Prep1 (pKnox1) Regulates Mouse Embryonic HSC Cycling and Self-Renewal Affecting the Stat1-Sca1 IFN-Dependent Pathway

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Abstract

A hypomorphic Prep1 mutation results in embryonic lethality at late gestation with a pleiotropic embryonic phenotype that includes defects in all hematopoietic lineages. Reduced functionality of the hematopoietic stem cells (HSCs) compartment might be responsible for the hematopoietic phenotype observed at mid-gestation. In this paper we demonstrate that Prep1 regulates the number of HSCs in fetal livers (FLs), their clonogenic potential and their ability to de novo generate the hematopoietic system in ablated hosts. Furthermore, we show that Prep1 controls the self-renewal ability of the FL HSC compartment as demonstrated by serial transplantation experiments. The premature exhaustion of Prep1 mutant HSCs correlates with the reduced quiescent stem cell pool thus suggesting that Prep1 regulates the self-renewal ability by controlling the quiescence/proliferation balance. Finally, we show that in FL HSCs Prep1 absence induces the interferon signaling pathway leading to premature cycling and exhaustion of fetal HSCs.

Introduction

Hematopoiesis is the production of blood cells in the embryo and throughout adult life. Hematopoietic stem cells (HSCs) produce and replace mature blood cells through self-renewal and differentiation. During embryonic development hematopoiesis occurs step-wise in different embryonic sites in the yolk sac around E7, in the aorta-gonad mesonephros (AGM) at E10.5 [1] and from E11 in the fetal liver (FL). At mid-late gestation in FL, HSCs undergo a massive expansion generating the stem cell pool that will contribute to mature blood cells during entire life. Around birth HSCs move to the bone marrow (BM) where they reside mainly in a quiescent state during adult life [2]. Intrinsic factors, such as transcription factors and chromatin modifiers, and extrinsic microenvironmental factors encircling the HSCs modulate their activity during both embryonic and adult life [3]. The BM niche and the factors controlling adult HSCs have been extensively studied [4–9], but developmental mediators of HSCs biology remain largely unknown. Identifying the mechanisms regulating HSCs during development is crucial since often cells undergoing malignant transformation reacquire properties distinctive of stem cells during developmental stages [10–14]. Thus the molecular details of fetal HSCs may be critical for further elucidation of HSCs malignancy and possible new targets for cancer therapy. Finally, factors regulating expansion and proliferation of FL HSCs might help developing protocols for ex vivo expansion of HSCs for clinical applications.

Prep1 is a TALE family homeodomain transcription factor and plays an essential role in embryonic development [15–19]. Prep1−/− embryos die before gastrulation around E6.25 due to p53-dependent apoptosis of epiblast cells [19]. A hypomorphic Prep1 (Prep1) mutation that causes the expression of 3 to 10% of Prep1 protein induces later embryonic lethality around E17.5 [20]. However, one quarter of these hypomorphs escape embryonic death and are important for identifying adult biological functions of Prep1. Prep1 acts as a tumor suppressor controlling genome stability and is an essential regulator of hematopoietic differentiation [21–23]. Prep1 controls both myeloid and lymphoid differentiation as Prep1−/− FL cells generate less CFU-GEMM colonies and differentiate less B cells [24]. The thymus of adult Prep1−/− mice is underdeveloped and the T cell lineage is compromised [25]. All these features of Prep1−/− FL and BM can be reproduced by transplanting FL cells into ablated hosts. Prep1−/− FL cells compete inefficiently with wild-type (wt) cells in competitive repopulation assays suggesting a defect in the HSC compartment [24]. However, how Prep1 affects HSCs biology and which HSCs properties are regulated remain unanswered.

