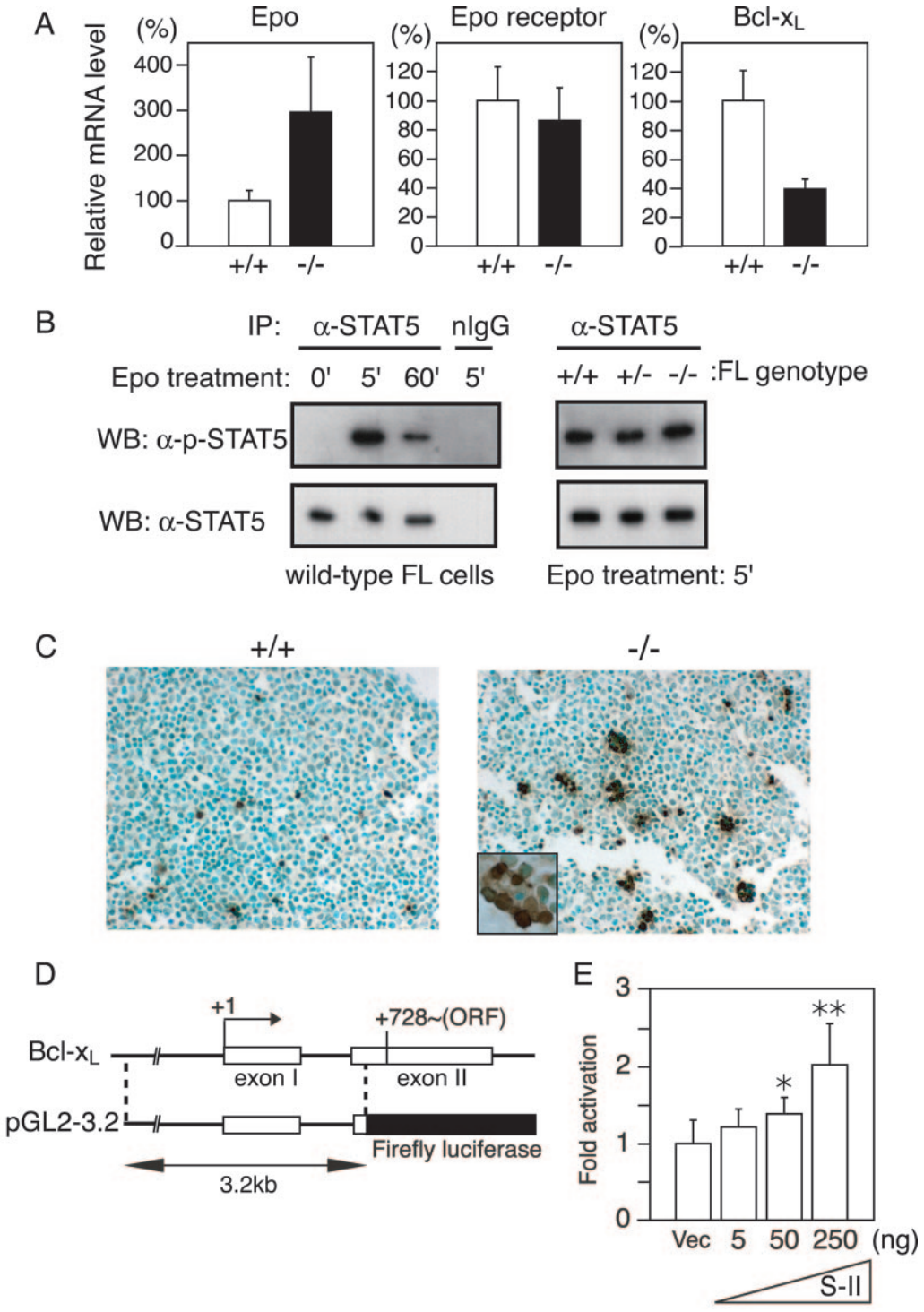

FIG. 5. Increased apoptosis and reduced *Bcl-x*$_L$ transcription in *S-II*$^{-/-}$ FL. (A) Real-time reverse transcription-PCR analyses were performed to assess the expression of the erythropoietin gene (*Epo*), its receptor gene (*Epo receptor*), and *Bcl-x*$_L$ in *S-II*$^{+/+}$ and *S-II*$^{-/-}$ FLs at E13.5. Data are means with standard deviation ($n = 5$ for each genotype). (B) Epo-dependent activation of STAT5 determined by Western blot analysis. In the right panels, FL cells of the indicated genotypes were stimulated with Epo for 5 min, and Epo-dependent phosphorylation of STAT5 was analyzed by immunoprecipitation followed by Western blotting with anti-phospho-STAT5 antibody (top right). A blot probed with anti-STAT5 antibody is also shown (bottom right). The left panels indicate that phosphorylation detected by the anti-phospho-STAT5 antibody was Epo dependent. (C) TUNEL staining of *S-II*$^{+/+}$ and *S-II*$^{-/-}$ FL at E13.5. Scattered TUNEL-positive nuclei were observed in *S-II*$^{+/+}$ FL (left panel), whereas many clusters of TUNEL-positive nuclei were detected in *S-II*$^{-/-}$ FL (right panel). The inset shows a magnified image of a cluster of TUNEL-positive cells. (D and E) S-II positively affected *Bcl-x*$_L$ transcription. (D) Schematic representation of the *Bcl-x*$_L$ reporter construct. The upper line shows the endogenous murine *Bcl-x*$_L$ locus; +1 indicates the transcription start site, and open boxes indicate exons. (E) Dose-dependent activation of luciferase activity from the *Bcl-x*$_L$ reporter construct by cotransfection of a plasmid encoding mouse S-II. Values on the horizontal axis were the amounts of the S-II expression vector used. The data are presented as fold activation, where the values with firefly luciferase activity were normalized to that of the internal control (*Renilla* luciferase). Activity for the empty vector (Vec) was set at 1.0. Data are means with standard deviations ($n = 4$ each). *, $P < 0.05$, and **, $P < 0.01$ compared with the control (empty vector).

