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The homeodomain transcription factor *Prep1* (pKnox1) is required for hematopoietic stem and progenitor cell activity

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Abstract

Most of the hypomorphic *Prep1*$^{i/i}$ embryos (expressing 3–10% of the Prep1 protein), die between E17.5 and P0, with profound anemia, eye malformations and angiogenic anomalies [Ferretti, E., Villaescusa, J.C., Di Rosa, P., Fernandez-Diaz, L.-C., Longobardi, E., Mazzieri, R., Miccio, A., Micali, N., Selleri, L., Ferrari G., Blasi, F. (2006). Hypomorphic mutation of the TALE gene *Prep1* (pKnox1) causes a major reduction of Pbx and Meis proteins and a pleiotropic embryonic phenotype. Mol. Cell. Biol. 26, 5650–5662]. We now report on the hematopoietic phenotype of these embryos. *Prep1*$^{i/i}$ fetal livers (FL) are hypoplastic, produce less common myeloid progenitors colonies (CFU-GEMM) in cytokine-supplemented methylcellulose and have an increased number of B-cells precursors that differentiate poorly. *Prep1*$^{i/i}$ FL is able to protect lethally irradiated mice only at high cell doses but the few protected mice show major anomalies in all hematopoietic lineages in both bone marrow (BM) and peripheral organs. *Prep1*$^{i/i}$ FL cells compete inefficiently with wild type bone marrow in competitive repopulation experiments, suggesting that the major defect lies in long-term repopulating hematopoietic stem cells (LTR-HSC). Indeed, wt embryonic expression of *Prep1* in the aorta–gonad–mesonephros (AGM) region, fetal liver (FL), cKit$^+$Sca1$^+$Lin$^-$AA4.1$^+$ (KSLA) cells and B-lymphocytes precursors agrees with the observed phenotype. We therefore conclude that *Prep1* is required for a correct and complete hematopoiesis.

Keywords: Homeobox; Prep1; Hematopoiesis; Hematopoietic stem cells; Repopulating activity

Introduction

The function of *Hox* cofactors *Pbx1*, *Meis1* and *Prep1* is directly linked to hematopoiesis and leukemia. Pbx1 was identified since in pre-B leukemia it was translocated at the C-terminus of the E2A transcription factor (E2APbx1) (Kamps et al., 1990; Nourse et al., 1990). Ablation of the *Pbx1* gene in mouse causes an embryonic lethal phenotype with severe homeotic malformations, hypoplasy (or absence) of many organs, but also lymphoid, myeloid and erythroid deficiencies (Selleri et al., 2001; Dimartino et al., 2001; Sanyal et al., 2007). Likewise, while *Meis1* (and *Hoxa9*) overexpression causes leukemia in mice (Fujino et al., 2001; Nakamura et al., 1996; Schnabel et al., 2000; Wang et al., 2006), *Meis1* ko embryos show ophthalmic and angiogenic anomalies and are deficient in all hematopoietic lineages including LTR-HSC (Hisa et al., 2004; Azcoitia et al., 2005). An embryonic lethal hypomorphic *Prep1* mutant (*Prep1*$^{i/i}$), which produces 3–10% of the normal Prep1 protein level, show hypoplasy of most organs, anemia, eye malformations and angiogenic anomalies (Ferretti et al., 2006). Some *Prep1*$^{i/i}$ mice escape embryonic death but adult mice show a defective T cell development (Penkov et al., 2005).

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Meis1, Meis2 and Meis3 and the two members of the Prep sub-family, Prep1 and Prep2, bind the amino terminus of all Pbx proteins (Moens and Selleri, 2005). Prep1 was identified as a stable interactor of Pbx proteins (Berthelsen et al., 1998a), controls the half-life of Pbx1, Pbx2 and Meis1 and the expression of *Pbx3*, *Pbx4*, *Meis2* and *Meis3* genes (Longobardi and Blasi, 2003; De Florian et al., 2004; Ferretti et al., 2006). Prep and Meis proteins contain homologous, conserved Pbx-interacting-motifs (Shen et al., 1997; Knoepfler et al., 1997; Berthelsen et al., 1998b) and all of them are therefore expected to bind to Pbx. Prep (or Meis) and Pbx can form trimeric complexes with anterior Hox proteins required for the expression of specific genes (Jacobs et al., 1999; Ryoo et al., 1999; Ferretti et al., 2000, 2005; Berkes et al., 2004).

During development, blood cells are derived from the mesodermal layer of the embryo through an inductive, still not totally understood process. Throughout mouse embryogenesis, blood cells are generated sequentially from several distinct vascular tissues. These include the yolk sac blood islands (E7.5–E11), which produce blood cells with erythroid and/or myeloid potential, the intraembryonic AGM (E10–E11), and finally the FL (E11/12–birth). At birth, the BM becomes the major hematopoietic tissue (Dzierak and Medvinsky, 1995; Orkin, 2000; Choi, 2002; Bertrand et al., 2005).

Several key transcription factors are required at different stages of hematopoiesis and some can switch the lineage phenotype of cells in which they are introduced. In this way, progenitor cells can be reprogrammed, since not only the nature but also the level of expression of these regulators is important in the choice of lineage (Orkin, 2000). The erythropoietic deficiency of *Prep1*$^{i/i}$ embryos with the reduction of cMyb-positive cells and T cells' impairment in adult *Prep1*$^{i/i}$ mice (Penkov et al., 2005; Ferretti et al., 2006) suggests that Prep1 might be involved more deeply in hematopoiesis, affecting common progenitors or stem cells.

In this paper, we show that, in addition to the previously described deficiency in erythropoiesis and T cell development (Penkov et al., 2005; Ferretti et al., 2006), *Prep1*$^{i/i}$ hypomorphic embryos are also deficient in B-lymphoid differentiation. We also show that *Prep1*$^{i/i}$ FL cells fail to compete (0.5%) with wild type in competitive repopulation assays, in essentially all hematopoietic lineages. Moreover, we show that *Prep1* is expressed in KSLA and B-cells progenitors, and by cells located in embryonic hematopoietic regions that co-express markers of hematopoietic stem/progenitors cells. These data show that Prep1 is required for embryonic hematopoiesis and suggest that in its absence active long-term repopulating hematopoietic stem cells (LTR-HSC) are decreased in activity and/or in number.

Materials and methods

Prep1 targeting

Prep1^{i} mice and embryos, as well as the PCR genotyping strategy, have been described (Penkov et al., 2005; Ferretti et al., 2006).

Antibodies

Anti-Pbx1b and Pbx2 antibodies were obtained from Michael Cleary. A commercial anti-Prep1 monoclonal antibody (Upstate Biotechnology, Upstate House, Dundee, UK) was used in some experiments. Polyclonal anti-Prep1 antibodies were described previously (Berthelsen et al., 1998b).

Radioprotection assay

CD45.1^{+} C57BL/6J (Jackson Labs., Ann Arbor, MI) recipient mice (6–8 weeks old) were lethally irradiated (1100 cGy delivered in a single dose) using an X ray source (150 kV, 11.9 mA). Single-cell suspensions from CD45.2^{+} E14.5 wt, heterozygous *Prep1*$^{+/i}$ or homozygous *Prep1*$^{i/i}$ FL were prepared by passage through a cell strainer (40 μm nylon) and graded numbers of cells were transplanted by injection into the tail vein of irradiated recipients. After transplantation, recipient mice were maintained on antibiotic water containing neomycin sulfate (0.016 g/L). The survival of mice was monitored daily for 30 days and subsequently less frequently for 8 months after transplantation.

