Stem Cell Research (2013) 11, 1129–1136

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SHORT REPORT

PRDM11 is dispensable for the maintenance and function of hematopoietic stem and progenitor cells

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Received 19 November 2012; received in revised form 17 June 2013; accepted 30 July 2013
Available online 9 August 2013

Abstract Hematopoietic stem cells (HSC) [1] supply organisms with life-long output of mature blood cells. To do so, the HSC pool size has to be maintained by HSC self-renewing divisions. PRDM3 and PRDM16 have been documented to regulate HSC self-renewal, maintenance and function. We found *Prdm11* to have similar expression patterns in the hematopoietic stem and progenitor cell (HSPC) compartments as *Prdm3* and *Prdm16*. Therefore, we undertook experiments to test if PRDM11 regulates HSC self-renewal, maintenance and function by investigating the $Prdm11^{-/-}$ mice. Our data shows that phenotypic HSPCs are intact in bone marrow (BM) of one-year-old $Prdm11^{-/-}$ mice. In addition, $Prdm11^{-/-}$ mice were able to fully regenerate the hematopoietic system upon BM transplantation (BMT) into lethally irradiated mice with a mild drop in lymphoid output only. Taken together, this suggests that PRDM11, in contrast to PRDM3 and PRDM16, is not directly involved in regulation of HSPCs in mice.

Introduction

The hematopoietic system relies on constant replenishment of mature cells from the HSC compartment. In order to maintain tissue homeostasis, HSCs are tightly regulated by both intrinsic and extrinsic signals (Warr et al., 2011).

The PRDM family is characterized by the presence of a PR-domain that has similarities with the SET domain of histone methyl transferases. The PR-domain is followed by repeated zinc finger motifs in all members of the PRDM family with the exception of PRDM11 that has a zinc knuckle that probably is involved in protein–protein interactions (Briknarova et al.,

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[1] Hematopoietic stem cell (HSC), hematopoietic stem and progenitor cell (HSPC), bone marrow (BM), bone marrow transplantation (BMT), acute myeloid leukemia (AML), peripheral blood (PB), multipotent progenitor (MPP), pre-megakaryocyte/erythroid (preMegE), megakaryocytic progenitor (MkP), pre-granulocyte/macrophage (preGM), granulocyte/macrophage progenitors (GMP), common lymphoid progenitors (CLP), colony forming unit erythroid (CFU-E), proErythroid (proE), colony forming unit megakaryocyte (CFU-Mk), colony forming unit granulocyte macrophage (CFU-GM), megakaryocyte (Mk), LSK ($Lineage^-$, $Sca1^+$, $c\text{-}Kit^{hi}$).

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http://dx.doi.org/10.1016/j.scr.2013.07.009

2011; Fog et al., 2012). Several members of the PRDM family have been shown to be important in regulation of the HSC compartment and to be linked to hematological malignancies. Hematopoietic-specific knockout of PRDM16 results in reduced numbers of phenotypic and functional HSCs due to increased apoptosis and cell cycle activity (Aguilo et al., 2011; Chuikov et al., 2010). In contrast, overexpression of *Prdm16* in HSCs causes an expansion of HSCs *in vitro* that upon transplantation generates a myeloproliferative disorder (Deneault et al., 2009).

PRDM3/EVI1–MDS1/MECOM is the fusion protein created by intergenic splicing of EVI1 with MDS1 (Fears et al., 1996). It was recently suggested to be responsible for the impairment in HSC functions previously documented to be caused by EVI1 alone (Zhang et al., 2011), although this is still debated (Kataoka et al., 2011). EVI1 alone has been shown to play an important role in both fetal and adult hematopoiesis and has proved important for the survival, maintenance and proliferation of HSPCs in mice (Goyama et al., 2008; Kataoka et al., 2011). High expression of *EVI1* in acute myeloid leukemia (AML) patients is associated with poor prognosis. *RPN1* is a fusion partner found upstream of *EVI1* in AML patients (Suzukawa et al., 1994) and *RPN1–EVI1* was in the latest WHO classification of hematopoietic and lymphoid neoplasms included as a new separate group (WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, Fourth Edition, 2008). *RPN1* has also been reported to translocate to other members of the PRDM family like *PRDM16* (Duhoux et al., 2012), *PRDM3* (Suzukawa et al., 1994) and interestingly, translocation to *PRDM11* has also been reported (Lugthart et al., 2010).