We used this assay to examine the presence of erythroid precursors in *S-II*$^{+/+}$ and *S-II*$^{-/-}$ FL at E13.5. In wild-type embryos, TER119^{high} cells expressed CD71 at various levels, that is, CD71^{high} to CD71$^{medium\text{-}low}$, indicating that erythroblast differentiation progressed beyond the late erythroblast stage (Fig. 4B, +/+). FL cells from heterozygotes had a similar expression pattern (data not shown). In contrast, the TER119 and CD71 expression profile of *S-II*$^{-/-}$ FL cells was strikingly different (Fig. 4B, −/−): the population of TER119^{high} CD71$^{medium\text{-}low}$ cells was almost absent, and the population of TER119^{high} CD71^{high} cells was increased over that in *S-II*$^{+/+}$ liver cells. These findings clearly indicated that erythropoiesis is disrupted at the erythroblast stage, causing severe anemia in *S-II*$^{-/-}$ embryos.

Reduced *Bcl-x*$_L$ gene expression in *S-II*$^{-/-}$ FL. The erythropoietin (Epo)-dependent signaling pathway is essential for the production of definitive erythrocytes (61). Therefore, we next analyzed whether the signaling pathway functions properly in *S-II*$^{-/-}$ FL. Real-time reverse transcription-PCR analyses of gene expression revealed that *Epo* expression was not decreased, but rather upregulated, in *S-II*$^{-/-}$ embryos (Fig. 5A). Expression of the *Epo receptor* gene (Fig. 5A) and *Stat5* (data not shown), by which the Epo signal is transduced (52, 61), was not affected by the S-II null mutation. Epo stimulation induces transient activation of STAT5 protein by phosphorylation (41). We examined Epo-dependent STAT5 phosphorylation by immunoprecipitation of STAT5 protein followed by detection with anti-phosphorylated STAT5 antibody (Fig. 5B, left panels) and determined that STAT5 phosphorylation was not impaired in *S-II*$^{-/-}$ FL cells (Fig. 5B, right panels). In contrast, the mRNA level of *Bcl-x*$_L$, which is a target gene of the activated STAT5 (51), was reduced to less than half that of the wild type (Fig. 5A).