Competitive repopulation assay

We transplanted 6- to 8-week-old lethally irradiated CD45.1^{+} C57BL/6J mice with a mixture of cells. Competitor cells (200,000 per mouse) were obtained from the bone marrow of a double positive CD45.1^{+}/CD45.2^{+} mouse; 150,000, 300,000 or 500,000 immunologically labeled (CD45.2^{+}) donor (wt or *Prep1*$^{i/i}$) FL cells were mixed to the competitor cells and injected into irradiated mice. A total of 6 mice was used per genotype and dose (i.e. a total of 36 mice). After about 28 weeks the blood was withdrawn from the tail vein and the cells tested for their surface antigens by flow cytometry (CD45 to distinguish donors and host cells, and other markers to distinguish cell lineages). The antibodies are described below. Calculation of RU (repopulating units) was performed as described (Ema and Nakauchi, 2000).

Flow cytometry analysis of LTR-HSC in FL and cell sorting

In FL, the AA4.1^{+}, cKit^{+}, Sca1^{+}, Lin^{-} cells were sorted to study the HSC-enriched sub-population, and the cKit^{+} population for *in vitro* B cell assays. For peripheral blood analysis, erythrocytes were removed from peripheral blood using lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2–7.4). After blocking of specific binding with antibodies to CD16/CD32 (Pharmingen, San Diego, CA), the following antibodies were used in cytofluorimetry: PE-conjugated anti-mouse CD45.1 and biotinylated anti-mouse CD45.2 (Pharmingen, San Diego, CA), Tricolor-conjugated streptavidin (Caltag Laboratories, Burlingame, CA, USA).

For flow cytometry analysis of fetal liver cells, cKit, AA4.1, Ter119, CD71, Sca-1 and IL-7R antibodies were from Pharmingen (San Diego, CA). For bone marrow, lymph nodes or spleen cells, PE-conjugated rat anti-mouse Sca-1 (Pharmingen, San Diego, CA), the Lin cocktail of FITC-conjugated antibodies for lineage markers (B220, Gr-1, Mac-1, Ter119 and CD3, Pharmingen, San Diego, CA) were used. For the analysis of *in vitro* propagated B cells, we used PE-Cy5-conjugated anti-B220 (Pharmingen, San Diego, CA) and FITC-conjugated anti-IgM (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Flow cytometry analysis of B cell progenitors and cell sorting

FL cells from 14.5 dpc embryos were stained with the following antibodies by standard procedures after blocking the non-specific binding by anti-mouse CD16/CD32 (Pharmingen, San Diego, CA): PE-conjugated anti-mouse CD43, APC-conjugated anti-mouse B220 (Pharmingen, San Diego, CA), FITC-conjugated CD24 (Pharmingen, San Diego, CA). The CD43^{+}/B220^{+}/CD24^{-} (precursor), CD43^{+}/B220^{+}/CD24^{+} (proB) and the CD43^{-}/B220^{+} cells from 14.5 dpc FL were then sorted to evaluate the expression level of Prep1 mRNA during B cell development.

In vitro B cell expansion. Hematopoietic progenitor cells were isolated from E14.5 FL cells. Sorted cKit$^+$ cells (4×10^3/well) were seeded into 6-well plates containing a confluent monolayer of OP9 cells. All cocultures were performed in the presence of 5 ng/ml IL-7 and 5 ng/ml Flt3L. Cells were collected by forceful pipetting after 12 days, counted and analyzed by FACS. An anti-CD45 antibody was used to discriminate hematopoietic from stromal cells.

Colony-forming assays

Colony assays were carried out incubating 2.5×10^4 *Prep1*$^{+/+}$, *Prep1*$^{+/i}$ or *Prep1*$^{i/i}$ FL single-cell suspensions in 1 ml methylcellulose enriched with recombinant cytokines (Methocult GF M-3434, Stem Cell Technologies, Vancouver, Canada), in triplicate. CFU-GM and BFU-E were scored after 10 days of culture. CFU-GEMM (granulocytes, erythrocytes, megakaryocytes, macrophages CFU) after 13 days.

Immunofluorescence and immunohistochemistry

For whole-mount immunofluorescence, embryos or dissected dorsal aorta were fixed with methanol/DMSO (4:1), blocked overnight at 4 °C in blocking solution (3% BSA, 5% FBS, 20 mM $MgCl_2$, 0,3% Tween-20, in PBS containing 5% goat serum) overnight and then incubated for 1 h at room temperature with anti-Prep1 antibody (from Upstate Biotechnology, Upstate House, Dundee, UK) in 1:100 dilution, or with biotinylated anti-cKit (1:50 dilution, from Pharmingen, San Diego, CA). Embryos were then washed with PBS 0.1% Triton X-100, and incubated for 2 h at room temperature with secondary antibodies (anti-mouse Cy3-conjugated and anti-rat Cy5-conjugated, 1:400 dilution), washed and fixed with paraformaldehyde 2% for 5 min in PBS, and washed again extensively. Finally, they were incubated with biotinylated anti-Sca1 (1:50 dilution, from Pharmingen, San Diego, CA), washed and incubated with streptavidin–FITC (Pierce, Perbio), ending by an incubation with DAPI for 5 min, and final washes. Then, embryos were mounted in PBS–Glicerol (50%) and analyzed by confocal microscopy.

For immunohistochemistry, FL from E14.5 embryos were fixed, dehydrated and embedded in paraffin. Deparaffinated sections (7 μm) were blocked as for immunofluorescence, and incubated for 1 h at room temperature with anti-Prep1 antibody (from Upstate) in 1:100 dilution, or with anti-Ter119 (1:50 dilution, from Pharmingen, San Diego, CA), washed in PBS Triton X-100 (0.5%), and incubated with biotinylated secondary anti-mouse or anti-rat IgG (Vectastain® ABC Kit, Vector Laboratories, Inc.) following manufacturer's protocol. Finally, Ter119 was detected with DAB substrate kit and Prep1 with Vector Red Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Inc.).

RNA extraction, RT–PCRs and two-step PCR from B-lymphoid progenitors and KSLA cells RNA

Total RNA was extracted (see below) from sorted FL cells according to standard procedures using the Quiagen kit. Ten ng of total RNA was retrotranscribed with the Invitrogen Superscript Choice System protocol using random examers for first strand cDNA synthesis. Amplification of specific amplicons were obtained with the following primers pair:

PREP-1for, 5-ACAGACGCTAAGTATAGACAG-3
PREP-1rev, 5-AATCTGCTGGGATTGCACA-3

Amplification consisted in a first denaturation step at 97 °C for 2 min followed by 38 cycles each consisting of a denaturation step at 97 °C for 30 s, an annealing step at 60 °C for 30 s, and an extension step at 72 °C for 30 s. To end the reaction, an extension step of 10 min at 72 °C was used. 2 μl of this first step of amplification were then used in a second PCR that was performed basically as the first, but in 45 cycles. The identification of the ~500 bp amplified fragment as Prep1 mRNA was confirmed by restriction enzyme mapping with the AAT2 and DDE1 enzymes.

To detect Prep1 mRNA from KSLA cells, total RNA was isolated and reverse-transcribed from 150 sorted wt embryo cells as described above. The RNA was concentrated using a Speed Vac Concentrator (Labconco). All the RNA were retrotranscribed in a single reaction using SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and random primers. For PCR amplification, the specific primers for Prep1 have been described above. Those for Pbx1, and β-actin were:

Pbx1 forward: 5′-AGCTGGAGAAGTATGAGCAGGCATGC-3′
Pbx1 reverse: 5′-ACTGTACATCTGACTGGCTGC-3′
Actin forward: 5′-GGCATCCTGACCCTGAAGT-3′
Actin reverse: 5′-CGGATGTCAACGTCACACTT-3′

Conditions for PCR amplification were the same for all the primers: denaturation at 94 °C for 5 min, 35 cycles at 94 °C (15 s), 60 °C (30 s), 72 °C for 30 s, with a final elongation step at 72 °C for 10 min.