Despite the striking effects on hematopoiesis of other PRDM-family members, very little is known about the potential function of PRDM11 within the hematopoietic system. We therefore took advantage of the *Prdm11*$^{-/-}$ mouse line to investigate the role of PRDM11 in the HSPC compartment.

Material and methods

Mice

The *Prdm11*$^{-/-}$ mice will be described elsewhere (manuscript in preparation). Briefly, *Prdm11* exons 2 and 3 were targeted by loxP sites and excised using a germline CMV-cre mouse and then backcrossed more than 10 generations onto C57BL/6 (CD45.2). WT mice B6.SJL (CD45.1) were used for transplantation studies as recipients and support.

Phenotypic and cell cycle analysis of the bone marrow

BM cells from femur and tibia were prepared into single cell suspension and stained with the appropriate antibodies and nucleic acid stains (for details on fluorescent conjugate, clone and company see Supplemental Table 1). For cell cycle analysis, BM cells from femur and tibia were prepared into single cell suspension and stained with primary antibodies (Supplemental Table 1) and subsequently fixed using 2% paraformaldehyde (Sigma-Aldrich). Cells were next permeabilized with 0.01% saponin (AppliChem) and stained with intracellular Ki67 (B56, BD) antibody when appropriate. Finally, cells were stained with DAPI (Invitrogen). All FACS analysis was done on the LSRII (BD).

Bone marrow transplantation

For Competitive BMT experiments, donor (CD45.2) and competitor (CD45.1) cells were mixed in a 1:1 ratio and transplanted into 10–12 week-old lethally irradiated (900 rads) recipients (CD45.1). Peripheral blood (PB) was collected at indicated time points, red blood cells lysed with PharmLyse (BD) and remaining white blood cells were stained with the appropriate antibodies (anti-mouse CD45.1, CD45.2, CD4, CD8, NK1.1, Mac1 and CD19, for details see Supplemental Table 1) and analyzed by FACS. All FACS analyses of PB were run on the LSRII (BD) and data analysis was performed using FlowJo (TreeStar). Experiments were approved by the "Dyreforsogstilsynet" in Denmark.

qRT-PCR

The Lineage^{-}, Sca1^{+}, c-Kit^{hi} (LSK), MkP and CLP Flt3^{+} cells were sorted using similar gating strategies as depicted in Fig. 1A. We defined B cells by the expression of B220 and T cells by expression of CD4 and CD8. Cells of interest were FACS sorted into RLT buffer containing 2-mercaptoethanol followed by RNA extraction using RNeasy Micro Kit (Qiagen) according to manufacturer's instructions. RNA was reverse transcribed into cDNA using ProtoScript M-MuLV first strand cDNA synthesis kit (NEB). Next, qPCR on cDNA from 200 cell equivalent using Light Cycler 480 SYBR Green 1 Master (Roche) was run on a Light Cycler 480 (Roche). Delta CT values were calculated by $2^{-(\text{CT 'Gene of interest'} - \text{CT 'House keeping gene'})}$.