Increased apoptosis in *S-II*$^{-/-}$ FL. Bcl-x$_L$, an antiapoptotic factor, is involved in the terminal differentiation of definitive erythrocytes (6), and targeted disruption of the gene in mice indicates that homozygous mutation results in midgestational lethality with a massive increase in apoptotic cell death in FL (28). Thus, we next examined whether there was an increase in apoptosis in *S-II*$^{-/-}$ FL by TUNEL staining of liver sections of E13.5 embryos. We observed more TUNEL-positive cells in *S-II*$^{-/-}$ than in *S-II*$^{+/+}$ FL, indicating increased apoptosis in the *S-II*$^{-/-}$ embryos (Fig. 5C). In addition, TUNEL-positive cells were clustered in *S-II*$^{-/-}$ FL, whereas they were scattered in *S-II*$^{+/+}$ FL. Closer examination revealed that the cluster of TUNEL-positive cells surrounded a TUNEL-negative cell (Fig. 5C, inset in the right panel). This structure possibly represents an erythroblastic island in which a central macrophage is surrounded by several erythroblasts undergoing terminal differentiation (3). These results suggest a massive increase in erythroblast apoptosis at the terminal differentiation step, which is consistent with the absence of viable TER119^{high} CD71$^{medium\text{-}low}$ cells in *S-II*$^{-/-}$ FL (Fig. 4B).

S-II-induced activation of *Bcl-x*$_L$ gene transcription. We next investigated whether S-II has positive effects on *Bcl-x*$_L$ transcription. For this purpose, we utilized reporter gene assays in embryonic fibroblast cells prepared from an *S-II*$^{-/-}$ embryo at E13.5. The reporter construct pGL2-3.2 contained a 3.2-kb genomic fragment from the mouse *Bcl-x*$_L$ gene upstream of the translation initiation codon in exon II fused to the firefly luciferase gene (Fig. 5D) (22). Cotransfection of *S-II*$^{-/-}$ EF cells with an S-II expression vector resulted in activation of the *Bcl-x*$_L$ reporter activity in a dose-dependent manner (Fig. 5E), suggesting that S-II positively affects *Bcl-x*$_L$ transcription.

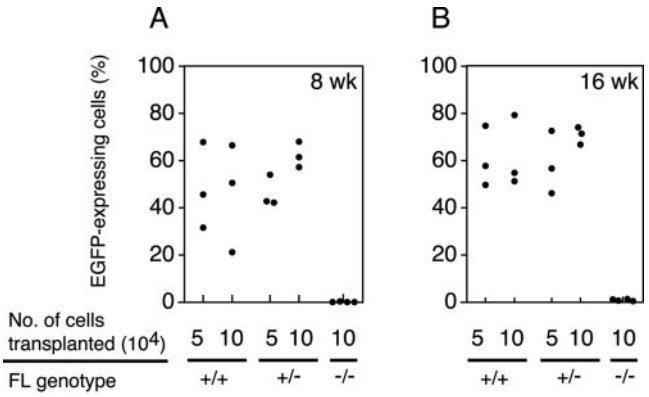

FIG. 6. Loss of repopulating potential of *S-II*$^{-/-}$ FL cells. Frequencies of EGFP-expressing cells at (A) 8 weeks and (B) 16 weeks posttransplantation in the peripheral blood of irradiated mice that received the indicated numbers of FL cells of the indicated genotypes. The symbols represent the values for individual recipient mice (+/+, $n = 3$; +/−, $n = 3$; −/−, $n = 4$).

Loss of repopulating potential of S-II-deficient hematopoietic cells. The results presented so far indicate that S-II is required for definitive erythropoiesis in FL. To further define the role of S-II in HSCs and hematopoietic lineages other than the erythroid lineage, we investigated the ability of *S-II*$^{-/-}$ FL cells to contribute to hematopoietic lineages in transplantation assays. We transplanted enhanced green fluorescent protein-expressing FL cells from E13.5 embryos into lethally irradiated adult mice. To correct for differences in liver cellularity between the three genotypes, equal amounts of FL cells (10^5 cells) were injected. Because the frequencies of phenotypically defined HSCs and multilineage hematopoietic progenitors were similar among the three genotypes (Fig. 3D and 4A), the numbers of HSCs and multilineage progenitors injected were comparable in the transplantations.