Statistical analysis

The significance of the data is calculated with homoscedastic two-tailed Student's *t*-test. However, significance did not change with unequal variance *t*-test.

Results

Prep1$^{i/i}$ *FL is deficient in B-lymphoid lineage maturation*

The phenotype of *Prep1*$^{i/i}$ embryos has variable penetrance and expressivity, but includes a striking pallor, smaller size, edema, anemia and a smaller liver spot that are observed in about 60% of the embryos (Ferretti et al., 2006). We also previously observed that in the presence of erythropoietin and in the absence of other cytokines, the growth of CFU-E and BFU-E colonies in methylcellulose was strongly reduced in *Prep1*$^{i/i}$ FL (Ferretti et al., 2006). The anemic phenotype was found in many but not all embryos at E14.5–16.5 and is in agreement with the death *in utero* of three quarters of the *Prep1*$^{i/i}$ embryos between E17.5 and P0 (Ferretti et al., 2006). In order to analyze other hematopoietic lineages, we tested the progenitor activity of *Prep1*$^{i/i}$ FL cells, carrying out colony-forming assays in cytokine-enriched (SCF, IL-3, IL-6, EPO) methylcellulose medium. At E14.5, the *Prep1*$^{i/i}$ FL was generally much smaller than wt (see total cell number in Table 1). The total number of colonies formed, after plating equal number of cells, by *Prep1*$^{i/i}$ FL cells was reduced but the result was not statistically significant. Also the number of CFU-GM and BFU-E colonies in the three phenotypes showed a trend towards a decrease in *Prep1*$^{i/i}$ but did not reach statistical significance. However, CFU-GEMM colonies (more immature common myeloid progenitors, granulocyte, erythrocyte, macrophage and megakaryocyte) were reduced to less than half compared to both wt and heterozygous FL ($p=0.02$ or 0.01, respectively). The data of Table 1 were obtained from a total of 5 wt, 12 *Prep1*$^{+/i}$ and 6 *Prep1*$^{i/i}$ embryos.

We then investigated whether single-lineage progenitors were affected. B cell lineage progenitors were studied by flow cytometry using the Il7R, Sca1 and cKit markers (Table 2). While the percentage of CMP (including also GMP and MEP, IL7R$^-$, cKit$^+$, Sca1$^-$) in the FL was comparable in the three phenotypes, the population enriched in more undifferentiated progenitors ($p=0.03$) (IL7R$^-$, cKit$^+$, Sca1$^+$) and the CLPs ($p=0.02$) (IL7R$^+$, cKit^{lo}, Sca1^{lo}) were in fact relatively

Table 1
Colony-forming activity of E14.5 FL cells in semi-solid methylcellulose medium

Genotype	N[a]	No. cells/FL[b]	CFC/25,000[c]	CFU-GM[c]	BFU-E[c]	CFU-GEMM[c]
+/+	5	26.2±5.7 (100%)	31.6±5.48	8.00±3.69	20.2±4.21	3.44±1.41
+/i	12	17.8±5.2 (67.9%)	35.6±6.00	7.94±2.97	23.7±4.26	3.93±1.86
i/i	6	10.9±5.2 (41.6%)	26.7±6.31	6.92±3.51	18.3±2.72	1.48±0.85
p-value	i/i vs. +/i	0.02	0.01	0.52	0.01	0.01
p-value	i/i vs. +/+	0.001	0.21	0.62	0.39	0.02

[a] Number of independent $Prep1^{i/i}$, $Prep1^{+/i}$ and $Prep1^{+/+}$ embryos analyzed.
[b] Number of fetal liver cells $\times 10^{-6}$ ± standard deviation. The number in brackets in the lower row represents the percentage (100% for wt FL).
[c] Twenty-five thousand FL cells of the indicated genotype were plated in semi-solid methylcellulose medium supplemented with SCF, IL-3, IL-6 and EPO and the number of colonies formed scored after 10 days (CFU-GM and BFU-E) or 13 days (CFU-GEMM). The results represent the average number of colonies/plate± standard deviation scored from the indicated number of experiments, each carried out in triplicate. The *p*-values were calculated by the homoscedastic *t*-test (see Materials and methods).

increased in the $Prep1^{i/i}$ FL cells. These results suggest a delay in B-cells progenitors maturation in the $Prep1^{i/i}$ FL.

We also studied whether $Prep1^{i/i}$ cells were impaired in their myeloid and B lymphoid differentiation, as was previously shown for the T (Penkov et al., 2005) and erythroid (Ferretti et al., 2006) lineages, performing *in vitro* differentiation experiments in sorted $cKit^+$ FL cells. No difference in neutrophils and macrophage differentiation was observed by culturing cells with recombinant mG-CSF or mM-CSF (data not shown).

We then analyzed B cells differentiation by culturing four thousand sorted $cKit^+$ FL cells of the three genotypes *in vitro* on the OP9 stromal cell line for 12 days (Table 3). At this time, $Prep1^{i/i}$ cells produced a higher percentage of $B220^+$ cells (in agreement with the higher proportion of CLP in FL, Table 2), and a normal percentage of $B220^+IgM^+$ cells with respect to wt and heterozygous embryos. Since the percentage of the $B220^+$ cells was higher in the $Prep1^{i/i}$ phenotype, this resulted in a decreased percent of differentiation to the IgM^+ stage (Table 3). Therefore, this experiment also points to an accumulation of precursors and a delayed B cell differentiation in $Prep1^{i/i}$ cells.

Finally, we examined the B cells progenitors at E14.5 in the FL of $Prep1^{i/i}$ embryos using flow cytometry with B220, CD43 and CD24 markers. Overall, the average number of very immature progenitors ($B220^+CD43^+CD24^-$), pro-B ($B220^+CD43^+CD24^+$) and pre-B ($B220^+CD43^-CD24^+$) cells was lowest in the wt, intermediate in the heterozygous and highest in the homozygous $Prep1^{i/i}$ embryos. However, while the data from wt and heterozygous embryos fell into a narrow range, those from the homozygous $Prep1^{i/i}$ embryos were very different from each other. In particular, the percent of Pro-B in the three different $Prep1^{i/i}$ embryos was 2.5, 2.7 and about 37% of the total, while Pre-B represented 1.5%, 1.5% and 8.7% (data not shown). Hence, one of the embryos showed a strong block of differentiation at the Pro-B to Pre-B stage. In any case, the values of the other embryos were always higher than in the heterozygous or wt embryos. The same type of variability was previously observed in a similar analysis of the erythroid differentiation in $Prep1^{i/i}$ embryos, in which again only a minority of the embryos showed a block of differentiation (Ferretti et al., 2006).

These three experiments, although performed on different embryos and directed to testing different aspects of B cells differentiation, are in essential agreement with each other and show that there is a delay/block of B cell differentiation, possibly at the Pro-B to Pre-B stage.

To support the function of Prep1 in B cells differentiation, we have analyzed the presence of Prep1 mRNA in the B-cells progenitors, sorting Pro-B, Pre-B and earlier progenitors from wt FL by FACS, and determining the presence of Prep1 mRNA by RT–PCR (see Materials and methods). The results show that Prep1 mRNA is present in very early precursors, Pro-B and Pre-B cells (Fig. 4A). The identity of the RT–PCR products was determined by restriction enzyme digestion (with AAT2 and DDE1 enzymes) of the band shown in Fig. 4A (data not shown).