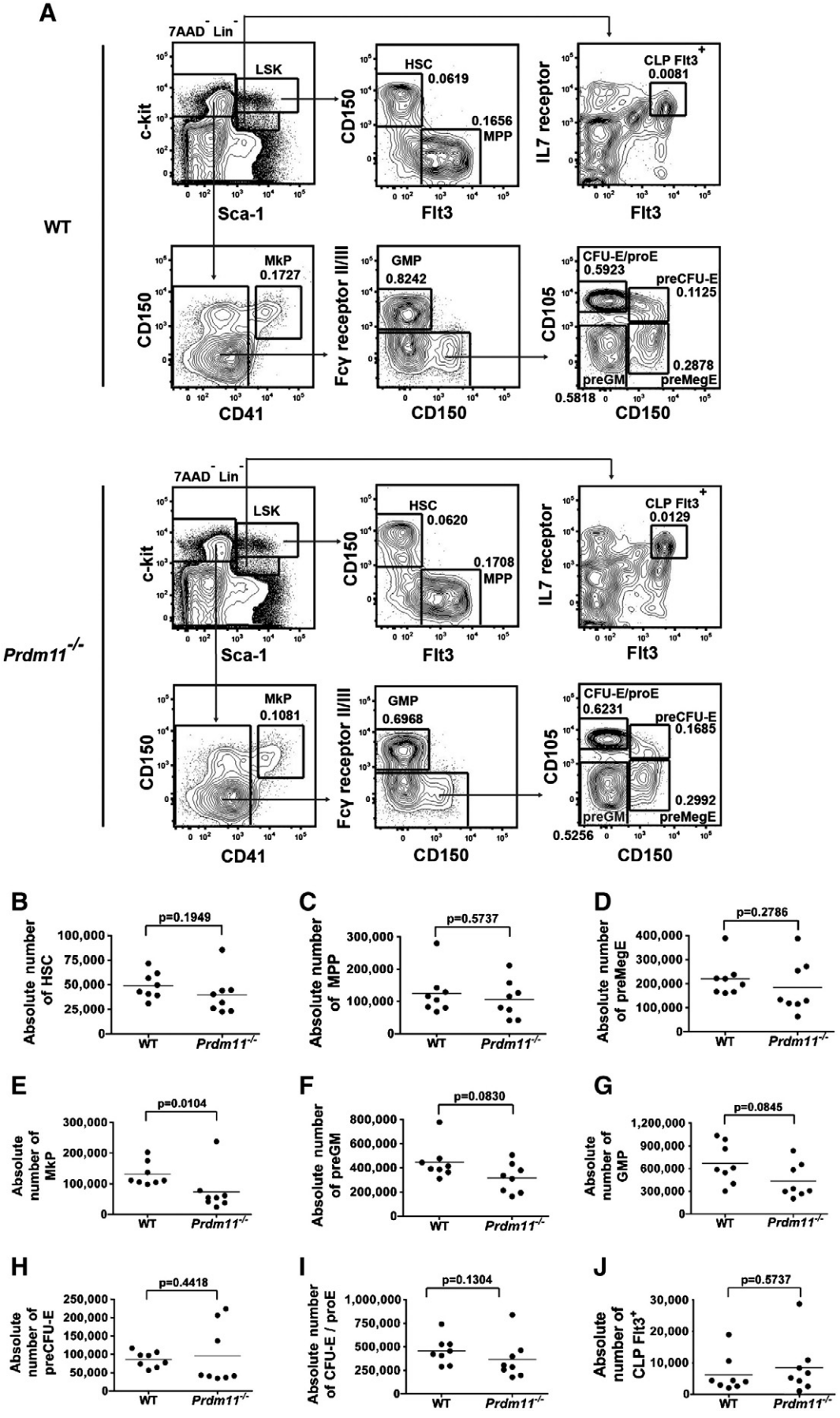
Primers used for qRT-PCR:

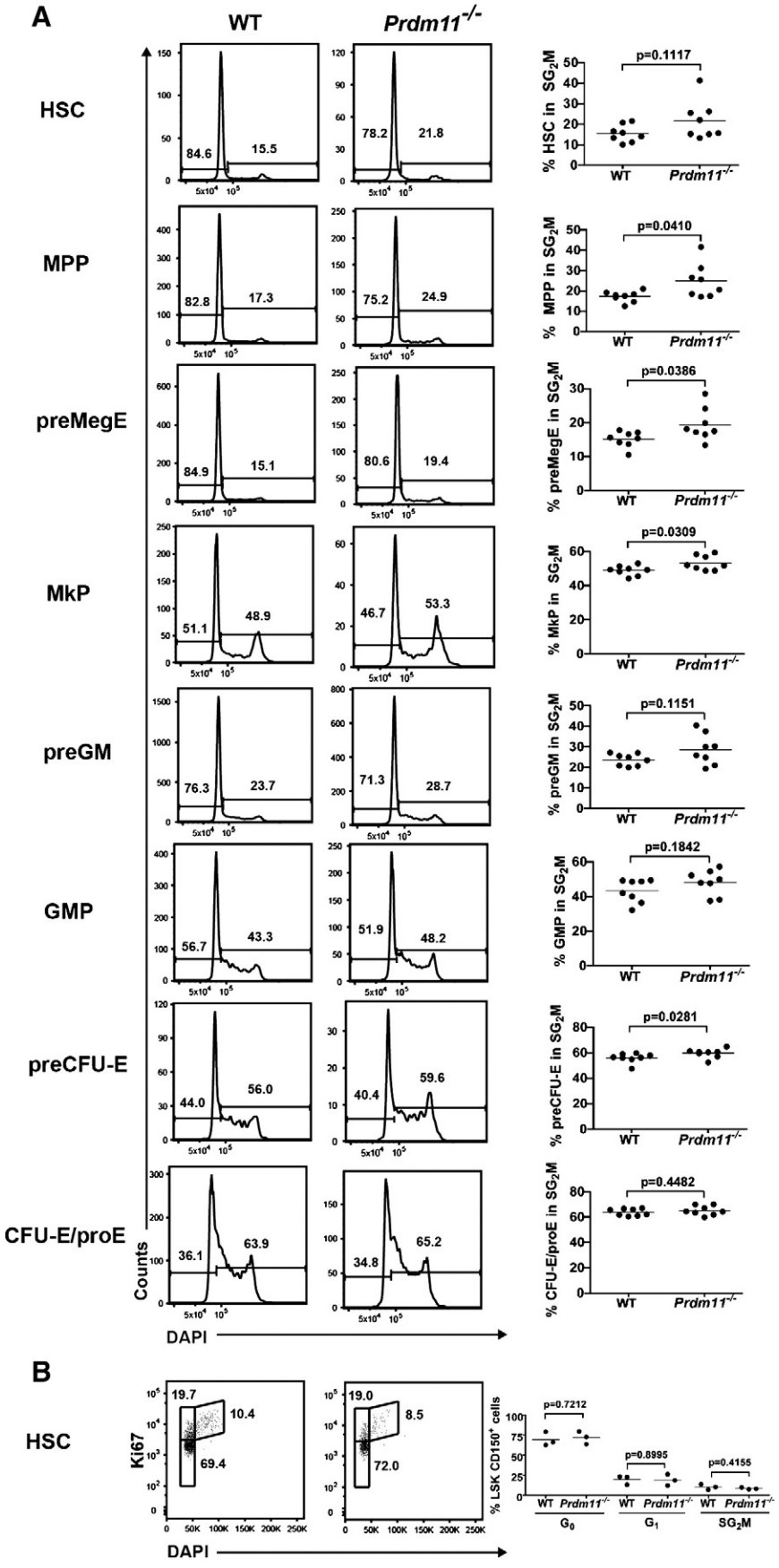
Prdm3 forward 5′-GCTATGATCAGCACAACCTTGTTG-3′
Prdm3 reverse 5′-TGCTGCCGTTCTTCGTGGATG-3′
Prdm11 forward 5′-TCATCCGACCCATCTATGGAC-3′
Prdm11 reverse 5′-TCAGCTGGCTTCTTCTCATGG-3′
Prdm16 forward 5′-GACCATACCCGGAGGTGTGT-3′
Prdm16 reverse 5′-GCGTCGGCTCCAAAGCTAAC-3′
B-actin forward 5′-CTCTTCCAGCCTTCCTTCCT-3′
B-actin reverse 5′-TGCTAGGGCTGTGATCTCCT-3′.

In vitro megakaryocyte colony assay and platelet counts

Whole BM cells (100,000/culture slide) from WT or *Prdm11*$^{-/-}$ mice were cultured using the MegaCult culture assay (StemCell Technologies) according to manufacturer's instructions. The following cytokines were added to the media: rh Thrombopoietin 50 ng/mL, rh IL-6 20 ng/mL, rh IL-11 50 ng/mL and rm IL-3 10 ng/mL (PeproTech). After 7 days of culture, the

Figure 1 Phenotypic analysis of bone marrow sub-populations of WT and *Prdm11*$^{-/-}$ mice. A) BM of one-year-old *Prdm11*$^{-/-}$ mice and WT controls was phenotypically analyzed by FACS. Representative FACS plots with the average frequency shown. Absolute numbers were calculated for B) HSCs, C) MPPs, D) preMegEs, E) MkPs, F) preGMs, G) GMPs, H) preCFU-E, I) CFU-E/proE and J) CLP Flt3^{+}. Horizontal bar indicates mean value. n = 8.


