

# Polycomb Group Gene *rae28* Is Required for Sustaining Activity of Hematopoietic Stem Cells

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## Abstract

The *rae28* gene (*rae28*), also designated as *mph1*, is a mammalian ortholog of the *Drosophila polyhomeotic* gene, a member of Polycomb group genes (*PcG*). *rae28* constitutes PcG complex 1 for maintaining transcriptional states which have been once initiated, presumably through modulation of the chromatin structure. Hematopoietic activity was impaired in the fetal liver of *rae28*-deficient animals (*rae28*<sup>-/-</sup>), as demonstrated by progressive reduction of hematopoietic progenitors of multilineages and poor expansion of colony forming units in spleen (CFU-S<sub>12</sub>) during embryonic development. An in vitro long-term culture-initiating cell assay suggested a reduction in hematopoietic stem cells (HSCs), which was confirmed in vivo by reconstitution experiments in lethally irradiated congenic recipient mice. The competitive repopulating units (CRUs) reflect HSCs supporting multilineage blood-cell production. CRUs were generated, whereas the number of CRUs was reduced by a factor of 20 in the *rae28*<sup>-/-</sup> fetal liver. We also performed serial transplantation experiments to semiquantitatively measure self-renewal activity of CRUs in vivo. Self-renewal activity of CRUs was 15-fold decreased in *rae28*<sup>-/-</sup>. Thus the compromised HSCs were presumed to reduce hematopoietic activity in the *rae28*<sup>-/-</sup> fetal liver. This is the first report to suggest that *rae28* has a crucial role in sustaining the activity of HSCs to maintain hematopoiesis.

Key words: Polycomb group genes • *rae28* • hematopoiesis • hematopoietic stem cells • self-renewal

## Introduction

The Polycomb group genes (*PcG*)\* were originally identified from *Drosophila* mutants with impaired anteroposterior patterning which is elicited by ectopic *homeotic complex* (*HOMC*) expression. Spatial regulation of *HOMC* genes in these mutants is correctly initiated, but it is not maintained

properly, indicating that *PcG* are required, not for the initiation of transcription, but for the maintenance of repression (1–3). *PcG* products form multimeric protein complexes and epigenetically maintain the repressed state through mitosis. *PcG* are conserved from *Drosophila* to mammals. The *rae28* gene (*rae28*) is a mammalian homologue of the *Drosophila polyhomeotic* gene, which is a member of *PcG* (4, 5). Mammalian *PcG* protein complexes can be classified into two distinct sets of complexes, Polycomb complex 1 and 2 (6). *rae28* is a constituent of Polycomb complex 1 with M33, *bmi1* or *mel18*, and *Scmh1* (7–10). *Drosophila* Polycomb repressive complex 1, which possibly corresponds to mammalian *PcG* complex 1, have an ability to competitively inhibit chromatin remodeling complex, SWI/SNF (11), and also interacts with >30 proteins including general transcription factors, sequence specific-DNA binding fac-

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\*Abbreviations used in this paper: BFU-E, erythroid burst-forming unit; CFU-C, CFU culture; CFU-E, erythroid CFU; CFU-GM, granulocyte/macrophage CFU; CFU-Mix, multilineage CFU; CFU-S<sub>12</sub>, day-12 CFU spleen; CRU, competitive repopulating unit; dpc, d post coitus; HDAC, histone deacetylase; HSC, hematopoietic stem cell; LTC-IC, long-term culture-initiating cell; *PcG*, Polycomb group.

tor, zeste, and histone deacetylase (HDAC) to repress transcription from the target loci (12, 13). On the other hand, Polycomb complex 2 may include *eed*, *Enx1* or *Enx2*, *Vav*, HDAC, and *YY1*, a mammalian homologue of *Pleiohomeotic* (14–17). HDAC may constitute the repressive domain, while *zeste* and *YY1* the DNA binding domain (12, 13, 16, 17). There may be transition from PcG complex 2 to 1 during development, which is suggested in early *Drosophila* development (18).

As we previously reported *rae28*-deficient mice (*rae28*<sup>-/-</sup>) displayed abnormal anteroposterior patterning in the skeletons and rhombomeres, which is reflected in altered *Hox* gene expression in the maintenance phase (19, 20). The similar abnormal anteroposterior patterning of the skeletons was observed in mutant mice deficient in the other mammalian PcG genes, *M33*, *bmi1*, and *mel18* genes (21–24), indicating that PcG are conserved in the function as well as in the structure. Interestingly, studies of all the mutant mice deficient in one of PcG showed that PcG have an important function in lymphopoiesis (19, 21, 23, 25–28). B cell maturation was arrested between the pro- and preB cell stages in *bmi1*<sup>-</sup>, *mel18*<sup>-</sup>, *M33*<sup>-</sup>, and *rae28*-deficient mice and T cell development was also impaired in some of these mice, while preB lymphoid progenitors increased in *eed* mutant mice and lymphoproliferative disorders appeared in the later stages of the mutants (29). Although evidence has been presented that *bmi1* and *eed* respectively act as positive and negative regulators of the proliferative activity of lymphohematopoietic cells (29), the role of PcG in hematopoiesis has not been well characterized.

Accumulating evidence suggests that self-renewal and commitment of hematopoietic stem cells (HSCs) to a distinct lineage is governed by complex external signals that modulate gene expression patterns through activation of specific transcription factors (30). However, much less is known about the intrinsic genetic factors regulating HSCs (31). In this context, PcG have recently attracted attention (6).

In this study, since *rae28*<sup>-/-</sup> were lethal at the perinatal period, we performed detailed examinations of hematopoiesis in *rae28*<sup>-/-</sup> embryos and provided in vivo as well as in vitro evidence indicating that HSC activity is not sufficient to maintain hematopoiesis in *rae28*<sup>-/-</sup> during embryonic development. These findings strongly suggest that PcG have a crucial role in the regulation of HSCs.

## Materials and Methods

***rae28*-deficient Mice.** The generation of mice deficient in *rae28* by homologous recombination has been described previously. Neonates and embryos obtained by the Cesarean sections were analyzed by PCR to identify the genotype and were subjected to further hematological examinations (19).

**Hematological Analysis of Peripheral Blood and Spleen Cells.** The peripheral blood of embryos was obtained by heart puncture and the counts were determined using hemocytometer. Cell suspensions from the spleen were prepared using metal mesh. Manual differential leukocyte counts of the blood cells were performed on smears or cytocentrifuge preparations stained with May-Grünwald-Giemsa.

***Methylcellulose Assay.*** To detect myeloid colony-forming cells (colony-forming units culture [CFU-Cs]) including erythroid CFU (CFU-E), erythroid burst-forming units (BFU-E), granulocyte/macrophage CFU (CFU-GM) and multilineage CFU (CFU-Mix), fetal liver cells were suspended in MethoCult M3230 (Stem Cell Technologies) supplemented with 3 U/ml human erythropoietin (Chugai), 10 ng/ml mouse stem cell factor (Genzyme), 10 ng/ml mouse GM-CSF, and 10 ng/ml mouse IL-3 (BD PharMingen), and then plated in 35-mm Petri dishes. Colonies were scored after 14 d of incubation at 37°C in a humidified incubator with 5% CO<sub>2</sub> in air (32, 33).