Table 2
CMP and CLP cells in the E14.5 $Prep1^{i/i}$ fetal liver[a]

Embryos genotype	n[b]	$IL7R^-cKit^+Sca1^+$	$IL7R^-cKit^+Sca1^-$ CMP	$IL7R^+cKit^{lo}Sca1^{lo}$ CLP
wt	12	100±9.2	100±6.3	100±4.4
i/+	15	93.5±17.7	92.5±7.9	110±18.1
i/i	8	172.3±34.5	100.9±17.4	200±50
p-value[c]		0.03	0.95	0.02

[a] Proportion of cells with the indicated phenotype divided by the total FL cellularity and related to the wt embryos, in which the value is set=100. Data represent the mean±SEM.
[b] Number of embryos FL analyzed.
[c] Student's *t*-test (*wt* vs. *i/i*).

$Prep1^{i/i}$ embryos are defective in hematopoietic stem cells

Hematopoietic stem cells are capable of repopulating the bone marrow of a lethally irradiated mouse. We performed repopulation experiments in lethally irradiated wt $CD45.1^+$ host mice transplanting different numbers of donor $CD45.2^+$ FL cells (250,000 to 2 million) from $Prep1^{+/+}$, heterozygous $Prep1^{+/i}$ or homozygous $Prep1^{i/i}$ embryos. Mice surviving at 30 days with wt and $Prep1^{i/i}$ FL reached almost the same degree of chimerism (not shown) (87% vs. 95%, not shown). Fig. 1 shows the differential ability of FL cells to short-term protect irradiated mice. Indeed, $Prep1^{i/i}$ FL cells were much less effective in their

Table 3
In vitro B-cell differentiation of E14.5 FL cells plated onto the OP9 stromal cell line

Embryos' genotype	*n*	Total B220$^+$ ($\times 10^{-3}$)	B220$^+$ (%)	Total B220$^+$ IgM$^+$ ($\times 10^{-3}$)	B220$^+$ IgM$^+$ (%)	IgM$^+$ in the B220$^+$ subset (%)
wt	7	801±147	34.68±2.71	17.31±2.76	0.94±0.21	2.78±0.64
+/i	6	958±249	44.02±4.4	22.6±5.22	1.44±0.56	3.68±1.5
i/i	4	2091±497	80.98±1.09	18.8±6.7	1.01±0.33	1.24±0.42
p-value [a]		0.07	<0.001	0.81	0.88	0.07

Four thousand sorted c-Kit$^+$ FL cells were cultured on the OP9 stromal cell line for 12 days, and then the cells were analyzed by cytofluorimetry for the indicated markers. Data represent the mean±SEM of the number (or of the percentage) of cells with the given phenotype.
[a] Student's *t*-test (*wt* vs. *i/i*).

protective activity at 30 days post transplantation. Fig. 1 compiles the data obtained from a total of 98 mice transplanted with various doses of FL cells of the various genotypes, that suggest that *Prep1*$^{i/i}$ FL contain fewer, or less functional, short-term repopulating progenitors (STR-HSC). Indeed, only at the highest cell dose (2 million cells per animal), *Prep1*$^{i/i}$ FL cells protected as efficiently as wt or heterozygous, while with 250,000–500,000 cells the protection activity was about one fourth than wt or heterozygous cells (Fig. 1 and data not shown). In any case, those mice still alive at 30 days lived for at least 8 months (not shown). This experiment allows us to conclude that while short-term protective activity is impaired in *Prep1*$^{i/i}$ FL cells, the long-term repopulation activity may still be normal but the number or activity of LTR-HSC may be reduced.

To test this point, we sacrificed the long term protected mice of the above experiment 6–8 months after transplantation and studied their BM and peripheral hematopoietic organs: the data indicated an overall deficient repopulating activity of *Prep1*$^{i/i}$ FL cells. In BM (Table 4) and spleen (data not shown), the number of *Prep1*$^{i/i}$ donor-derived cells (i.e. CD45.2$^+$) of the B-lineage (B220$^+$, p=0,008) and myeloid lineage (Gr1$^+$ and Mac1$^+$, p=0.04) was reduced 30% to 50% with respect to wt. No difference in donor contribution to Ter119$^+$ cells was observed in either BM or spleen. The total number of cells in BM was decreased about 20% in the *Prep1*$^{i/i}$ FL cells transplanted mice. The deficient repopulation in BM thus affects mostly B (57% of wt) and less the myeloid (75% of wt)

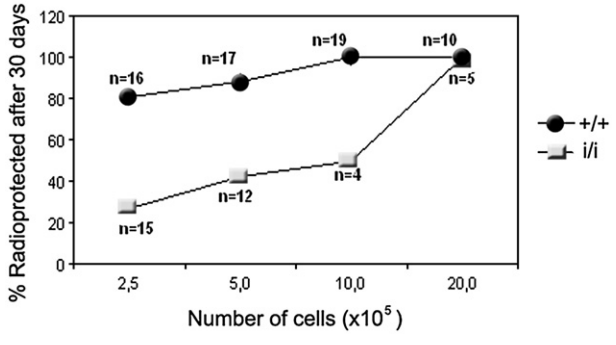

Fig. 1. Repopulation activity of *Prep1*$^{i/i}$ E14.5 FL cells. Short-term (30 days) radioprotective activity of E14.5 wt and *Prep1*$^{i/i}$ FL cells. Lethally irradiated CD45.1$^+$ mice were transplanted with different doses of E14.5 FL cells from both wt and *Prep1*$^{i/i}$ CD45.2$^+$ embryos. Survival of mice (in percent) is shown at various cells doses of transplanted cells, 30 days after transplantation. Each single point represents the average of several mice, indicated by "*n*". The phenotypes of the transplanted cells are indicated.

population. In spleen and lymph nodes, in addition, *Prep1*$^{i/i}$ CD8$^+$/TCRαβ$^+$ and CD4$^+$/TCRαβ$^+$ T-lymphocytes were reduced by two thirds (not shown). This agrees with our previous data showing a similar deficiency in donor *Prep1*$^{i/i}$ FL cells in thymus with accumulation of double positive T-cells and a decrease in single positive CD4$^+$ and CD8$^+$ cells (Penkov et al., 2005). Peripheral blood analysis also showed a decreased number of mature CD4$^+$ and CD8$^+$ T-cells, an almost normal number of B220$^+$ cells, and decreased Gr1$^+$ as well as Ter119$^+$ cells of *Prep1*$^{i/i}$ (i.e. CD45.2$^+$) origin (Table 5). These data show that repopulated mice are deficient in most hematopoietic lineages.

We also analyzed the colony-forming activity of BM cells from 10 transplanted mice in cytokine-supported methylcellulose colony assays. BM from *Prep1*$^{i/i}$ FL cells-transplanted mice formed a normal number of CFU-GM and BFU-E and a much smaller number of common myeloid progenitor colonies (CFU-GEMM, 33% of wt, p=0.059), as observed (Table 1) with embryonic cells. The total colony-forming activity of the bone marrow (two femurs and two tibiae) was reduced by 10% (not shown) but CFU-GEMM colonies were reduced about fourfold (Table 6).

The above results suggest that the *Prep1*$^{i/i}$ FL has a deficient activity of both STR-HSC (Fig. 1) as well as LTR-HSC (Tables 4–6). We therefore used flow cytometry to sort highly enriched hematopoietic stem cells from the E14.5 FL. We determined the number of cells displaying cKit$^+$Sca1$^+$Lin$^-$ AA4.1$^+$ (KSLA cells) surface properties, after gating out of Ter119$^+$ (Hsu et al., 2000). In the two tested embryos, the absolute number of KSLA cells per E14.5 *Prep1*$^{i/i}$ FL was reduced four fold compared to wt (3500 vs. 13,500) but in both cases KSLA cells were about 0.06% of the total FL cells. Since KSLA cells are highly enriched in LTR-HSC (Hsu et al., 2000), this result does not support a specific reduction of these cells in *Prep1*$^{i/i}$ FL. However, although highly enriched in LTR-HSC, KSLA cells cannot be assimilated to LTR-HSC. Hence our data suggest that the deficient repopulation activity of *Prep1*$^{i/i}$ FL may not be due to a decrease in the absolute number of LTR-HSC but rather to their lower activity (survival or other).