We now demonstrate that Prep1 regulates the number of HSCs in FLs as well as their functionality to de novo generate the hematopoietic system in ablated hosts. Prep1 controls self-renewal of the FL HSC compartment and governs the balance between quiescent and proliferating fetal HSCs. The absence of Prep1 derepresses the interferon (IFN) signaling pathway, Stat1 is...
**Figure 1. Prep1 affects stem and progenitors compartments in E14.5 FLs.** (A–D) Representative FACS analyses of Prep1+/+ and Prep1−/− FLs are shown on the left. Graphs describing percentage and absolute numbers are reported on the right. Numbers in the FACS plots indicate the percentage of cells in parental gates. (A) Lin− Sca1− cKit+ population is shown. FACS plots are referred to Lineage− gate (n = 8 for each genotype; % L Lin− Sca1− K cKit+ = 0.01; # L Lin− Sca1− K cKit+ = 0.01). (B) HSC population is identified as Lin− Sca1− K cKit+ CD150+ CD41− CD48−. FACS plots are referred to L Lin− Sca1− K cKit+ gate (n = 8 for each genotype; % HSCs p = 0.0000056; # HSCs p = not significant). (C) CLPs are identified as Lin− Sca1− Il7R− int cKit− int. FACS plots are referred to Lin− L Lin− Sca1− K cKit+ gate. (n = 4 for each genotype; % CLPs p = 0.00014; # CLPs p = 0.00023). (D) FACS plots regarding myeloid progenitors refer to Lin− Sca1− cKit+ gate. CMPs are FcγR− CD34+ (n = 4 for each genotype; % CMPs p = 0.005; # CMPs p = 0.007); CMPs are FcγR− CD34− (n = 4 for each genotype; % CMPs p = 0.005; # CMPs p = 0.007); MEPs are FcγR+ CD34− (n = 4 for each genotype; % MEPs p = not significant; # MEPs p = not significant).

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activated and FL HSCs cycle more frequently. We also identify Prep1 target genes likely mediating these effects.

**Methods**

**Mice**

Prep1<sup>i/i</sup> have been described [23,26]. Congenic CD45.1 mice (B6.SJL-Ptprc<sup>BoyCrl</sup>; Ly5.1) were purchased from Charles River. All animal experiments were performed in accordance with Institutional Animal Care and Use Committee of IFOM and approved by the Italian Ministry of Health (project #110/11). Animals were kept on a 12/12-h light/dark cycle (lights on at 07:00 h) with free access to food and water. All animal handlings (transplantation, sacrifice etc.) were in accordance with the guidelines established by EU (directive 2010/63/EU). Topical anesthesia (ELMA cream) applied to the site 30 minutes prior to intravenous injection of cells. Mice were sacrificed by carbon dioxide euthanasia.

**Cells**

Cell suspensions were obtained from E14.5 FLs as already described [27]. BM cells were harvested by flushing femurs and tibias in PBS with 2% heat-inactivated fetal bovine serum (staining buffer). Peripheral blood (PB) was obtained by tail bleeding. EML1 cells were the kind gift of Dr. Schickwann Tsai and were cultured as described [28]. pLKO.1 vectors encoding shRNA targeting murine Prep1 or nontargeting control were obtained from Sigma (St Louis, MO). Lentiviral infection was obtained by 2 cycles of spinoculation (1800 rpm, 45 minutes per cycle).

**Flow cytometry**

Stainings of surface markers were performed in staining buffer with the following conjugated monoclonal antibodies purchased from BD (BD Pharmingen) or eBioscience: Gr1 (RB6-8C5), B220 (RA3-6B2), Ter119 (TER-119), CD3e (145-2C11), Mac1 (M1/70), Sca1 (D7 or E13–161.7), cKit (2B8), AA4.1 (AA4.1), CD41 (MWReg30), CD48 (HM48–I), CD150 (mShad150), Il7R (SB/199), CD45.2 (104), CD45.1 (A20). The lineage cocktail (Lin) was composed of Gr1, B220, CD3e and Ter119. Mac1 was included only in the Lin used for BM cells. Stainings of intracellular markers were performed as follows: after surface marker staining, cells were fixed and permeabilised by Cytofix/Cytoperm kit (BD) and stained for pSTAT1 (#9271, Cell signaling) or Ki67 (#5136524, BD) for 45 min. Then, fluorochrome-conjugated secondary antibodies were added. For cell cycle staining Hoechst33342 (1 μg/mL, Sigma) was added to stain DNA. Apoptosis staining was performed using AnnexinV Binding Buffer (BD), FITC-AnnexinV (Sigma) according to the manufature’s protocol. Stained cells were analyzed by FACScalibur (BD Biosciences) or FACSCantoII (BD Biosciences). Cell sorting was performed by FACS/Aria (BD Biosciences). CellQuest, Diva and FlowJo (Tree Star) software were used for data acquisition and analyses.