***Long-Term Culture-initiating Cell (LTC-IC) Assay.*** Fetal liver cells were suspended in the myeloid long-term culture media (Methocult M5300; Stem Cell Technologies) containing 10<sup>-6</sup> M freshly dissolved hydrocortisone sodium hemisuccinate (Sigma-Aldrich) and placed on S17 feeder layer in 60-mm Petri dishes. Cultures were incubated at 33°C in humidified incubator with 5% CO<sub>2</sub> in air and fed weekly by half-media exchange. The cultures were recovered by exposure to 0.25% trypsin solution in EDTA for 5 min. The cells were washed once and were assayed for the presence of myeloid CFU-Cs (33, 34).

***Day 12 CFU-Spleen (CFU-S<sub>12</sub>) Assay.*** The day 12 CFU-spleen (CFU-S<sub>12</sub>) content of cell suspensions was determined as described originally. Appropriate dilutions of cells were injected into congenic recipients that has been irradiated with 9.0 Gy given in a single dose from a <sup>137</sup>Cs γ-ray source. The spleen was excised 12 d later, fixed in Telleyesniczky's solution, and macroscopic surface colonies were counted (32, 35).

***Long-Term Reconstitution Assay.*** C57BL/6 mice were used as recipients. The fetal liver cells (4 × 10<sup>5</sup>) were injected into lethally irradiated recipient mice and their survival was monitored. To quantitatively measure competitive repopulating units (CRUs) test cells were prepared from 14.5-d post coitus (dpc) fetal liver genetically labeled by EGFP. Lethally irradiated recipients were cotransplanted with the test cells and 10<sup>5</sup> competitor cells from the bone marrow of normal congenic mice. Reconstitution by the test HSCs was assessed for each recipient 3 mo after transplantation by analysis of peripheral blood. Peripheral blood was obtained by retroorbital sinus puncture. Animals showing >1% of EGFP<sup>+</sup>-multilineage cells 3 mo after transplantation, as determined by two-color FACS<sup>®</sup> analysis with mAbs against Gr-1, Mac-1, B220, and CD3 (BD PharMingen), were considered to be positive for repopulation by the test HSCs. The frequency of CRUs in the test cells was determined by limiting dilution analysis. A line of best fit was generated by the maximal-likelihood method, and the frequency of CRUs determined using standard statistical methods by interpolation of the number of test cells required to obtain a 37% negative response (36, 37).

For serial bone marrow transplantation, EGFP-labeled fetal liver cells containing predetermined number of CRUs were injected into lethally irradiated first recipient mice. Secondary transplantation was performed 1 mo after the primary transplantation to estimate expansion of CRUs in the primary recipients. Increasing number of bone marrow cells from the primary recipients were coinjected with competitor bone marrow cells. Animals showing both EGFP<sup>+</sup>-lymphoid and myeloid cells at a frequency of >1% were considered to be repopulated by CRUs present in the primary recipients as described above. Expansion of CRUs in the primary recipients was calculated by CRU frequency in the recipients, which was estimated by limiting dilution analysis described above.

***FACS<sup>®</sup> Analysis.*** Single cell suspensions of the fetal liver and bone marrow were incubated with specific mAbs to murine cell

surface antigens: Mac-1, Gr-1, Ter-119, CD3, CD4, CD8, B220, Sca-1, and c-kit (BD PharMingen). The antibodies were directly coupled to FITC or biotin, the latter being visualized with R-phycoerythrin-streptoavidin. Analysis was performed on a FACSCalibur™ (Becton Dickinson) and dead cells were excluded by PI (1  $\mu$ g/ml) staining and gating forward angle and side scatter of light (25).

**Preparation of Subpopulations of Hematopoietic Cells.** 10–14-wk-old C57BL/6 mice and 14.5 dpc embryos were dissected to obtain bone marrow and fetal liver cells. These cells were pooled, washed twice with PBS, made into single-cell suspension by repeating pipetting, and filtered through 40- $\mu$ m nylon mesh. Cells reacting to antibodies against Gr-1, B220, CD4, CD8, and Ter-119 were removed by using immunomagnet beads. The resulting Lin<sup>-</sup> cells were stained with FITC-conjugated anti-Sca-1 antibodies. Cell sorting was performed on a FACS Vantage™ (Becton Dickinson), to purify Lin<sup>-</sup> Sca-1<sup>+</sup> and Lin<sup>-</sup> Sca-1<sup>-</sup> fractions.

**Cell-Cycle Analysis.** Cells were pulse labeled with bromodeoxyuridine (BrdU) (Sigma-Aldrich) at 10  $\mu$ g/ml for 15 min at 37°C, harvested, washed with PBS, and fixed in cold 70% ethanol. Cells were treated with 1 mg/ml RNase at 37°C for 20 min. Cells were subjected to ice-cold 0.1 M HCl for 10 min. The washed cells were heated to 95°C. The tubes were suspended in ice-cold PBS containing 0.5% Tween 20 (PBST). The cells were stained with anti-BrdU antibody (BD PharMingen) diluted 1:100 in PBST containing 0.5% BSA at room temperature for 30 min. After washing twice with PBST, the cells were stained in 0.4 ml FITC-conjugated goat anti-mouse IgG antibody (BD PharMingen) diluted 1:100 in PBST containing 0.5% BSA. After 20 min the cells were washed and resuspended in PBS containing 10  $\mu$ g/ml PI to stain DNA. Cells were analyzed on a FACSCalibur™ (reference 38; Becton Dickinson).

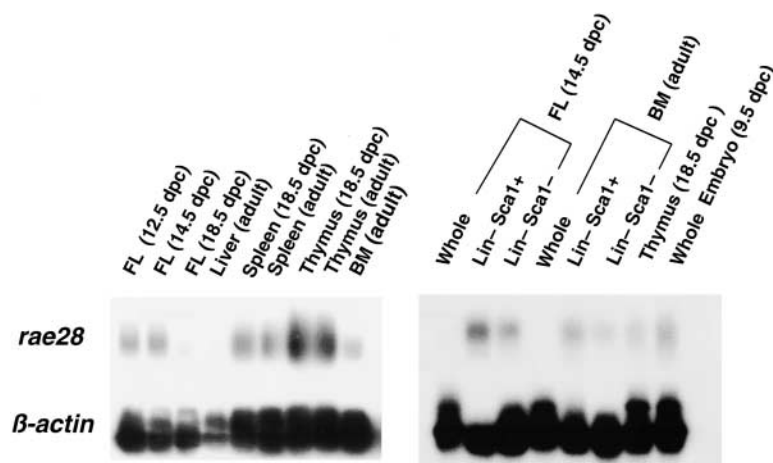
**Total cDNA Amplification and Southern Blot Analysis of the cDNA.** Total cellular RNAs were extracted from fetal liver cells and purified hematopoietic cells by the acid guanidinium thiocyanate-phenol-chloroform method. cDNA was synthesized with a 60-mer primer containing a polythymidine stretch. A short polyadenosine tail was added to the first strand of the cDNA using terminal deoxynucleotidyltransferase (GIBCO BRL). Second-strand synthesis and PCR amplification were done with the same primers. Amplified total cDNA was size fractionated on a 1% agarose gel, transferred to nylon membranes, and subsequently hybridized with <sup>32</sup>P-labeled probes (39).