If Prep1 has a role in hematopoietic stem cells physiology, it should be expressed in KSLA cells. Therefore, we examined the expression of Prep1 in these cells preparing total RNA and performing RT–PCR on the sorted cells. As shown in Fig. 4B, these cells produced Prep1 (and Pbx1b) mRNA.

To test the hypothesis of a hematopoietic stem cells deficiency in the *Prep1*$^{i/i}$ embryos, we performed competitive

Table 4
Analysis of the bone marrow of mice transplanted with *Prep1*$^{i/i}$ E14.5 FL cells shows a deficient long-term repopulation activity

Donor	N[a]	CD45.2$^+$ B220$^+$ ($\times 10^6$)[b]	CD45.2$^+$ Gr1$^+$/Mac1$^+$ ($\times 10^6$)[b]	CD45.2$^+$ Ter119$^+$ ($\times 10^6$)[b]	Total cell number ($\times 10^6$)[b]
+/+, +/*i*	11/57	18.1±6.08	41.1±11.06	40.0±13.9	111±19.7
i/*i*	7/16	10.3±4.05	30.5±8.5	43.2±7.37	89.4±17.7
p-value		0.008	0.04	0.53	0.03

Lethally irradiated CD45.1$^+$ mice were transplanted with 200,000 to 2,000,000 CD45.2$^+$ FL cells of the indicated donor genotype. Cell suspensions of the bone marrow of surviving mice (mostly transplanted with 2 million cells in the case of the *Prep1*$^{i/i}$ FL) were analyzed at 6–8 months after transplantation. A total of 98 mice were transplanted with wt and heterozygous (total of 11 embryos) or homozygous *Prep1*$^{i/i}$ FL cells (7 individual embryos), and 73 survived. The data are taken from the animals of the experiment shown in Fig. 1.

[a] The first number shows the number of embryonic FL employed for each genotype. The second, the total number of surviving mice examined. Data are taken from the surviving mice of the experiment shown in Fig. 1. The *p*-values were calculated by the homoscedastic *t*-test (see Materials and methods).

[b] Data show the average number of CD45.2$^+$ cells expressing the different cell surface markers scored by flow cytometry±standard deviation. Each measurement was performed in triplicate.

repopulation experiments transplanting lethally irradiated CD45.1$^+$ mice with a combination of cells: 200,000 competitor BM cells from wt doubly labeled CD45.1$^+$/CD45.2$^+$ mice, along with 150,000, 300,000 or 500,000 E14.5 FL donor cells from either CD45.2$^+$ wt or *Prep1*$^{i/i}$ littermate embryos. The E14.5 FL donor cells were derived from 1 wt and 1 *Prep1*$^{i/i}$ embryo (total number of cells/liver: 27 and 8 million, respectively). Each cell dose was transplanted into a total of 6 mice per group and the surviving mice (32/36) were analyzed about 28 weeks post-transplantation. Peripheral blood from the transplanted mice was analyzed by flow cytometry for the expression of the CD45.1 and CD45.2 markers, and the relative contribution of the three populations (assessed by the number of CD45.1$^+$/CD45.2$^+$ competitor, and CD45.1$^-$/CD45.2$^+$ donor cells), was used to evaluate chimerism. The (very low) contribution of the CD45.1$^+$ host cells (equal in all mice) was not included in the calculations. The data from all cell doses were pooled for each genotype and expressed as RU per 10^5 transplanted cells. Repopulating units (RUs) were calculated from the percentage of CD45.2$^+$ versus CD45.1$^+$CD45.2$^+$ circulating cells (Ema and Nakauchi, 2000), as indicated in the legend to Table 7. The data show that *Prep1*$^{i/i}$ FL had about fivefold less repopulating activity than wt (2.18 vs. 10.8, $p=0.0001$). Since the total number of FL cells in *Prep1*$^{i/i}$ was about one third than in wt, the overall repopulating activity of the *Prep1*$^{i/i}$ FL was reduced over sixteen fold compared to wt (174 vs. 2880 RU). This decrease is much higher than that of KSLA cells (see above) and therefore may suggest that *Prep1*$^{i/i}$ FL has a major deficiency in the activity, rather than number, of LTR-HSC.

We also analyzed peripheral blood for the competitive repopulating activity of different hematopoietic lineages. As shown in Table 8, *Prep1*$^{i/i}$ FL-transplanted mice had a consistently lower number of RU than wt, in agreement with the overall lower repopulating activity. The statistical significance was high for the lymphoid lineage, but represented a trend for the myeloid and erythroid lineages. Of particular interest, and in agreement with the previously observed T cells deficiency of *Prep1*$^{i/i}$ adult mice (Penkov et al., 2005), *Prep1*$^{i/i}$FL competed 16-fold less for the reconstitution of the CD4$^+$ T-lineage ($p=0.0001$) and 6-fold less for the CD8$^+$ T-cells ($p=0.0002$). Moreover, they competed 3- to 4-fold less for the B-cells ($p=0.008$) reconstitution.

In conclusion, despite the variability among *Prep1*$^{i/i}$ embryos, all data indicate that the reduction of Prep1 is accompanied by a profoundly deficient hematopoiesis, in particular in the B-lymphoid lineage.

Expression of Prep1 in embryonic hematopoietic stem/progenitor cells

The multiple-lineage phenotype of the *Prep1*$^{i/i}$ embryos points out that the defect may lie in LTR-HSC or in early hematopoietic progenitors. We therefore used immunofluorescence and immunohistochemistry and analyzed the wt embryos to search for *Prep1* expression in active hematopoietic sites. The AGM region at E10.5–12.5 and the FL at E14.5, are sites where embryonic hematopoietic activity and precursors are concentrated (Hsu et al., 2000). At E10.5 we detected by whole-mount confocal immunofluorescence a Prep1-positive area around and

Table 5
Peripheral blood analysis of mice transplanted with *Prep1*$^{i/i}$ FL[a]

Genotype	N[b]	Cells/ml[c]	% Chimerism	CD45.2$^+$ CD4$^+$ TCRβ$^+$/ml[a]	CD45.2$^+$ CD8$^+$ TCRβ/ml[a]	CD45.2$^+$ B220$^+$/ml[a]	CD45.2$^+$ Gr1$^+$ Mac1$^+$/ml[a]	CD45.2$^+$ Ter119$^+$/ml[a]	Total Ter119$^+$/ml[d]
i/+, +/+	7/42	13,140±4468	96.1±1.61	1198±499.4	947±349	6940±834	1867±670.2	7734±2630	7986±2699
i/*i*	9/18	8227±2816	86.1±5.21	561±395	244±175	5049±139	1145±235.7	3731±2074	5359±1437
p-value		0.02	0.0001	0.015	0.0003	0.16	0.017	0.005	0.048

The *p*-values were calculated by the homoscedastic *t*-test (see Materials and methods).

[a] CD45.1$^+$ mice were lethally irradiated and transplanted with different doses of FL cells of CD45.2$^+$ mice (see Fig. 1). Analyses were performed 8 months after transplantation.

[b] The first number indicates the embryonic FL employed per genotype; the second number, the mice in which they were transplanted.