**Long Term Culture-Initiating Cell (LTC-IC) assay**

A feeder layer of freshly isolated wt BM cells was established plating 3×10<sup>5</sup> cells/well (96 well plate). Cells were cultured in MyeloCult (Stem Cell Technologies) supplemented with 10<sup>–6</sup> M Hydrocortisone (Sigma). Proliferation was blocked by X-Ray irradiation (15 gray) at 70–80% confluency. HSCs purified from FLs were plated on the established feeder layer as specified in the

![Figure 2](https://example.com/figure2.png)  
**Figure 2. Impaired clonogenic activity of Prep1 deficient stem cell populations.** (A) LTC-IC assay was performed plating 100, 300 or 500 L<sup>S</sup>S<sup>K</sup>A<sup>+</sup> cells purified from Prep1<sup>+/+</sup> and Prep1<sup>i/i</sup> FLs. Mean value of scored colonies after 12 days in methylcellulose at each cell dose are shown (n = 9 at each cell dose for each genotype; 500 L<sup>S</sup>S<sup>K</sup>A<sup>+</sup> p = 0.003, 300 and 100 L<sup>S</sup>S<sup>K</sup>A<sup>+</sup> p = not significant). (B) LTC-IC was performed with 50, 100, 200 L<sup>S</sup>S<sup>K</sup>A<sup>+</sup>CD150<sup>+</sup>CD48<sup>−</sup>CD41<sup>−</sup> from both genotypes and colonies scored after 12 days in methylcellulose. (n = 4 at each cell dose for each genotype; 200 cells p = 0.001, 100 cells p = 0.01, 50 cells p = not significant). (C) Representative images of LTC-IC colonies obtained from Prep1<sup>+/+</sup> and Prep1<sup>i/i</sup> L<sup>S</sup>S<sup>K</sup>A<sup>+</sup> cells (Prep1<sup>+/+</sup> colony: 4x original magnification; Prep1<sup>i/i</sup> colony: 10x original magnification; scale bars = 100 μm).

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Cultures were kept for 5 weeks in MyeloCult and then transferred into 35 mm plates in methylcellulose (MethoCult, Stem Cell Technologies). Colonies were scored after 12 days.

Transplantation assays

8 to 12 weeks old CD45.1 mice were lethally irradiated with a 6.5 gray dose of X-rays (experimentally determined as minimal lethal dose). Four hours after irradiation, unfraccionated or purified FL cells (CD45.2) in competition with BM cells were injected into the tail vain of CD45.1 mice. The injected cell doses are indicated in the text for each transplantation. For secondary and tertiary transplantations, $2 \times 10^6$ unfraccionated BM cells were collected from recipients and injected into the tail vain of new hosts. Repopulation activity was evaluated by repopulating units

Figure 3. Prep1 controls the number and the functionality of HSCs in E14.5 FLs. (A) 50, 100 or 200 HSCs sorted from Prep1$^{+/+}$ and Prep1$i/i$ FLs were transplanted into lethally irradiated mice in competition with $2 \times 10^5$ CD45.1 BM cells. Mice showing more than 2% CD45.2$^+$ cells in the PB were considered as positively repopulated. HSCs are identified as CD45.2$^+$ L$^-$ S$^+$ K$^+$ CD150$^+$ CD48$^-$ CD41$^-$ cells. The graph represents the percentage of positively repopulated mice 16 weeks after transplantation (n = 4 for each genotype). (B) The graph indicates the mean chimerism shown by transplanted mice at each cell dosage in the PB 16 weeks after transplantation. (C) 2000 LSK cells purified form Prep1$^{+/+}$ or Prep1$i/i$ FLs were transplanted in competition with $1 \times 10^6$ BM cells into lethally irradiated CD45.1 recipients. (D) PB analyses to detect donor-derived (CD45.2$^+$) cells performed at 7, 12, 16 and 20 weeks after transplantation. In the graph, black bars represent the mean of CD45.2$^+$ cells in the PB of Prep1$^{+/+}$ or Prep1$i/i$ reconstituted mice (n = 4 for each genotype; p < 0.001). (E–F) RUs (on the left) were calculated on FACS data (on the right) obtained from BMs of repopulated mice 20 weeks after transplantation. (E) Mean of RUs in Prep1$^{+/+}$ or Prep1$i/i$-transplanted mice; p = 0.05. (F) Mean of HSCs RUs in Prep1$^{+/+}$ or Prep1$i/i$-transplanted mice; p = 0.01. HSCs are identified as CD45.2$^+$ L$^-$ S$^+$ K$^+$ CD150$^+$ CD48$^-$ CD41$^-$ cells. doi:10.1371/journal.pone.0107916.g003
as follows: RU = (%chimerism) x (n. competitors cells/10^5)/ (100-%chimerism) [29].