## Results

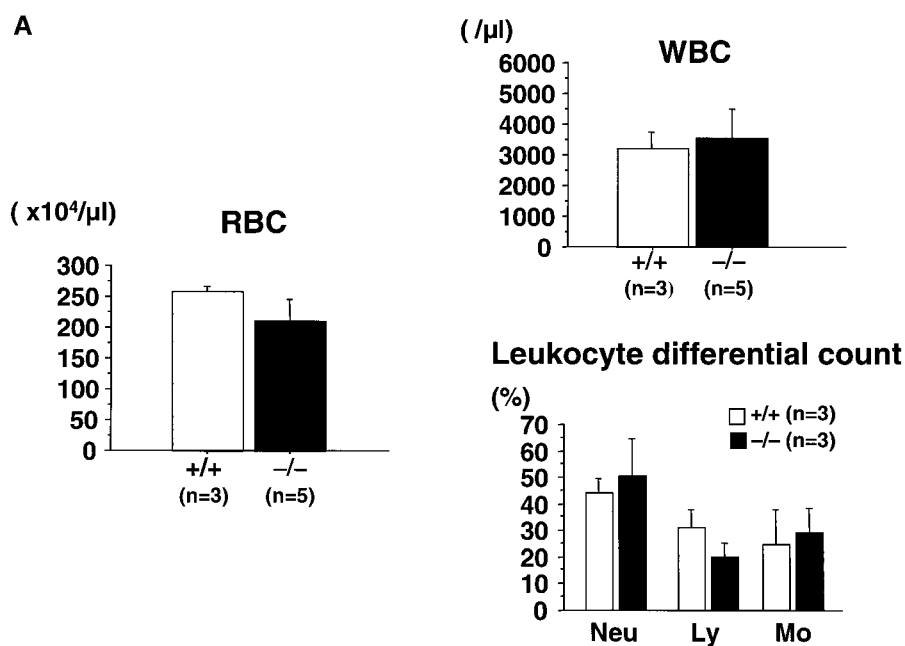
**Expression of *rae28* in Hematopoietic Cells.** As we reported previously, expression of *rae28* is predominant in the embryonic stage and almost disappears after birth except for the genital organs, brain, and thymus (4). We performed detailed examinations of *rae28* expression in the hematopoietic organs and subpopulations of the fetal liver and bone marrow cells. Total cellular RNAs were extracted from the fetal liver, spleen, thymus, and from the FACS®-purified subpopulations of the fetal liver and bone marrow cells. PCR-amplified total cDNAs were generated from mRNAs extracted from these organs and subpopulations, and it was confirmed that the amplified cDNAs preserved quantitative differences in mRNA abundance (data not shown).

The amplified cDNAs were subjected to Southern blot hybridization for analysis of *rae28* expression. This expression was detected in abundance in the fetal liver at 12.5 and 14.5 dpc, while it decreased at 18.5 dpc and was undetectable in the adult liver probably due to emigration of the hematopoietic cells (Fig. 1). *rae28* expression levels were much higher in the Lin<sup>-</sup> Sca-1<sup>+</sup> subpopulation enriched for primitive hematopoietic cells including HSCs than in the whole fetal liver and bone marrow cells (Fig. 1). It should be noted that a relatively high expression of *rae28* was preserved in the HSC-enriched subpopulation of the hematopoietic cells.

**Hematological Examination of the Peripheral Blood Cells and Spleen.** The hematological findings for the peripheral blood showed no significant differences in myeloid and erythroid lineages in *rae28*<sup>-/-</sup> neonates, although lymphoid cells tended to be reduced (Fig. 2 A). No significant differences were also observed in the 10.5-dpc primitive erythrocytes, peripheral blood and fetal liver cells at the cytological level (data not shown). The spleen in *rae28*<sup>-/-</sup> neonates was hypoplastic, although the severity varied among individuals (Fig. 2 B). The number of nucleated cells was reduced to 12.5% of the wild-type in the spleen of the mutant neonates (data not shown) but cell numbers were not reduced in the fetal liver. FACS® analysis of the spleen cells showed a reduction in B cells, but cells with the other lineage markers were conserved (data not shown).



**Figure 1.** Expression of *rae28* in hematopoietic tissues and purified subpopulations of fetal liver and bone marrow cells. PCR-amplified total cDNAs were prepared from total RNAs extracted from the hematopoietic tissues and fractionated cells and were analyzed by Southern blot analysis with a *rae28* probe. Note that high *rae28* expression was detected in Lin<sup>-</sup>, Sca-1<sup>+</sup> subpopulations. BM, bone marrow; FL, fetal liver; Whole, whole fetal liver or bone marrow cells; Lin<sup>-</sup>, Sca-1<sup>+</sup>, Lin<sup>-</sup>, Sca-1<sup>-</sup> subpopulations of fetal liver and bone marrow cells. Expression of  $\beta$ -actin is shown to confirm equal amount of cDNA on the filter.