[c] Number of cells/ml blood expressing the CD45.2$^+$ cell surface marker.

[d] This column shows the total number of Ter119$^+$ cells (CD45.1$^+$ plus CD45.2$^+$ cells).

Table 6
Colony-forming activity of bone marrow cells from mice transplanted with *Prep1*$^{i/i}$ FL[a]

Genotype	*N*[b]	CFU-GM	CFU-GEMM	BFU-E	Cells per BM[c]	CFU-GM per BM[d]	CFU-GEMM per BM[d]	BFU-E per BM[d]
+/+ or +/*i*	5/10	27.7±9.75	2.03±1.45	20.4±5.43	119.6±16.1	61,023	4466	44,941
i/i	5/10	27.2±5.65	0.67±0.54	21.7±3.1	96.5±14.8	47,416	1192	38,626
p-value[e]		0.90	0.059	0.56	0.13	–	–	–

[a] Fifty thousand cells of the indicated genotype were plated in semi-solid methylcellulose medium and the number of colonies formed scored after 10 days (CFU-GM and BFU-E) or 13 days (CFU-GEMM). The results represent the average number±standard deviation, of colonies/plate scored from the indicated number of experiments (*N*), each carried out in triplicate on independent BM from mice transplanted with 2,000,000 of FL cells of the indicated genotype (Fig. 1). Mice were sacrificed and analyzed 8 months after transplantation.
[b] Number of independent *Prep1*$^{i/i}$, *Prep1*$^{+/i}$ and *Prep1*$^{+/+}$ FL (2 million cells per mouse) employed for transplantation. The second number shows the total number of mice analyzed (i.e., each FL was used for two mice).
[c] Total number of cells $\times 10^{-6}$, obtained from two femurs and two tibiae of five animals/group, in the two groups of transplanted mice.
[d] Calculated from the measured average values reported in the third, fourth and fifth columns.
[e] *p*-values are calculated with a paired *t*-test. When calculated with a homoscedastic *t*-test, the values (corresponding to the third, fourth and fifth columns) were 0.93, 0.08 and 0.74, respectively.

within the dorsal aorta and somites, outlining intersomitic vessels (Figs. 2A, B, D). E10.5 *Prep1*$^{i/i}$ embryos gave no staining (not shown). In this area, Prep1 was expressed in most cells, also expressing cKit (Figs. 2E, F). At higher magnification (Fig. 2C), cells within the aorta were shown to co-express Prep1 with Sca1 and cKit (Figs. 2G–L), antigens commonly used to identify HSC (Orelio et al., 2004). Thus, this experiment reveals the presence of Prep1 in the AGM region, and its co-expression of Prep1, Sca1 and cKit, in a large fraction of cells, in keeping with the possibility that Prep1 is expressed by HSC.

Since *Prep1*$^{i/i}$ embryos often die of anemia, we also tested for the co-localization of Prep1 and Ter119, a marker of erythropoietic progenitors, in the FL. At E14.5, coronal FL sections were analyzed by immunohistochemistry with antibodies for Prep1 (red) and Ter119 (black). As shown in Fig. 3, Prep1 was also expressed in the FL in a fraction of cells positive for Ter119 (compare for example panels G and H), as well as in Ter119-negative cells (panel I). We also found co-localization of Prep1 with CD31, a marker of both hematopoietic and (mostly) endothelial cells (data not shown). These data confirm previous data showing co-expression of Prep1 in cMyb-positive and cKit-positive cells in the FL (Ferretti et al., 2006), and show that Prep1 is expressed in a region of active hematopoiesis.

To finally test for the expression of Prep1 also in adult hematopoietic stem and/or progenitor cells, we also sorted cells from the adult bone marrow (BM) in populations enriched in HSCs (Sca-1$^+$/Lin$^-$), or progenitor cells (Sca-1$^+$/Lin$^+$) or more differentiated cells (Sca-1$^-$/Lin$^+$) (Pineault et al., 2002), and immunoblotted nuclear and cytoplasmic extracts from 5×10^6 cells from each sorted population with anti-Prep1 and anti-Pbx1b antibodies (Fig. 4C). Prep1 was found in the nuclear as well as cytoplasmic extracts of Sca-1$^+$/Lin$^-$ cells. In Sca-1$^+$/Lin$^+$ subpopulations, Prep1 was still present but mostly in cytoplasmic extracts, while it was almost undetectable in the Sca-1$^-$/Lin$^+$ subpopulation. Pbx1b was detected in the nucleus of the Sca-1$^+$/Lin$^-$ bone marrow cell population, as expected (Selleri et al., 2001; Dimartino et al., 2001), but disappeared upon their maturation to precursors and more differentiated cells.

All these data support the expression of Prep1 in the hematopoietic compartment, both in the embryo (AGM and FL) and in the adult (BM), in cells that represent hematopoietic stem and/or progenitor cells.

Table 7
Prep1$^{i/i}$ FL contains a lower number of repopulation units (RU)[a]

	n wt[b]	RU[c]	*n Prep*$^{i/i}$	RU[c]	*p*-value[d]
Total RU/10^{-5}[e]	17	10.8±1.64	15	2.18±0.4	0.0001
RU/FL[f]		2880		174	

[a] C57BL/6, CD45.1$^+$ mice were lethally irradiated and then transplanted with a combination of 200,000 BM cells (competitor cells) from double-positive CD45.1$^+$/CD45.2$^+$ and different amounts of single-positive CD45.2$^+$ donor cells (from one E14.5 wt or *Prep1*$^{i/i}$ FL). The blood from surviving mice was drawn after about 28 weeks, and the presence and percent of the CD45.1$^+$ and CD45.2$^+$ surface markers measured by flow cytometry on circulating blood cells. In the calculation of RUs, the contribution of the CD45.1$^+$ host was not taken into account as it represents a small and fixed percentage (around 3%) in all mice.
[b] Number of transplanted mice surviving at the time of the analysis. Overall, 32 out of 36 mice survived.
[c] Results are presented as mean±SEM.
[d] The *p*-value was calculated with the homoscedastic *t*-test.
[e] Repopulating units. This number was calculated as percentage of circulating CD45.2$^+$ cells divided by percentage of circulating competitor-derived cells (CD45.1$^+$/CD45.2$^+$). Each value was then normalized to 100,000 competitor cells (Ema and Nakauchi, 2000).
[f] Average RUs per fetal liver. In this case, the wt FL contained 26.7 million cells, and the *Prep1*$^{i/i}$ FL 8 million cells.

Discussion

TALE class homeodomain transcription factors (Pbx, Prep and Meis) are key regulators of embryonic development in all

Table 8
Repopulating activity of *Prep1*$^{i/i}$ (CD45.2$^+$) FL-repopulated mice are deficient in all hematopoietic lineages[a]

Marker	RU/10^{-5} wt	RU/10^{-5} i/i	*p*-value
CD8$^+$	3.37±0.47	0.21±0.05	0.0001
CD8$^-$ CD3$^+$	5.83±1.06	0.95±0.21	0.0002
Ter119$^+$	1.37±0.46	0.61±0.23	0.16
B220$^+$	9.67±2.18	2.66±0.98	0.008
Gr1$^+$/Mac1$^+$	5.4±1.56	2.19±0.98	0.1

[a] Data were collected in the experiment illustrated in Table 7 and details are given in the legend to Table 7, in Footnote b. Results are presented as mean±SEM. *p*-values were calculated by the homoscedastic *t*-test.