Real-Time quantitative PCR
HSCs were sorted from Prep1+/+ and Prep1i/i FLs and kept on ice. Cell lysis, RNA extraction, reverse transcription and pre-amplification were performed by Taqman kit #4387299 (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. Taqman Assays-on-demand were purchased from Life Technologies.

Each PCR reaction was run in triplicate and GAPDH was used as housekeeping gene. Real-time PCRs were carried out on the ABI/Prism 7900 HT Sequence Detector System (Applied Biosystems, Foster City, CA).

Statistical analysis
Values are expressed as mean and error bars represent SEM. The significance of differences was determined by two-tailed Student’s t test. P value (p) ≤0.05 were considered significant (*p≤0.05; **p≤0.01; ***p≤0.001).

Results
Prep1 regulates the number of functional HSCs in E14.5 FLs
To investigate how Prep1 regulates the stem and progenitor compartments during development, we used E14.5 Prep1+/+ FL cells. Initially we determined the immunophenotype of stem and progenitors populations in Prep1i/i FLs in comparison to wt controls. The Lin^2 Sca1^+ cKit^+ (L^2 S^+ K^+ ) population, mainly enriched in progenitors and to a lesser extent in stem cells, showed a 2-fold increase in Prep1 deficient embryos (Figure 1A).

Further dissection of the L^2 S^+ K^+ population with the CD150, CD48 and CD41 markers revealed a 50% reduction in the frequency of Prep1i/i L^2 S^+ K^+ cells that express stem markers (CD150^-cKit^-CD41^-) compared to the wt counterpart (Figure 1B). From now on and throughout the paper we will refer to the L^2 S^+ K^+ cells as HSC as they represent an almost pure HSC population [30]. The absolute number of the Prep1i/i HSCs was similar to the wt because of the relative expansion of the L^2 S^+ K^+ population (Figure 1B). Importantly Prep1 is expressed in the HSC compartment (see below).

We also analyzed lymphoid and myeloid committed progenitor populations. Prep1i/i common lymphoid progenitors (CLPs) showed a statistically significant 3.3 fold increase compared to wt controls (Figure 1C). Conversely, common myeloid progenitors (CMPs) and granulocyte/macrophage progenitors (GMPs) were
reduced in frequency and absolute number when Prep1 was underexpressed (38% and 38% reduction, respectively) (Figure 1D). These data indicate a role for Prep1 in regulating the first steps of the hematopoietic differentiation during fetal development, from stem cells to lineage-primed progenitors, and that Prep1 exerts a peculiar differentiation stage-specific function.

To functionally characterize the impact of Prep1 on HSCs we investigated their ability to form colonies in vitro in LTC-IC assay. We tested the HSC population (Fig. 1B) and the L. S’K’A4.A.1+ cells (Figure S1). The latter is another cell population enriched for HSCs [31,32]. Comparing the numbers of scored colonies at all plated cell doses, a strong decrease in the clonogenic potential of Prep1i/i+ stem/progenitor cells was observed (figures 2A and 2B). This agrees with our previous finding that myeloid, erythroid and immature CFU colonies were reduced in the absence of Prep1 [24]. Hypomorphic Prep1i/i+ generated smaller colonies in comparison to wt cells (Figure 2C). Thus, Prep1 acts on the functionality of stem/progenitor cells.

To investigate whether the reduced frequency and clonogenic potential of Prep1i/i+ HSCs depends on the lower number of HSCs, we analyzed the frequency of functional stem cells by an in vivo transplantation experiments with purified HSCs (Figure 3A). Wt HSCs repopulated all the recipients already at the lowest cell dosage (50 cells) whereas Prep1i/i+ HSCs fully repopulated recipients only at the highest dosages (Figure 3A; mice showing more than 2% donor-derived cells in the PB were considered as positively repopulated). The mean chimerism shown by transplanted mice at each cell dosage is shown in Figure 3B.

This set of experiments demonstrates that Prep1 controls the number of HSCs in FL and points to a role for this transcription factor in regulating also their functionality.