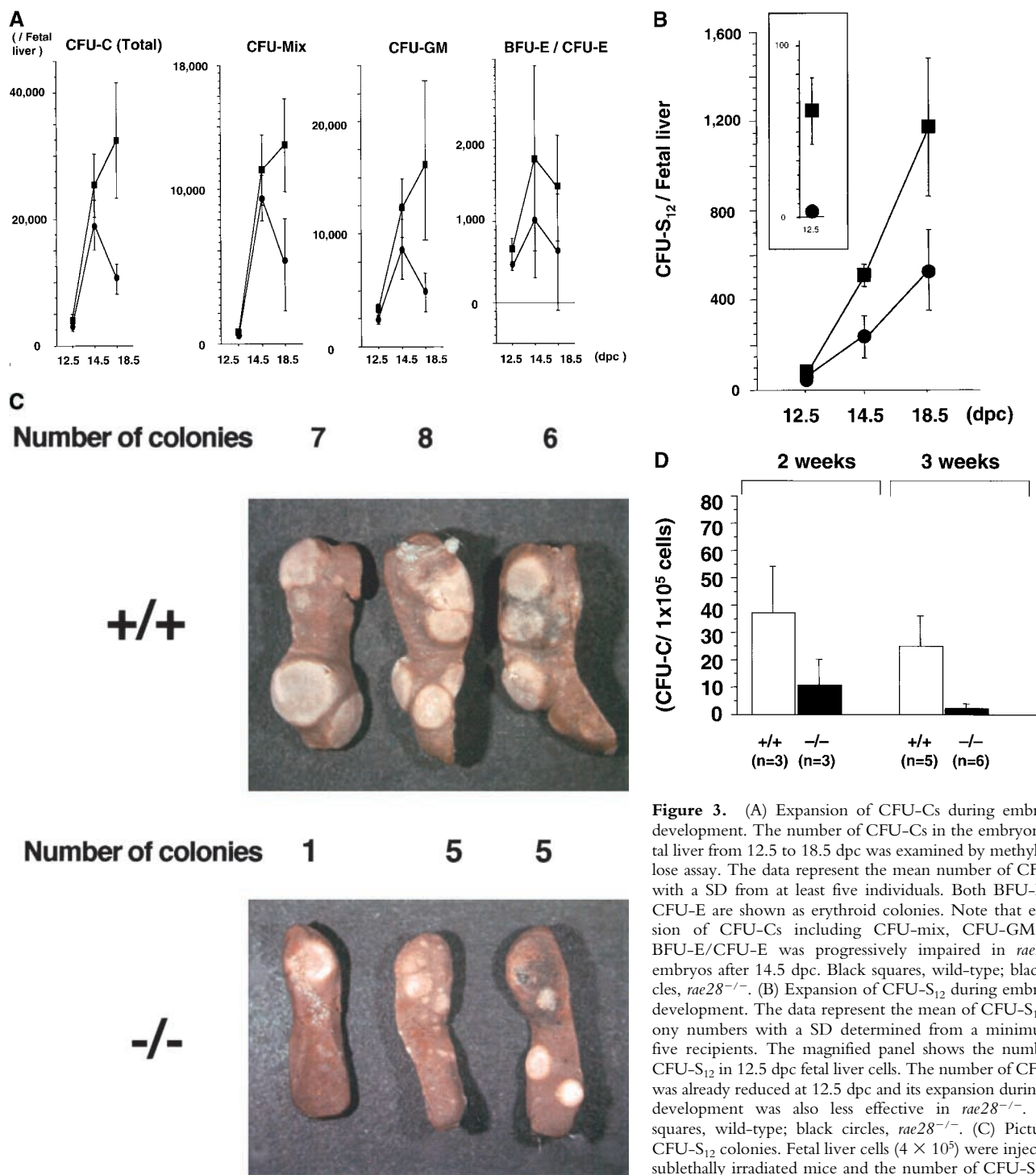
**Figure 2.** Peripheral blood cells and the spleen. (A) Peripheral blood cell counts. The data represent mean values with a SD from at least three independent individuals. (B) Picture of spleen from +/+, wild-type and -/-, *rae28*<sup>-/-</sup> mice.

*Expansion of CFU-C and CFU-S<sub>12</sub> during Embryonic Development.* Splenic hypoplasia and the reduced number of spleen cells described above suggested the presence of hematopoietic defects in *rae28*<sup>-/-</sup> embryos, because the spleen is one of the major hematopoietic organs in the neonatal period. We first examined the increase in CFU-Cs during fetal development. Although clonogenic progenitors including CFU-Mix, CFU-GM, CFU-E, and BFU-E were generated and increased until 14.5 dpc in *rae28*<sup>-/-</sup> embryos, expansion of all these hematopoietic progenitors was impaired later in the fetal development (Fig. 3 A). We next examined more premature hematopoietic progenitors in *rae28*<sup>-/-</sup> embryos, and found that CFU-S<sub>12</sub> was markedly reduced in number to one-tenth of the normal level as early as 12.5 dpc and expansion of CFU-S<sub>12</sub> was affected during embryonic development (Fig. 3 B). The size of CFU-S<sub>12</sub> colonies was also significantly reduced in *rae28*<sup>-/-</sup> (Fig. 3 C).

*LTC-IC Assay.* Activity of HSCs in the mutants at 14.5 dpc was examined in vivo as well as in vitro. First, we performed the LTC-IC assay. In this assay we determined the number of clonogenic progenitors in fetal liver cells

cultured on an S17 feeder layer for 2–3 wk. Although the limiting dilution assay to determine the frequency of LTC-IC was not performed, the number of CFU-C colonies can be assumed to reflect the frequency and proliferative capacity of LTC-IC on the feeder layer. The reduction in the number of colonies was evident in *rae28*<sup>-/-</sup> (Fig. 3 D). The number was reduced more remarkably in 3-wk than in 2-wk cultured fetal liver cells, suggesting that reduction of LTC-IC is more prominent in the upper level of the hematopoietic hierarchy in *rae28*<sup>-/-</sup>.

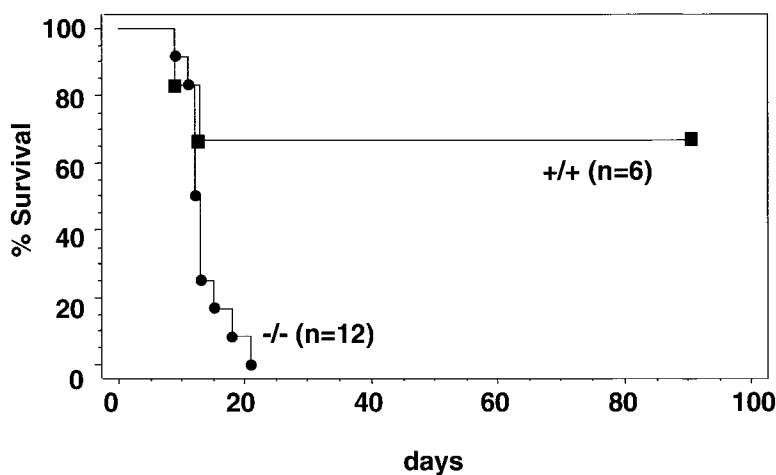
*Long-Term Reconstitution Assay.* The most rigorous test of the function of HSCs is the ability to reconstitute the hematopoietic system of recipients in which endogenous hematopoiesis has been ablated. Since the methylcellulose clonogenic, CFU-S<sub>12</sub> and LTC-IC assays showed reduction of premature hematopoietic cells in *rae28*<sup>-/-</sup> as mentioned earlier, we assessed the ability of the fetal liver cells to reconstitute the hematopoietic system in lethally irradiated mice. The fetal liver cells ( $4 \times 10^5$ ) from 14.5 dpc wild-type and *rae28*<sup>-/-</sup> embryos were injected into lethally irradiated congenic recipient mice and their survival was monitored. All the recipients injected with *rae28*<sup>-/-</sup> fetal