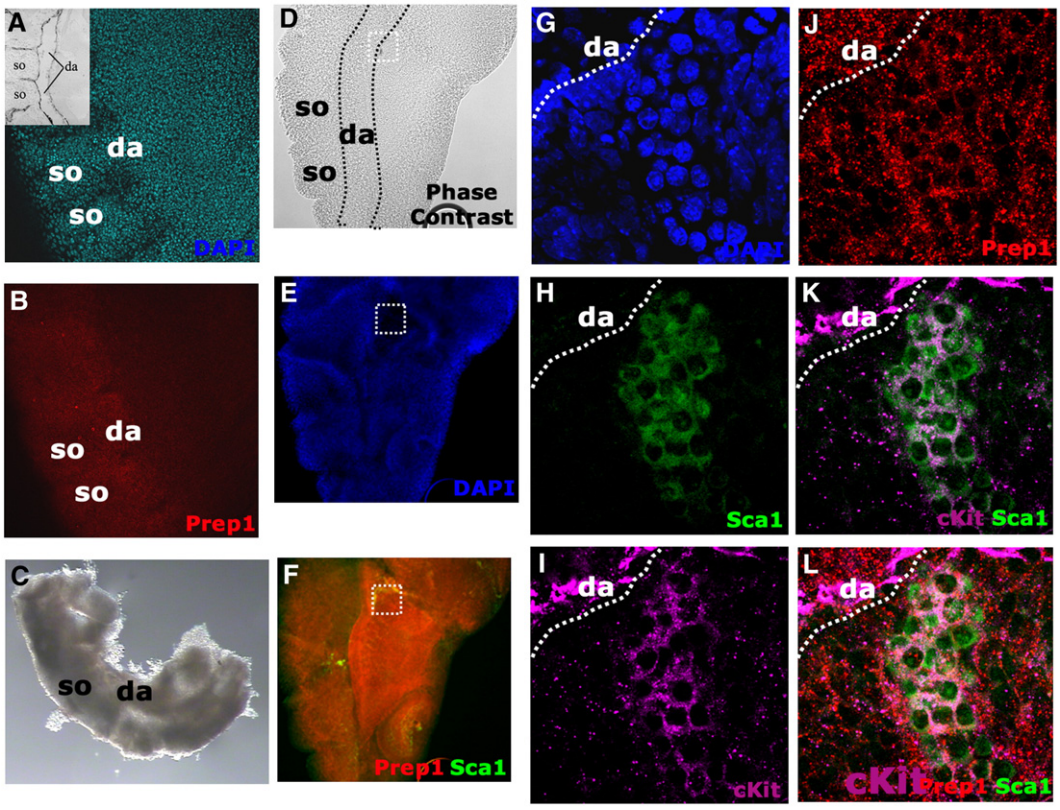

Fig. 2. Immunofluorescence analyses of *Prep1* expression in the AGM region. (A–B) Confocal expression analysis of Prep1 in whole-mount E10.5 embryos in the region of the dorsal aorta (da) and somites (so) in wild-type embryo, showing red staining for Prep1 (B) and DAPI in blue (A). Notice the dorsal aorta and intersomitic vessel regions showing *Prep1* expression (see also scheme in gray, inside A). (C) Dissected AGM region used for whole-mount analysis. (D) Phase contrast analysis of the region, showing schematically the aorta. (E, F) Same region as in panel D, stained for DAPI and Prep1, respectively. The square depicts the region magnified in panels G–L. (G–L) AGM region stained with DAPI, Prep1, Sca1 and/or cKit antibodies, as indicated. Notice $Sca1^{+}cKit^{+}Prep1^{+}$ cells inside and lining the aorta. Panel L shows the merging of the Sca1, cKit and Prep1 staining. Magnification: A–B=40×; C, D–F=20×; G–L=63× with a zoom of 2.

organisms (Moens and Selleri, 2005). While the ablation of the Hox genes generally has a local phenotype, the ablation of some TALE genes has a pleiomorphic, embryonic lethal phenotype (Hisa et al., 2004; Azcoitia et al., 2005; Ferretti et al., 2006; Selleri et al., 2001), in agreement with TALE proteins regulating the activity of multiple Hox genes and proteins. *Meis1* and *Pbx1* ko embryos die at E14.5 (1, 17, 35), *Pbx3* ko at P0 (Rhee et al., 2004) while *Pbx2* ko has no apparent phenotype (Selleri et al., 2004). Among TALE genes, the *Prep1* ko has the most drastic phenotype with intra-uterine death at E7.5, i.e. before gastrulation (Fernandez LC, Jenkins N, Copeland NG and Blasi F, in preparation). Indeed, the analysis of the $Prep1^{i/i}$ hypomorphic embryos reveals that *Prep1* controls the level of Meis and Pbx family members either at the pre- or at the post-transcriptional stage and hence the entire TALE–Hox pathway and the expression of at least some *Hox* genes (De Florian et al., 2004; Penkov et al., 2005; Ferretti et al., 2006).

In this paper, we have shown that $Prep1^{i/i}$ embryos display a major FL hypoplasy (Table 1) which affects the number of KSLA cells, highly enriched in LTR-HSC cells (Hsu et al., 2000). When analyzed in detail, $Prep1^{i/i}$ FL cells showed a deficient proliferation of CFU-GEMM colonies in methylcellulose (Table 1). Moreover, we have observed by three different approaches that $Prep1^{i/i}$ FL cells have a tendency to a delayed or blocked differentiation of the B-lymphocytes precursors (Tables 2 and 3). These results are in agreement with the expression of Prep1 in stem cells and pro-B and pre-B progenitors (Fig. 4).

The defect in hematopoietic progenitors became even more evident when we analyzed the repopulating activity of $Prep1^{i/i}$ FL cells. Indeed, the 10-fold less short-term radio-protection (Fig. 1) agrees with the reduced number of CFU-GEMM and with the reduced ability to differentiate of B cells progenitors. However, even if only at high doses of $Prep1^{i/i}$ cells, mice were long-term radio-protected. However, these mice showed deficiencies in essentially all lineages, in peripheral blood and hematopoietic organs (Tables 4–6). These data complement the previously observed deficiency in erythropoiesis during the embryonic life (Ferretti et al., 2006) and in T-cells development of the adult $Prep1^{i/i}$ mice (Penkov et al., 2005). Hence $Prep1^{i/i}$ embryos are deficient in the development of essentially all lineages, possibly because of defects in the LTR-HSC. Indeed, competitive repopulation assays (Tables 7 and 8) showed that $Prep1^{i/i}$ FL cells had a lower RU activity than wt. This assay gives no information on the actual number of LTR-HSC (Ema and Nakauchi, 2000) as no dilution titration was carried out due to the variable inter-embryonic penetrance and expressivity of the $Prep1^{i/i}$ mutation (Ferretti et al., 2006). However, since the percentage of $Prep1^{i/i}$ KSLA cells reflected the overall FL hypoplasy, while the reduction in RU was much more drastic, the results likely indicate a reduced activity rather than number of