Prep1 affects HSCs self-renewal by controlling their proliferation state

To analyze the role of Prep1 in regulating the functionality of fetal HSCs, we assessed the ability of Prep1-deficient cells to de novo generate the stem/progenitor compartments in vivo. We transplanted CD45.2 Prep1i/i+ or wt FL L. S’K’A4.A.1+ cells in competition with wt CD45.1 BM cells (Figure 3C) and followed the kinetic of repopulation in the PB of the hosts exploiting the CD45.2 phenotype of the donor cells (Figure 3D). Prep1i/i+ cells generated less mature hematopoietic cells in PB compared to wt controls at all time points (Figure 5D). This agrees with the decrease of both mature myeloid and lymphoid cells in the PB of Prep1i/i+ recipients [24]. Twenty weeks after transplantation, we analyzed the repopulation efficiency of Prep1i/i+ v wt HSCs by counting the number of repopulating units (see Methods). This revealed a dramatic (86%) decrease in the ability of Prep1i/i+ HSCs to repopulate the entire hematopoietic system (Figure 3E). We also measured the ability of FL Prep1i/i+ HSCs to give rise to new stem cells, investigating the donor-derived HSCs compartment in the host. Again, the number of Prep1i/i+-derived RUs dropped compared to wt controls (Figure 3F). The progenitor compartments were also negatively affected by the absence of Prep1 in repopulated mice (Table S1). The time of 20 weeks after transplantation, chosen to assess PB reconstitution, does not conflict with malignant transformation occurring in Prep1i/i+ mice at least 7–8 months after transplantation [21].

Functional impairment or decreased HSCs number in Prep1-deficient FLs might be due to increased apoptosis. However, the experiment shown in figure 4A rules out this hypothesis since wt and Prep1i/i+ HSCs displayed comparable apoptosis level as measured by Annexin V binding. Nonetheless, when we moved to the analysis of the proliferative state of HSCs, we noticed a slight increase in the percentage of Prep1i/i+ HSCs in G1 and S-G2-M phases compared to wt and, importantly, a statistically significant 50% reduction in the Prep1 deficient G0 quiescent pool (Figure 4B). The depletion of the G0 pool in the stem cell compartment in the absence of Prep1 indicates that the functional defects observed in hypomorphic FLs might depend on a deregulated balance between quiescent and proliferative states which may lead to the exhaustion of the stem cell pool. To verify this hypothesis, we tested the self-renewal ability of fetal HSCs through serial transplantsations into ablated hosts (Figure 5A–5D).

BM cells from Prep1i/i+ transplanted mice were less efficient than wt in repopulating PB in secondary recipients (Figure 3B). 25% of Prep1i/i+ transplanted mice (2/8) showed no reconstitution at all after long-term repopulation. Eight weeks after secondary transplantation, reconstitution by donor cells was analyzed with respect to the myeloid, B-lymphoid and T-lymphoid lineages (Figure 5C). Prep1i/i+ donor cells were much less efficient than wt littermates in generating all the lineages, with a 30% decrease of myeloid CD45.2+Gr1+Mac1+ and 75% reduction of both CD45.2+B220+ and CD45.2+CD3+ lymphoid cells (Figure 5C). When we transferred BM cells from secondary to tertiary recipients (Figure 5D), Prep1i/i+-derived cells lost their repopulation capacity as only a minor fraction of the tertiary transplanted hosts (2/8) showed a low repopulation activity (Figure 5E–F).

In summary, Prep1i/i+ HSCs initially repopulate primary recipients as wt controls. However, in secondary and tertiary recipients the Prep1i/i+ stem cell pool decreases tending to premature exhaustion (Figure 5F).

To directly support the above conclusion, we re-transplanted primary recipient’s BM cells after readjusting the CD45.2/CD45.1 ratio in donor cells by FACS sorting (six mice for wt and 2 for hypomorphic). The result was unequivocal with a mean 42 v 5% donor-derived cells in wt and Prep1i/i+ secondarily transplanted mice (Figure S2A). Similarly, in PB donor-derived myeloid, B-lymphoid and T lymphoid cells were dramatically
Figure 6. Prep1 modulates the IFN signaling pathway of FL HSCs. (A) The expression of Sca-1 on the cell surface of Prep1\(^{+/+}\) and Prep1\(^{+/−}\) L\(^{−}\)K\(^{−}\)CD150\(^{−}\)CD48\(^{−}\)CD41\(^{−}\) cells is quantified by mean fluorescent intensity (MFI). The representative FACS histogram on the left refers to L\(^{−}\)K\(^{−}\)CD150\(^{−}\)CD48\(^{−}\)CD41\(^{−}\) gate (n = 8 for each genotype; p = 0.003). (B) qRT-PCR was performed on sorted Prep1\(^{+/+}\) and Prep1\(^{+/−}\) L\(^{−}\)S\(^{−}\)K\(^{−}\)CD150\(^{−}\)CD48\(^{−}\)CD41\(^{−}\) cells to detect Sca1 transcripts. Values are normalized on GAPDH expression (n = 4 for each genotype; p = 0.02). (C) The histograms show the expression of the indicated genes in sorted Prep1\(^{+/+}\) and Prep1\(^{+/−}\) L\(^{−}\)S\(^{−}\)K\(^{−}\)CD150\(^{−}\)CD48\(^{−}\)CD41\(^{−}\) cells as detected by qRT-PCR. (n = 4 for each genotype; Stat1 p = 0.02; Adar1 p = 0.02; Irf7 p = 0.003; Prep1 p = 0.00002). (D) The representative FACS histogram on the left refers to the L\(^{−}\)S\(^{−}\)K\(^{−}\)CD150\(^{−}\) gate. The negative control (grey histogram) tracing represents a sample stained for all the surface markers plus the fluorochrome-conjugated secondary antibody used to detect pStat1, but without the pStat1 antibody. (n = 3 for each genotype; p = 0.01). The phosphorylation of Stat1 (pStat1) was analyzed by MFI and quantitated on the right.