**Figure 3.** (A) Expansion of CFU-Cs during embryonic development. The number of CFU-Cs in the embryonic fetal liver from 12.5 to 18.5 dpc was examined by methylcellulose assay. The data represent the mean number of CFU-Cs with a SD from at least five individuals. Both BFU-E and CFU-E are shown as erythroid colonies. Note that expansion of CFU-Cs including CFU-mix, CFU-GM, and BFU-E/CFU-E was progressively impaired in *rae28*<sup>-/-</sup> embryos after 14.5 dpc. Black squares, wild-type; black circles, *rae28*<sup>-/-</sup>. (B) Expansion of CFU-S<sub>12</sub> during embryonic development. The data represent the mean of CFU-S<sub>12</sub> colony numbers with a SD determined from a minimum of five recipients. The magnified panel shows the number of CFU-S<sub>12</sub> in 12.5 dpc fetal liver cells. The number of CFU-S<sub>12</sub> was already reduced at 12.5 dpc and its expansion during fetal development was also less effective in *rae28*<sup>-/-</sup>. Black squares, wild-type; black circles, *rae28*<sup>-/-</sup>. (C) Picture of CFU-S<sub>12</sub> colonies. Fetal liver cells ( $4 \times 10^5$ ) were injected to sublethally irradiated mice and the number of CFU-S<sub>12</sub> was counted after fixation with Teleyesniczky's solution. The size as well as the number of CFU-S<sub>12</sub> colonies was reduced in *rae28*<sup>-/-</sup>. +/+, wild-type; -/-, *rae28*<sup>-/-</sup>. (D) The number of LTC-IC in the 14.5 dpc fetal liver. Fetal liver cells were cultured 2 and 3 wk on S17 feeder layer cells and cultured cells were subjected to methylcellulose assay. The data represent the mean of CFU-C colony numbers with a SD determined from at least five individuals. The number is presumed to reflect the frequency and expansion of LTC-IC during the culture. Note that the reduction of LTC-IC was more severe in 3-wk culture than in 2-wk culture. White squares, wild-type; black squares, *rae28*<sup>-/-</sup>.

liver cells died within 3 wk, while 70% of those with wild-type cells survived >12 wk, indicating that the ability to reconstitute the hematopoietic system was severely reduced in *rae28*<sup>-/-</sup> fetal liver cells (Fig. 4).

Quantitative measurement of CRUs by limiting dilution analysis was then performed to allow direct comparison of the frequency and activity of HSCs. First, we intercrossed *rae28*<sup>+/-</sup> heterozygous mice with a transgenic line ubiqui-



**Figure 4.** Kaplan-Meier survival curve of lethally irradiated congenic mice transplanted with fetal liver cells. Fetal liver cells ( $4 \times 10^5$ ) prepared from 14.5-dpc embryos were injected into congenic recipient mice and survival of the mice was investigated in the conventional facility. Black squares, wild-type; black circles, *rae28*<sup>-/-</sup>.

tously expressing EGFP (40) and prepared *rae28*<sup>-/-</sup> homozygous and wild-type embryos expressing EGFP to distinguish the test cells from the competitors. Increasing doses of fetal liver cells from the EGFP-labeled 14.5 dpc *rae28*<sup>-/-</sup> and wild-type embryos were mixed with  $10^5$  wild-type bone marrow cells as competitors and transplanted into lethally irradiated congenic recipients. Limiting dilution analysis revealed >20-fold reduction in CRU frequency in *rae28*<sup>-/-</sup> compared with the wild-type (1 per  $4 \times 10^5$  vs. 1 per  $2 \times 10^4$ , respectively,  $P < 0.01$ ; Fig. 5 A). Thus total numbers of CRUs in the *rae28*<sup>-/-</sup> and wild-type fetal liver were estimated to be 50 and 1,000, respectively, because the number of fetal liver cells was  $2 \times 10^7$  in either of *rae28*<sup>-/-</sup> and wild-type.

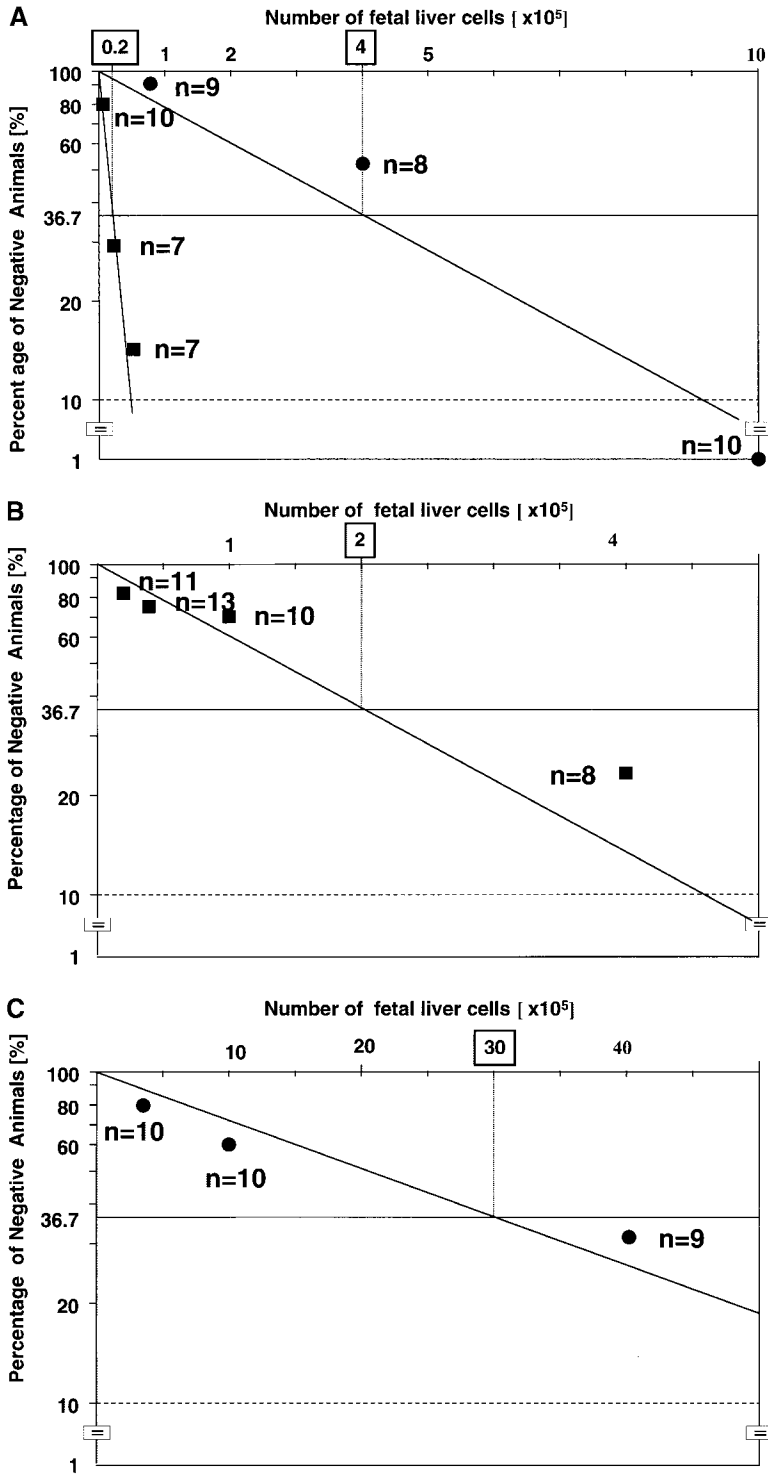
**Serial Transplantation Experiment.** To further assess the self-renewal ability of *rae28*<sup>-/-</sup> HSCs, we performed serial transplantation experiments. Fetal liver cells containing 20 CRUs, as calculated in terms of the frequency of CRUs determined as described above, were transplanted into lethally irradiated primary recipient mice. 1 mo after transplantation, bone marrow cells from the femurs and tibias of the primary recipient mice were assayed for CRU number by limiting dilution analysis in secondary recipients. The frequency of CRUs was 1 per  $2 \times 10^5$  cells in primary recipients reconstituted with wild-type CRUs (Fig. 5 B) compared with 1 per  $3 \times 10^6$  in those with *rae28*<sup>-/-</sup> (Fig. 5 C), indicating a markedly impaired regenerative capacity for CRUs in the absence of *rae28* (Table I). The number of total bone marrow cells was  $2.4 \times 10^7$  in the femurs and tibias of primary recipients reconstituted with either the wild-type or *rae28*<sup>-/-</sup> CRUs. Since bone marrow cells in the femurs and tibias are estimated to correspond to 18.7% of total marrow cells (41), 1 mo after reconstitution the numbers of CRUs in the total marrow of primary recipients reconstituted with the wild-type and *rae28*<sup>-/-</sup> CRUs were  $\sim 640$  and 43, respectively. The increase in CRUs was estimated to be 32 times within 1 mo in the wild-type, but that in *rae28*<sup>-/-</sup> was, surprisingly, limited to no more than 2.2 times (Table I). These in vivo results clearly dem-