The contribution of donor cells was analyzed by quantifying the percentage of EGFP-expressing cells in peripheral blood by flow cytometry. Because lineage-committed progenitors differentiate rapidly in vivo (56), we first analyzed the contributions of donor cells in peripheral blood at 9 days after transplantation. We detected EGFP-expressing cells derived from *S-II*$^{-/-}$ FL cells, albeit at reduced frequencies compared with those from *S-II*$^{+/+}$ and *S-II*$^{+/-}$ FL (+/+ or +/−, 1.0% ± 0.4%, $n = 6$; −/−, 0.2% ± 0.1%, $n = 4$; $P = 0.004$). The result was consistent with that of in vitro colony formation assays (Fig. 3) and further supported the notion that S-II is not essential for the generation and maturation of lineage-committed progenitors. At 8 weeks posttransplantation, however, virtually no EGFP-positive donor-derived cells were detected in the peripheral blood of recipients transplanted with *S-II*$^{-/-}$ FL cells, whereas recipients that received *S-II*$^{+/+}$ and *S-II*$^{+/-}$ FL cells showed massive contributions of donor-derived cells, even when 5×10^4 cells were transplanted (Fig. 6A). Consequently, we could not detect *S-II*$^{-/-}$ FL-derived blood cells at

16 weeks after transplantation (Fig. 6B). The findings indicated that *S-II*$^{-/-}$ FL did not contain HSCs with long-term repopulating potential, although they contained phenotypically defined HSCs and multilineage hematopoietic progenitors.

DISCUSSION

The present study demonstrated that homozygous inactivation of the S-II gene in mice leads to embryonic lethality with impaired definitive erythropoiesis in FL. We also provide evidence indicating that S-II ablation results in functionally defective HSCs. While S-II is ubiquitously expressed, these results indicate that S-II has critical roles in definitive hematopoiesis but is not essential for the gross development of other tissues until midgestation.

S-II$^{-/-}$ embryos died at E13.5 to 16.5 with severe anemia. Enough nucleated primitive red blood cells were produced in the homozygous mutants to sustain fetal life, indicating that S-II is not crucial for primitive hematopoiesis. In contrast, the production of definitive erythrocytes was severely impaired. While the frequencies of the earliest erythroid committed progenitors (BFU-E), multilineage progenitors, and myeloid-committed progenitors were not significantly different between *S-II*$^{+/+}$ and *S-II*$^{-/-}$ FL, the frequency of late erythroid progenitors (CFU-E) was increased in *S-II*$^{-/-}$ FL (Fig. 3). Consistently, flow cytometry analysis indicated that the population of TER119^{high} CD71^{high} cells, corresponding to proerythroblasts and early erythroblasts, was increased but that of TER119^{high} CD71$^{medium\text{-}low}$ (late erythroblasts) was almost absent in *S-II*$^{-/-}$ FL (Fig. 4B). These results suggested that S-II is not required for the commitment of HSCs to the erythroid lineage but has an essential function in erythropoiesis at the erythroblast stage.

Epo and its signaling components are essential for definitive erythropoiesis during differentiation of erythroid-committed progenitors to erythroblasts (39, 61). Although *Epo* and *Epo receptor* gene expression and Epo-dependent activation of STAT5 were not reduced, the *Bcl-x*$_L$ expression level was significantly lower in *S-II*$^{-/-}$ FL cells (Fig. 5A and B). Bcl-x$_L$ is critical for erythroblast survival (19, 53), and its expression increases drastically during Epo-dependent terminal differentiation of primary erythroid progenitors (12). Targeted disruption of the *Bcl-x*$_L$ gene leads to embryonic lethality with widespread apoptosis of FL hematopoietic cells (28). These studies indicate that the regulation of *Bcl-x*$_L$ expression by Epo is a principal component of its signaling during erythroid terminal differentiation. Relatively small changes in *Bcl-x*$_L$ expression might perturb the balance between pro- and antiapoptotic Bcl proteins, leading to cell death. Consistent with this notion, Socolovsky et al. reported that the amounts of annexin V, one of the apoptotic cell markers, are negatively correlated to *Bcl-x*$_L$ expression levels in erythroblasts (53). Thus, increased apoptosis in *S-II*$^{-/-}$ FL is likely to be a result of reduced *Bcl-x*$_L$ expression in erythroblasts.