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reduced in Prep1i/i compared to wt reconstituted mice (Figure S2B–D).

Prep1 modulates the interferon signaling pathway in FL HSCs

The reduced quiescent stem cell pool and the premature exhaustion of HSCs in the Prep1i/i FL suggest that Prep1 regulates self-renewal by controlling the quiescence/proliferation balance. To gain insight into the signaling cascades and the molecular mechanisms regulated by Prep1 in FL HSCs, we investigated the increased expression of the surface marker Sca-1 in L2SK+ cells (Figure 1C). Sca-1 is known to regulate the repopulation activity and self-renewal of HSCs in both FL and BM [33,34]. We measured in Prep1i/i FL HSCs a 2-fold increase in Sca-1 antigen compared to wt control littermates (Figure 6A) and a comparable increase in its transcript level (Figure 6B). Since Sca-1 is an IFN-inducible gene [35] and is upregulated by IFNs in FL HSCs [33], we investigated whether other components of this signaling pathway are perturbed in Prep1i/i HSCs measuring the mRNA level of a panel of genes implicated in the IFN response (Figure 6C). We observed an increase in the expression of Stat1, Stat2 and Irf7 in Prep1i/i HSCs compared to wt littermates. Moreover we also noticed a statistically significant reduction in the expression of Adar1 in Prep1i/i cells.

Furthermore a ChIP sequencing analysis performed on mouse embryos [36] (and embryonic stem cells) revealed that Irf1, Irf2 and Irf8 are direct Prep1 targets (Figure 7). The peaks, having a highly significant intensity, are located about from 10 Kbp to few bp (Irf8) before the transcription start site of the genes. These data together with the increased expression of signaling mediators like Stat1, Stat2 and of Irf7 and the decrease of the suppressor of the IFN response (Adar1) indicate that Prep1 regulates the IFN pathway. To corroborate the involvement of STATs, we investigated the activation of Stat1 comparing its phosphorylation state in wt and Prep1i/i HSCs by flow cytometry and observed a statistically significant 1.6 fold-increase in pStat1 in Prep1i/i HSCs (Figure 6D).

Moreover, since the ablation of Pbx1 in adult HSCs influences the TGFbeta pathway [37], we investigated whether Prep1 acts in a similar way in FLs. To this aim, we knocked down (KD) Prep1 by shRNA in the EML1 progenitors cell line and assessed their responsiveness to TGFbeta. We found no significant alteration in SMAD levels at both RNA and protein level (Figure 8B–C). However, the phosphorylation of Smad2 and Smad3 was reduced in Prep1KD (Figure 8C). This suggests that Prep1 may partly act modulating TGFbeta.

We next investigated the status of the IFN pathway in Prep1i/i HSCs in the adult wt environment after transplantation. Neither the activation of Stat1 (pStat1) (Figure S3A) nor the overexpression of Sca-1 was reproduced in the BM niche of transplanted mice (Figure S3B). Moreover, since Sca-1 overexpression is a distinguishing marker of proliferative v. quiescent HSCs [34], we investigated the cell cycle status of these cells. In the adult environment, Prep1i/i and wt HSCs did not show any statistically significant difference in the quiescent G0 stem cell pool (Figure S3C). This indicates that the effect of Prep1 on the Stat1/Sca1 proliferative pathway is specific to fetal cells and may be dispensable during adult hematopoiesis.

Discussion

We have shown that Prep1 controls the number and function of HSC during mouse embryo development. Prep1 regulates HSC self-renewal since Prep1i/i cells are deficient in generating the stem...
cell compartment upon transplantation into ablated hosts (Figure 3) and undergo faster exhaustion upon serial transplantation (Figure 5).