onstrated that the self-renewal ability of CRUs was severely impaired in *rae28*<sup>-/-</sup>.

**Cell-Cycle Analysis of Fetal Liver Cells.** To examine whether hematopoietic defects in the *rae28*<sup>-/-</sup> fetal liver reflected an impaired proliferation of fetal liver cells, we examined the cell cycle status of 14.5 dpc fetal liver cells. The relative DNA content of the fetal liver cells was assessed with PI staining, whereas BrdU incorporation by the cells was measured after in vitro BrdU pulse labeling for 1 h of the isolated cells. As shown in Fig. 6, no significant difference in cycling parameters was evident.

**Expression of Genes Involved in Hematopoiesis.** To identify possible downstream target genes causing the HSC defects in *rae28*<sup>-/-</sup>, we analyzed the expression of *Hox* and genes which could potentially be involved in hematopoiesis. Total cDNAs were synthesized from mRNAs extracted from fetal liver cells at 14.5 dpc, which proved to predominantly consist of hematopoietic cells (>95%; data not shown) and *Hox* expression was detected by Southern blot analysis. We focused on a- and b-clustered *Hox* genes which are suspected to be involved in myelopoiesis. Although the expressions of *Hoxa1*, *a2*, *a3*, *a4*, *a7*, *a9*, *a10*, *b1*, *b2*, *b3*, *b4*, *b5*, and *b8* were all examined, no significant differences in the expression levels of these genes could be detected (Fig. 7 A).

We similarly analyzed expression levels of representative receptors and transcription factors which are involved in hematopoiesis in the mutant fetal liver, including *gp130*, *Epo receptor*, *G-CSF receptor*, *GM-CSF receptor*, *c-erbA $\alpha$* , *c-Mpl*, *c-jun*, *c-fos*, *c-myc*, *c-myb*, *tal1/SCL*, *AML1*, *GATA1*, *GATA2*, and *PU.1*. Expression of none of these genes was found to have significantly changed in the *rae28*<sup>-/-</sup> fetal liver cells (Fig. 7 B). We also examined expression of the *ink4a-ARF* genes (*p16<sup>ink4A</sup>* and *p19<sup>ARF</sup>*), *p21* CDK inhibitor, *B-raf*, *bax*, and *Ubiquitin* genes. No significant differences could be detected in the expression of these genes in the *rae28*<sup>-/-</sup> fetal liver (Fig. 7 B). *ink4a-ARF* gene products were further detected by Western blot analysis to confirm the findings (data not shown).



**Figure 5.** Competitive repopulation assay. (A) Frequency of CRUs in the fetal liver. The peripheral blood cells in the congenic recipient mice injected with 14.5-dpc fetal liver cells were examined to assess the long-term reconstitution capacity of HSCs. 3 mo after transplantation, the proportion of mice exhibiting  $>1\%$  EGFP<sup>+</sup> cells was used to calculate the frequency of CRU. Black squares, wild-type; black circles, *rae28*<sup>-/-</sup> (B and C) Serial transplantation experiments. Lethally irradiated recipient mice were injected with fetal liver cells including 20 CRUs, and 1 mo after injection frequency of CRUs in the primary recipient mice was determined by CRU assay. Frequency of CRUs derived from the wild-type and *rae28*<sup>-/-</sup> cells was calculated from Figs. B and C, respectively. Note the number of injected cells shown in the top column of the figures is 10 times different between Figs. B and C.

## Discussion

We have previously shown that *rae28* is involved in the early stage of B cell differentiation in a gene dosage-dependent manner (25). *rae28* is, however, predominantly expressed during early ontogeny (4) and *rae28* expression is preserved at high levels even in the adult stage in cell populations that are enriched in HSCs (Fig. 1). The detailed

analysis of premature hematopoietic cells including HSCs in *rae28*<sup>-/-</sup> reported here reveals a crucial role for *rae28* in sustaining HSC activity. Fetal liver cellularity and mature cell components were, however, relatively conserved in *rae28*<sup>-/-</sup>, probably because premature hematopoietic cells, although limited in number, can compensate for the reduc-

**Table I.** Semiquantitative Analysis of Self-Renewal Ability of CRUs

Genotype	+/+	-/-
Injected CRUs	20	20
BM cells in the femurs and tibiae	$2.4 \times 10^7$	$2.4 \times 10^7$
Frequency of CRUs	$1/2 \times 10^5$	$1/3 \times 10^6$
CRUs in the femurs and tibiae	120	8
CRUs in the entire body	640	43
Increase of CRUs	32 $\times$	2.2 $\times$

The numbers of bone marrow cells and CRUs in the primary recipients were examined one month after transplantation. The numbers of bone marrow cells represent mean values from four independent individuals. Frequency of CRUs was estimated by competitive repopulating unit assay shown in Figure 5. The numbers of CRUs in the entire body were calculated based on the previous report that bone marrow cells in the femurs and tibiae correspond to 18.7% of those in the entire body. BM, bone marrow; +/+, wild-type; -/-, *rae28*<sup>-/-</sup>.