Multiple transcription factors positively regulate *Bcl-x*$_L$ gene expression (13, 14). Among these factors, STAT5 is activated by Epo-dependent phosphorylation (41). The activated STAT5 protein binds to sites located in the first intron of both the human and murine *Bcl-x*$_L$ genes and activates expression of these genes (51, 52). The *Stat5* expression level (data not shown) and the extent of Epo-dependent STAT5 activation (Fig. 5B) were comparable regardless of the *S-II* genotype, suggesting that the decreased *Bcl-x*$_L$ expression was not due to a lower amount of activated STAT5 proteins in *S-II*$^{-/-}$ embryos.

Reporter gene assays provided evidence that S-II participates in the activation of *Bcl-x*$_L$ transcription (Fig. 5E). It is conceivable that S-II stimulates the transcription elongation of the *Bcl-x*$_L$ gene and contributes to the production of the full-length transcript encoding functional Bcl-x$_L$ protein. It is also possible that S-II enhances transcription initiation to activate *Bcl-x*$_L$ expression. In previous studies, we demonstrated that S-II interacts directly with transcriptional activators via its N-terminal region and contributes to transcriptional activation (31, 44). Thus, recruitment of S-II to the *Bcl-x*$_L$ gene by a transactivator might be a prerequisite for efficient transcriptional activation and thus production of *Bcl-x*$_L$ transcripts during erythroblast differentiation.

The yeast *S-II* mutant strain has defects in inducing *IMD2*, *SSM1*/*SDT1*, and *GAL1* genes in response to the nucleotide-depleting drug 6-AU and galactose (40, 45, 50). *Drosophila* S-II protein is involved in efficient transcription elongation of the *hsp70* gene upon heat shock in *Drosophila* cells (1). In the present study, an Epo-responsive gene, *Bcl-x*$_L$, was down-regulated in *S-II*$^{-/-}$ FL. We propose that S-II is required for the expression of a specific subset of genes that are inducible in response to external signals and/or stimuli.

S-II deficiency also resulted in functionally defective HSCs. There was no significant difference in the frequencies of colony-forming hematopoietic progenitors and Lin$^+$ cells in FL between *S-II*$^{+/+}$ and *S-II*$^{-/-}$ embryos except for the erythroid lineage. Therefore, S-II is not required for the commitment and differentiation of the myeloid lineage. The frequencies of phenotypically defined HSCs were comparable between *S-II*$^{+/+}$ and *S-II*$^{-/-}$ FL (Fig. 4A), indicating that the generation of HSCs per se does not require S-II. The total numbers of these hematopoietic progenitors and HSCs per FL, however, were severely reduced in *S-II*$^{-/-}$ FL as a result of the hypocellularity in FL, suggesting that S-II-deficient HSCs could not expand efficiently in FL. Consistent with these findings in FL, in vivo transplantation experiments demonstrated that S-II-deficient cells have lost their capacity to reconstitute all hematopoietic lineages 8 and 16 weeks posttransplantation (Fig. 6). Thus, S-II is essential for the expansion of HSCs in vivo.

In addition to the hematopoietic defects, *S-II*$^{-/-}$ embryos developed pericardial edema at E13.5 (Fig. 1E). Targeted inactivation of the *Epo* or *Epo receptor* gene leads to an enlarged chest bulge with visible pericardial edema, an indicator of cardiac malfunction (60). In *Epo receptor* knockout mice, the myocardium and endocardium of the embryonic heart show extensive apoptosis (62). Increased cardiomyocyte death in the *S-II*$^{-/-}$ embryonic heart might lead to impaired cardiac function, resulting in pericardial edema.

Previous studies indicate that the transcription elongation factors ELL, elongin, and Spt5 are indispensable for proper embryonic development in fruit fly, mouse, and zebrafish (7, 11, 15, 27). The present study demonstrates a nonredundant role of S-II in embryonic development and extends knowledge

of the essential contribution of the RNAPII elongation machinery in the regulation of definitive hematopoiesis.

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