These results are coherent with the role in hematopoiesis of other TALE family members: Pbx1 and Meis1. Pbx1 regulates transcription mostly as a dimer with Prep1 or Meis1. In a mixed cells population Meis1-Pbx1 complex mainly associated with intragenic regions of developmental genes, whereas Prep1-Pbx1 bound promoters of genes involved in basic cell functions [36]. Pbx1 and Meis1 play an important role in the hematopoietic compartment as both FL Pbx1<sup>−/−</sup> [38] and Meis1<sup>−/−</sup> HSCs [39,40] are inefficient in establishing multi-lineage hematopoiesis when tested in transplantation experiments. Moreover, Pbx1 regulates BM HSC self-renewal by maintaining the quiescent stem cell pool [37]. Adult Pbx1<sup>−/−</sup> HSCs show a reduced G0 fraction and undergo exhaustion faster than wt. Pbx1 also has a lineage-specific role preventing myeloid differentiation and maintaining lymphoid potential [41]. As Prep1 is the most common partner of Pbx1 [36], the phenotype of Prep1<sup>−/−</sup> HSC may be similar to Pbx1<sup>−/−</sup>. However, CMPs and CLPs frequency in the Pbx1<sup>−/−</sup> background is different from Prep1<sup>−/−</sup>, where the frequency of lymphoid progenitors is drastically increased. Moreover, whereas Meis1 and Pbx1 act in concert to transcriptionally modulate TGFbeta response [37,42], Prep1 is mainly implicated in the regulation of the IFN-response pathway and may affect the TGFbeta pathway in the adult. The phenotypes may also depend on additional non-transcriptional effects of Prep1 as both Meis1 and Pbx1 are decreased in Prep1<sup>−/−</sup> FL [20].

In this paper we also show that Prep1 regulates the cell cycle state of the FL HSCs, maintaining cells in the G0 phase (Figure 4). Embryonic FL HSCs are mainly proliferating cells in comparison with the quiescent adult BM HSCs. At E14.5, FL HSCs cycle every 48 hours [43], while adult HSCs divide every 7 weeks [44,45]. As a consequence, G0 phase FL HSCs cannot aptly be considered the fetal counterpart of the adult HSCs which represent a “dormant” pool. Rather, the prolonged permanence in G0 is a requirement to assure that the modality of cell division corresponds to biological necessities. Furthermore, in terms of cell cycle the transit through G0 and the prolonged cell cycle are peculiarities of HSCs versus progenitors cells [43]. In this view, Prep1 might act as an intrinsic factor necessary at mid-gestation to regulate the cell cycle of HSCs and to preserve their self-renewal ability.

In homeostatic conditions quiescent BM HSCs enter the cell cycle dividing preferentially by asymmetric cell division with the aim to produce at the same time mature cells and to maintain a stem cell pool [46–48], FL HSCs mainly divide by symmetric self-renewing divisions in order to expand the HSC pool [49,50]. We show that, in E14.5 FLs, Prep1 deficiency causes a reduction in the number of HSCs and an increase in multipotent progenitors identified as L<sup>2</sup>S<sup>+</sup>K<sup>+</sup> cells. The inverse correlation between stem and progenitor cells Prep1<sup>−/−</sup> embryos might be caused by a change in the balance between symmetric and asymmetric cell divisions skewed towards the asymmetric mode, leading to a reduced stem cell pool and the increased progenitor compartment.

In addition, the increased cycling shown by Prep1<sup>−/−</sup> fetal HSCs positively correlates with the role of Prep1 as tumor suppressor. Indeed Prep1<sup>−/−</sup> FL cells cause lymphomas when transplanted in hosts after a long latency [21] and the deregulated cell cycle might contribute to make these cells prone to malignant transformation.

Furthermore, Prep1<sup>−/−</sup> FL HSCs display an activated IFN-induced response as demonstrated by the activation of STAT1, increased IFN-induced transcripts and decreased Adar1 (Figure 6). This feature positively correlates with the reduced number

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**Figure 8. Prep1 does not affect Smads expression but modulates their phosphorylation in the EML1 progenitor cell line.** (A) Prep1 knock-down (KD) was assessed by immunoblotting analysis. Actin was used as loading control. (B–C) Prep1 KD or control EML1 cells were incubated 4 h with (+) or without (−) TGFβ (10 ng/ml). (B) Histograms show fold induction of the indicated transcripts in Prep1 KD or control EML1 cells as measured by qRT-PCR. The data represent 2 independent experiments. (C) Smads and their phosphorylated forms (as indicated) were detected by Western blot analyses. Vinculin was used as loading control. The data were reproduced in 2 independent experiments.