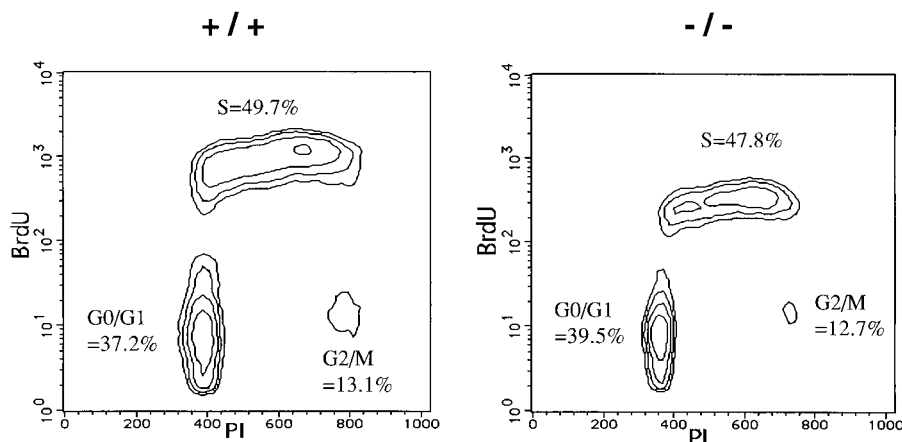
tion. The progressive reduction of clonogenic progenitors may thus be compatible with functional failure of HSCs to supply the progenitors. The number of CRUs in *rae28*<sup>-/-</sup> was only one-twentieth of the wild-type (1 per  $4 \times 10^5$  cells) (Fig. 5 A), suggesting that no more than 50 CRUs were generated in the *rae28*<sup>-/-</sup> fetal liver during the developmental period. Furthermore, *rae28*<sup>-/-</sup> CRUs underwent a net expansion of no more than 2.2-fold in transplant recipients in 1 mo, while wild-type CRUs expanded 32-fold (Table I), indicating that the transplanted *rae28*<sup>-/-</sup> CRUs were amplified much less efficiently under the conditions of this assay.

Although we could not determine whether wild-type HSCs can reconstitute the hematopoietic system in *rae28*<sup>-/-</sup> mice because *rae28*<sup>-/-</sup> embryos were lethal at the perinatal period, the result of a series of reconstituting experiments described in this study suggests that HSC defects in *rae28*<sup>-/-</sup> are manifest within the HSC population rather than in the nonhematopoietic accessory cells of fetal liver and spleen. This is also supported by the evidence that a secondary

CFU-S<sub>12</sub> assay with wild-type congenic recipients demonstrated a reduction in the self-renewal ability of *rae28*<sup>-/-</sup> CFU-S<sub>12</sub> (data not shown). The reduced activity of *rae28*<sup>-/-</sup> HSCs is presumed to be at least in part responsible for the poor expansion of CFU-S<sub>12</sub> and the progressive reduction of clonogenic progenitors during fetal development. However, the size of CFU-S<sub>12</sub> was significantly reduced and their self-renewal ability was diminished in the secondary CFU-S<sub>12</sub> assay as described earlier, suggesting that the progenies derived from *rae28*<sup>-/-</sup> HSCs were also affected in their ability.

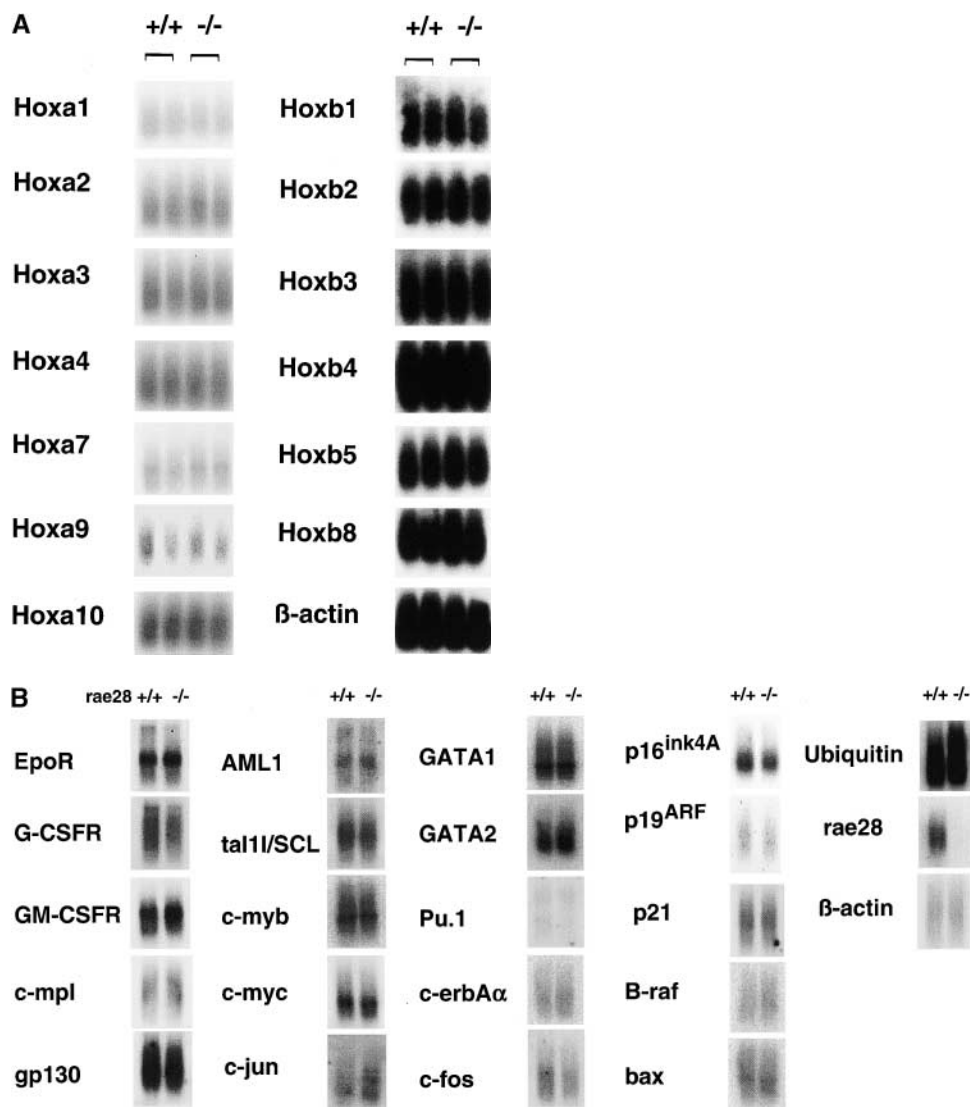
Thus, abrogated HSCs thus appear to cause a systemic defect in the hematopoietic system in *rae28*<sup>-/-</sup>, which can be expected to result in hyposplenia in *rae28*<sup>-/-</sup> neonates. Our study supports the notion that *rae28* is indispensable for sustaining the activity of HSCs. The function of *rae28* in anteroposterior patterning of the rhombomeres and paraxial mesoderm is mediated by its sustaining effect on *Hox* expression once initiated (19). Expression of multiple *Hox* genes from the three different clusters (*Hoxa3*, *a4*, *a5*, *b3*, *b4*, and *d4*) was found to be affected in the paraxial mesoderm of *rae28*<sup>-/-</sup> embryos and that of *Hoxb3* and *b4* was also affected in the rhombomeres during the maintenance phase (19, 20). Since *Hox* overexpression results in myelo- or lymphoproliferation (42–46), we examined the effect of *rae28* deficiency on the expression of *Hox* genes in the a and b clusters in the fetal liver cells, because these *Hox* genes are expressed in the myeloid cells (47). However, none of the *Hox* genes examined showed significant differences in their expression in *rae28*<sup>-/-</sup> fetal liver cells (Fig. 7 A).