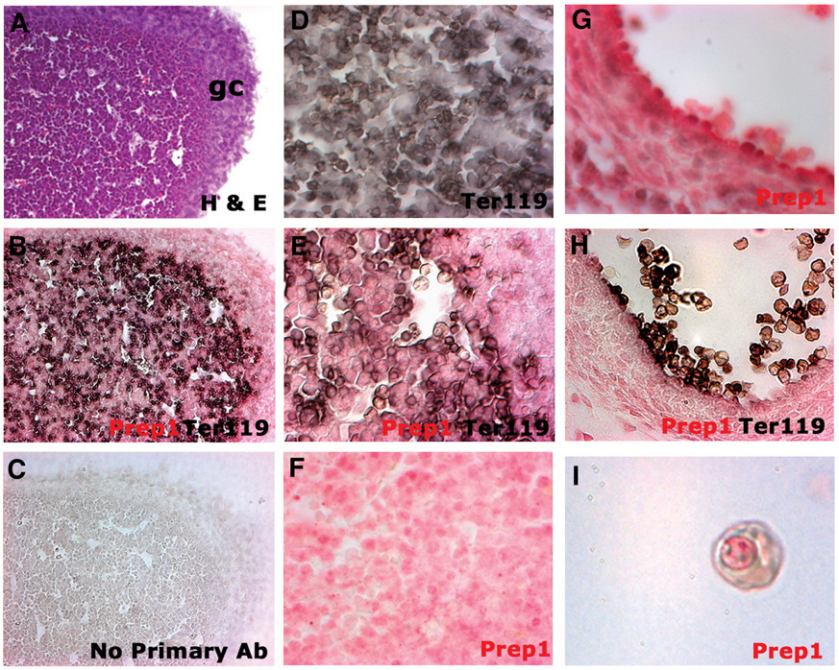

Fig. 3. Immunohistochemical analysis of *Prep1* expression on E14.5 FL sections. (A–F) Coronal sections of the FL showing immunohistochemistry of E14.5 FL stained with hematoxylin and eosin (A), with secondary antibodies only (C), with antibodies Ter119 (black) (D), with antibodies against Prep1 (red) (F, I). No $Ter119^{+}$ cells were detected in Glisson's capsule (gc, A). Ter119 staining is clearly located in the cytosolic membrane (D), while Prep1 shows both cytosolic and nuclear localization (F, G). Panels E and H show a section stained with both Prep1 and Ter119 antibodies. (G–H) Coronal sections of the aorta region showing a predominantly nuclear staining of Prep1. $Ter119^{+}Prep1^{+}$ cells are located inside aorta and in clusters located on the aorta endothelium (H), especially on the ventral side. (I) Magnification showing a nucleated blood cell with a clear nuclear Prep1 staining. Magnification: A–C=20×; D–H=63×; I=100×.

LTR-HSC. In any case, we can conclude that *Prep1* expression is required for the proper activity of the entire embryonic hematopoietic system. However, the lymphoid compartment appears to be more sensitive to the *Prep1* reduction. Erythroid and myeloid lineages also tended to be affected, but the significance were not always statistically supported (Table 7). However, those results may be affected by the high variability and penetrance of the $Prep1^{i/i}$ phenotype; conditional targeting studies will be required to better define the sensitivity of individual lineages to Prep1 reduction.

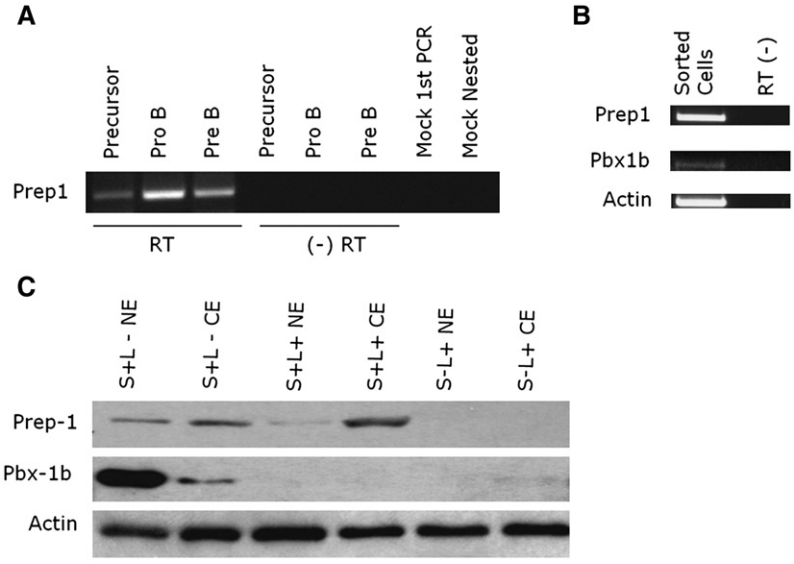

Fig. 4. Prep1 (and Pbx1b) mRNAs and proteins are present in sorted FL B-cells progenitors, KSLA cells and in adult BM hematopoietic cells populations. (A) RT–PCR analysis (see Materials and methods) in B-cells progenitors sorted from wt E14.5 FL. (B) RT–PCR analysis (see Materials and methods) of Prep1, Pbx1b and actin expression in KSLA cells sorted from wt E14.5 FL. (C) Immunoblotting analysis with Prep1, Pbx1b and actin antibodies on adult BM cells sorted for Sca1 and Lin surface markers as indicated on top (S=Sca1, L=Lin).

The deficient activity of the *Prep1*$^{i/i}$ LTR-HSC correlates well with the expression of this gene in KSLA cells (Fig. 4), in the embryonic hematopoietic tissues and with the co-expression with markers of LTR-HSCs or progenitors (Figs. 2 and 3). Confocal immunofluorescence in the AGM region and immunohistochemistry in FL show the presence of Prep1 staining in cells also expressing other markers of HSC like cKit and Sca1 (Figs. 1 and 2). Moreover, the demonstrated presence of Prep1 in adult BM Sca1^{+}Lin^{-} cells (Fig. 4) also agrees with a role of Prep1 in adult hematopoiesis. The presence of Prep1 in early T-cell progenitors had already been documented (Penkov et al., 2005).

Prep1 deficiency manifests with different degree of severity, depending on the level of expression. *Prep1* "null" embryos die at E7.5 (Fernandez LC, Jenkins N, Copeland NG and Blasi F, in preparation) while hypomorphic *Prep1*$^{i/i}$ embryos mostly die of anemia at E17.5–P0 with a phenotype that is stronger in the embryos producing less Prep1. The ability of one quarter of the embryos to survive and live at least 16–18 months (Ferretti et al., 2006) is likely due to the expression of slightly higher levels of residual Prep1. This suggests that different Prep1 threshold levels are required at different stages of the embryonic development.

The present analysis shows that the FL cells of *Prep1*$^{i/i}$ embryos have a deficient radioprotective and competitive repopulation activity but also present defects in the B-lymphoid lineages. Interestingly, these data also show a dependence of the B-lineage compartment differentiation on the *Prep1* gene dosage, as intermediate effects were observed in heterozygous embryos (see Results).

The hematopoietic phenotypes of the *Meis1* ko and of the hypomorphic *Prep1*$^{i/i}$ embryos is very similar (deficient hematopoiesis, angiogenesis and oculogenesis) (Hisa et al., 2004; Azcoitia et al., 2005; Ferretti et al., 2006). Since both proteins act through a common mediator, Pbx, this is not really expected. Prep1–Pbx and Meis1–Pbx dimers might have different targets or compensate for one another, hypotheses that are not in agreement with so similar phenotypes. However, since Prep1 controls the level of all *Pbx* and of *Meis1* (Ferretti et al., 2006), the *Prep1*$^{i/i}$ phenotype likely depends in part on the reduction of *Pbx* and *Meis1*. Therefore, part of the *Prep1*$^{i/i}$ phenotype depends on the reduction of Pbx and Meis1 levels. However, while *Pbx1* was shown to be required for B-lymphoid development at a stage between hematopoietic stem cells and pro-B progenitors (Sanyal et al., 2007), the low level expression of *Prep1* appears to affect not only differentiation of B-cells progenitors, but also earlier precursors, starting from the LTR-HSC.

We conclude that Prep1 is an important player in the activity of LTR-HSC and in the differentiation of the various progenitor lineages. Prep1 not only directly participates in this pathway by dimerizing with Pbx but also controls the expression of *Pbx* and *Meis*, and hence it should also affect the activity and expression of *Hox* and other genes regulating differentiation.

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