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of HSCs in Prep1<sup>−/−</sup> FLs. IFN signaling has been shown to regulate HSC properties in both adult and fetal tissues. Indeed, treatment of adult HSCs with IFNα induces their proliferation with the concomitant decrease of the G0 pool causing HSCs exhaustion, an effect mediated by activation of STAT1 and upregulation of Sca-1 [34]. In addition, Adar1 deaminase suppresses IFN signaling and is strongly down-regulated in FL HSCs [33]. Similarly to Prep1<sup>−/−</sup> HSCs, Adar1<sup>−/−</sup> FL HSCs upregulate IFN inducible genes (Sca-1, Stats and Irf6) and show a diminished number of HSCs [33].

Although not yet directly tested, it is tempting to speculate that the Prep1-absence-dependent activation of IFN response is connected to DNA damage accumulation. The induction of DNA damage in human and mouse cells triggers Atm-mediated DNA damage response that, in turn, through the activation of NFKB leads to the expression of IFN and IFN-induced genes such as Irf1 and Irf7 [51]. Interestingly, Prep1 acts as a tumor suppressor in maintaining genome stability in cells [22]. FL HSCs are exposed to proliferative stress with accumulation of mutations and genome instability per se, and Prep1<sup>−/−</sup> FL cells show DNA damage accumulation [22]. We speculate that the activation of the IFN response in Prep1<sup>−/−</sup> HSCs could possibly be induced by intrinsic DNA damage due to Prep1 deficiency. The direct binding of Prep1 to three interferon-response genes (Irf1, Irf2, Irf8), the increased expression of Irf7 and repression of Irfβ support the idea that Prep1 regulates the IFN pathway. These factors orchestrate the pathway and affect stem and progenitor cell proliferation and differentiation [52].

The present work also shows that the Stats/Scal proliferative axis is no longer activated in Prep1<sup>−/−</sup> HSCs isolated from adult transplanted BM niche (Figure S3). This is not a unique case in hematopoietic development as Mol−/− mice lose quiescent G0 HSCs and show repopulation defects. Importantly, the MII-dependent self-renewal defect is much stronger in the FL than in the adult BM niche [53]. The dual behavior of hypomorphic cells suggests that an embryonic niche-specific signal is required for the Prep1<sup>−/−</sup> fetal phenotype and possibly suggests different proliferative cues in FLs and adult quiescent BM niche. An additional regulator of HSC activity missing in Prep1<sup>−/−</sup> cells might be the TGFbeta-dependent activation of Smad2/Smad3, in agreement with the Pbx1<sup++++</sup> phenotype [37].

Conclusions

In this work, we demonstrate that the homeodomain transcription factor Prep1 controls the number and the biological activities of fetal HSCs. In particular, we show that Prep1 regulates the self-renewal ability and the cell cycle distribution of HSCs during fetal hematopoiesis by regulating the activation of Stat1, Scal via the IFN-dependent proliferative pathway.

Supporting Information

Figure S1 The HSC-enriched population L<sup>−</sup>S<sup>−</sup>K<sup>−</sup>A4.1<sup>+</sup> is affected by the absence of Prep1. (A) Representative FACS dot plots to identify L<sup>−</sup>S<sup>−</sup>K<sup>−</sup>A4.1<sup>+</sup> cells in Prep1<sup>−/−</sup> and Prep1<sup>−/−</sup> FLs. The plots show A4.1<sup>+</sup> cells in the L<sup>−</sup>S<sup>−</sup>K<sup>−</sup> population and the numbers indicate their percentage in the parental gate. (B) Graphs describe the percentage (left) and absolute numbers (left) of the L<sup>−</sup>S<sup>−</sup>K<sup>−</sup>A4.1<sup>+</sup> population (n = 8 for Prep1<sup>−/−</sup> and n = 12 for Prep1<sup>−/−</sup> FLs in both graphs; % L<sup>−</sup>S<sup>−</sup>K<sup>−</sup>A4.1<sup>+</sup>p = not significant; # L<sup>−</sup>S<sup>−</sup>K<sup>−</sup>A4.1<sup>+</sup>p = 0.02)).

Figure S2 Prep1<sup>+/+</sup> cells in a readjusted ratio with competitors show defective repopulation upon second-
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