We further analyzed expression of representative cytokine receptors (*gp130*, *Epo receptor*, *G-CSF receptor*, *GM-CSF receptor*, *c-erbA $\alpha$* , and *c-Mpl*), transcription factors (*c-jun*, *c-fos*, *c-myc*, *c-myb*, *tal1/SCL*, *AML1*, *GATA1*, *GATA2*, *PU.1*, and *Ikaros*; unpublished data), a tumor suppressor (*p19*) and a cyclin-dependent kinase inhibitor (*p16* and *p21*) in the *rae28*<sup>-/-</sup> fetal liver. *gp130*, a common signal transducer of LIF, IL-6, and oncostatin M, and *c-MPL*, a receptor for thrombopoietin, are assumed to have an important function in inducing self-renewal of HSCs (48–50).



**Figure 6.** Cell cycle status of fetal liver cells. Cells were pulse-labeled with 10  $\mu$ g/ml BrdU after harvest, stained with PI, and subjected to FACS<sup>®</sup> analysis. Representative BrdU-FITC/PI fluorescence profiles of wild-type and *rae28*<sup>-/-</sup> fetal liver cells are shown in the Figure. +/+, wild-type; -/-, *rae28*<sup>-/-</sup>.





**Figure 7.** Expression of a- and b-clustered *Hox* genes (A) and genes for receptors, signaling molecules and transcription factors (B) involved in hematopoiesis. Expression of the p16, p19, and p21 genes were also shown in the Figure. PCR-amplified total cDNAs were prepared from 14.5 dpc fetal liver cells and expression of the genes was analyzed by Southern blot analysis. Expression of  $\beta$ -actin is shown as an internal marker to confirm the equal amount of cDNAs on the filter. +/+, wild-type; -/-, *rae28*<sup>-/-</sup>.

*c-kit*, a receptor for SCF, synergizes with other cytokines to promote the formation of all lineages from HSCs (51). Exaggerated expression of *p16* and *p19* has been reported to cause abnormal lymphopoiesis in mutant mice overexpressing and lacking *bmi1* (52, 53), and *p21* has been shown to play a part in governing the entry of HSCs into the cell cycle (54). However, our study did not detect any significantly altered expressions of mRNAs for these molecules in *rae28*<sup>-/-</sup> fetal liver cells (Fig. 7 B). FACS<sup>®</sup> analysis confirmed that there were no differences in *c-kit*, *sca-1*, and *IL-7R $\alpha$*  expression in the fetal liver cells (data not shown).

These findings strongly suggest that development of hematopoietic cells with the lineage markers is well conserved in the *rae28*<sup>-/-</sup> fetal liver and that the affected cells are restricted to a small subpopulation of premature hematopoietic cells including HSCs. While it is not likely that abnormal expression of these genes mediates the functional defects of HSCs in *rae28*<sup>-/-</sup>, we cannot exclude the possibility that *rae28* deficiency affects expression of the genes exclusively in the specific subpopulations of hematopoietic

cells such as HSCs. Furthermore, TUNEL staining could not detect increased apoptosis in the 14.5 dpc fetal liver from *rae28*<sup>-/-</sup> (data not shown) and cell cycle status of *rae28*<sup>-/-</sup> fetal liver cells remained unaffected at 14.5 dpc (Fig. 6). Although compensatory mechanisms might preserve cell cycling, it is also unlikely that defective hematopoiesis in *rae28*<sup>-/-</sup> resulted from abnormal cell cycling and apoptosis in fetal liver cells.

To determine whether the hematopoietic defects in *rae28*<sup>-/-</sup> resulted from a defect in the development of HSCs, we examined hematopoiesis in the early stage of development. Histoanatomical examinations showed no significant abnormalities in primitive hematopoiesis in the *rae28*<sup>-/-</sup> yolk sac (data not shown). Since we could not detect CFU-S<sub>12</sub> even in the wild-type aorta-gonad-mesonephros region, development of premature hematopoietic cells in *rae28*<sup>-/-</sup> embryos at 10.5 dpc remains uncharacterized. CFU-S<sub>12</sub> was, however, already reduced in the 12.5 dpc fetal liver of the mutants and CRUs were remarkably underdeveloped at 14.5 dpc (Fig. 3 B). Although it appears

difficult to make a clear distinction as to whether or not *rae28* deficiency affects the development of HSCs, we detected CRUs, even at a low frequency, in the *rae28*<sup>-/-</sup> fetal liver. These CRUs were able to reconstitute the hematopoietic system by producing multilineage mature blood cells for >3 mo in the congenic recipients. Thus, it can be assumed that *rae28* is not essential for the development of HSCs but is required for sustaining the HSC activity for the amplification of HSCs after they are developed.

*eed* and *bmi1* were shown to regulate the proliferative activity of the mature and premature hematopoietic progenitors in, respectively, a positive and negative manner (29). However, HSCs were reported to be unaffected critically in *bmi1*-deficient mice, because erythropoiesis was not altered (23). The hematopoietic defect in *bmi1*-deficient mice was explained by the hypothesis that parallel pathways exist to compensate for the lack of *bmi1* in the fetal period and that the hematopoietic disorder becomes overt later on after the disappearance of the compensatory pathway (23). And another explanation of the hematopoietic defects was that it results from progressive overexpression of p16 CDK inhibitor from the *ink4A-ARF* locus (52). Abnormal expression of p16 and p19 from the *ink4A-ARF* locus, however, was not detected in *rae28*<sup>-/-</sup> fetal liver cells and impaired activity of HSCs is assumed to result in inefficient hematopoiesis during fetal development of *rae28*<sup>-/-</sup>. Since expression levels of transcription factors regulating lineage determination and those of lineage specific genes are conserved as far as we examined in this study, differentiation potential of HSCs seem to be relatively conserved except for B cell differentiation (25). The defect appears to be mainly focused on self-renewal ability of HSCs, which involves regulation of the cell cycle machinery and maintenance of the pluripotency.

Because PcG complex 1 appears to consist of multiple complexes varying in the constituents (6), that may reflect the functional specificity, each of the PcG members may display a different specific function in hematopoiesis. It is now possible to speculate about some plausible mechanisms underlying HSC defects in *rae28*<sup>-/-</sup> embryos. First, a PcG complex with *rae28* may play a part in maintaining the repressive state of genes which preserves the activity of HSCs, i.e., transcription of genes crucial for regulation of proliferation, commitment, and apoptosis of HSCs may be dysregulated, causing dysfunction of HSCs. This assumption is highly compatible with the expression profile of *rae28*, which shows that high *rae28* expression in primitive hematopoietic cells is progressively reduced in the course of hematopoietic cell differentiation. Second, since B cell defects in mice deficient in PcG family members are known to result at least partly from unresponsiveness to IL-7 (23, 25, 27), it may be possible that *rae28*<sup>-/-</sup> HSCs show no response or a poor response to cytokines crucial for the regulation of HSCs. In any case, since PcG-mediated repression system is presumed to play a crucial part in the regulation of HSCs, *rae28*<sup>-/-</sup> mutant mice can be expected to facilitate the clarification of molecular mechanisms underlying the regulation of HSCs.

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