A
WT
Prdm11-/-
7AAD- Lin-
LSK
c-kit
Sca-1
HSC
0.0619
0.1656
MPP
CD150
Flt3
CLP Flt3+
0.0081
0.0129
IL7 receptor
MkP
0.1727
0.1081
CD41
GMP
0.8242
0.6968
Fcγ receptor II/III
CFU-E/proE
0.5923
0.6231
preCFU-E
0.1125
0.1685
preGM
0.2878
0.2992
preMegE
0.5818
0.5256
CD105
B
Absolute number of HSC
p=0.1949
C
Absolute number of MPP
p=0.5737
D
Absolute number of preMegE
p=0.2786
E
Absolute number of MkP
p=0.0104
F
Absolute number of preGM
p=0.0830
G
Absolute number of GMP
p=0.0845
H
Absolute number of preCFU-E
p=0.4418
I
Absolute number of CFU-E / proE
p=0.1304
J
Absolute number of CLP Flt3+
p=0.5737
WT
Prdm11-/-


A
WT
Prdm11-/-
HSC
MPP
preMegE
MkP
preGM
GMP
preCFU-E
CFU-E/proE
84.6
15.5
78.2
21.8
82.8
17.3
75.2
24.9
84.9
15.1
80.6
19.4
51.1
48.9
46.7
53.3
76.3
23.7
71.3
28.7
56.7
43.3
51.9
48.2
44.0
56.0
40.4
59.6
36.1
63.9
34.8
65.2
Counts
DAPI
% HSC in SG2M
% MPP in SG2M
% preMegE in SG2M
% MkP in SG2M
% preGM in SG2M
% GMP in SG2M
% preCFU-E in SG2M
% CFU-E/proE in SG2M
p=0.1117
p=0.0410
p=0.0386
p=0.0309
p=0.1151
p=0.1842
p=0.0281
p=0.4482
B
HSC
Ki67
19.7
10.4
69.4
19.0
8.5
72.0
DAPI
% LSK CD150+ cells
p=0.7212
p=0.6995
p=0.4155
G0
G1
SG2M

slides were fixed in acetone and stained according to manufacturer's instructions.

Platelet levels in PB were measured using a Sysmex KX-21N (Sysmex). All samples were measured in duplicates.

Statistics

All data were screened for variance homogeneity using Bartlett's test and the assumption of normality was assessed by the D'Agostino and Pearson omnibus normality test. Student's unpaired two tailed *t*-test was used when data had a normal distribution with equal variance. Two sample t-tests were used for comparison, adjusted by Welch correction if the hypothesis of equal variances was rejected. Mann–Whitney test was applied if data were not normally distributed.

Results and discussion

PRDM11 is dispensable for steady-state maintenance of the hematopoietic system

To explore the expression pattern of *Prdm11* within the HSPC compartment we took advantage of the HemaExplorer web server (http://servers.binf.ku.dk/hemaexplorer) (Bagger et al., 2012; Bagger et al., 2013) and found that *Prdm3*, *Prdm16* and *Prdm11* show similar expression patterns in the HSPC compartments. All three transcripts displayed high expression in the HSCs and were progressively down-regulated following maturation (Supplemental Figs. 1A–C). To investigate if PRDM11 plays an important role in maintaining the HSPC compartment during steady-state hematopoiesis we phenotypically analyzed BMs of one-year-old *Prdm11*$^{-/-}$ mice by flow cytometry (Pronk et al., 2007). Despite the expression profile of *Prdm11* we observed no differences in either frequency or absolute numbers of phenotypically defined HSCs and multipotent progenitors (MPPs) between WT and *Prdm11*$^{-/-}$ animals (Figs. 1A–C) suggesting that PRDM11 is dispensable for the maintenance of the earliest HSPCs duringsteady-state hematopoiesis. Analysis of the further downstream progenitors, including pre-megakaryocyte/erythroid progenitors (preMegEs), megakaryocytic progenitors (MkPs), pre-granulocyte/macrophage progenitors (preGMs), granulocyte/macrophage progenitors (GMPs) as well as Flt3^{+} common lymphoid progenitors (CLP Flt3^{+}), did not reveal any major differences in one-year-old *Prdm11*$^{-/-}$ mice. Although we did observe a small decrease in both frequency and absolute number of MkPs these data suggests that PRDM11 is dispensable for early steady-state myelopoiesis or lymphopoiesis (Figs. 1A, D–G, J). In addition, the early erythroid progenitors pre-colony forming unit erythroid progenitors (preCFU-Es) and CFU-E/proErythroid progenitors (CFU/proEs) were unchanged in the *Prdm11*$^{-/-}$ mice in comparison to WT controls (Figs. 1A, H–I). Similarly, young 10–14 week-old *Prdm11*$^{-/-}$ mice do not differ from their age matched WT controls either (data not shown).

Next, we investigated the cell cycle activity of HSPCs within the BM since both PRDM3 and PRDM16 have previously been implicated in HSC cell cycle control (Aguilo et al., 2011; Chuikov et al., 2010; Zhang et al., 2011). Using flow cytometry we were unable to detect any major differences in the cell cycle distributions of HSPCs in the *Prdm11*$^{-/-}$ mice as compared to WT controls except in the MPP, preMegE, MkP and preCFU-E compartments where a marginal increase of cells in SG_2M was observed (Fig. 2A). This suggests that also PRDM11, in addition to PRDM3 and PRDM16, is involved in the regulation of progenitor cell proliferation, albeit mildly, but restricted to the MPP, preMegE, MkP and preCFU-E compartments. To investigate whether PRDM11 could be involved in regulating HSC quiescence we also co-stained for Ki67 and DAPI but found no difference in the distribution of cells between G_0 and G_1 (Fig. 2B). This suggests that PRDM11 is not directly involved in maintaining the balance between quiescence and proliferation within the HSC compartment. This notion is further supported by the previous finding that PRDM11 deficiency does not affect the total number of cells in any of the BM sub-compartments, with the exception of MkPs (Fig. 1). Taken together, these data suggests that PRDM11 is largely dispensable for steady-state maintenance of the hematopoietic system in one-year-old mice with only a marginal proliferative increase being observed in MPPs, preMegEs, MkPs and preCFU-E.

PRDM11 is dispensable for regeneration of the hematopoietic system following transplantation

HSCs deficient of PRDM3 or PRDM16 were previously shown to be unable to reconstitute the BM post-BMT (Aguilo et al., 2011; Chuikov et al., 2010; Zhang et al., 2011). To evaluate the regeneration capacity of HSCs from the *Prdm11*$^{-/-}$ mice we performed competitive BMTs by mixing whole BM from one-year-old *Prdm11*$^{-/-}$ or WT donors at 1:1 ratio with WT competitor cells and transplanted them into lethally irradiated recipients. Subsequently, we followed hematopoietic regeneration by FACS analysis of PB at 3, 8 and 16 weeks post-BMT. Initial short-term reconstitution at 3 weeks from *Prdm11*$^{-/-}$ donors did not differ in either total reconstitution or any of the mature lineages investigated (Figs. 3A–D). This suggests that PRDM11 is not required for HSC homing, niche lodgement and initial reconstitution. However, at 16 weeks post-BMT we observed a mild reduction of total cells derived from the *Prdm11*$^{-/-}$ donor that was due to decreases in mature B and T cells (Figs. 3A,C–D). In contrast, the short-lived myeloid cells, indicative of intact HSC function, were comparable with that

Figure 2 Cell cycle analysis of bone marrow sub-populations of WT and *Prdm11*$^{-/-}$ mice. A) Cell cycle analysis using DAPI to discriminate between G_0, G_1 and SG_2M of indicated BM sub-population in one-year-old *Prdm11*$^{-/-}$ mice and WT controls. Representative FACS plots with the average frequency shown. n = 8. B) Cell cycle analysis using DAPI and Ki67 to discriminate between G_0, G_1 and SG_2M in HSCs. Representative FACS plots with the average frequency shown. Horizontal bars indicate mean value. n = 3.

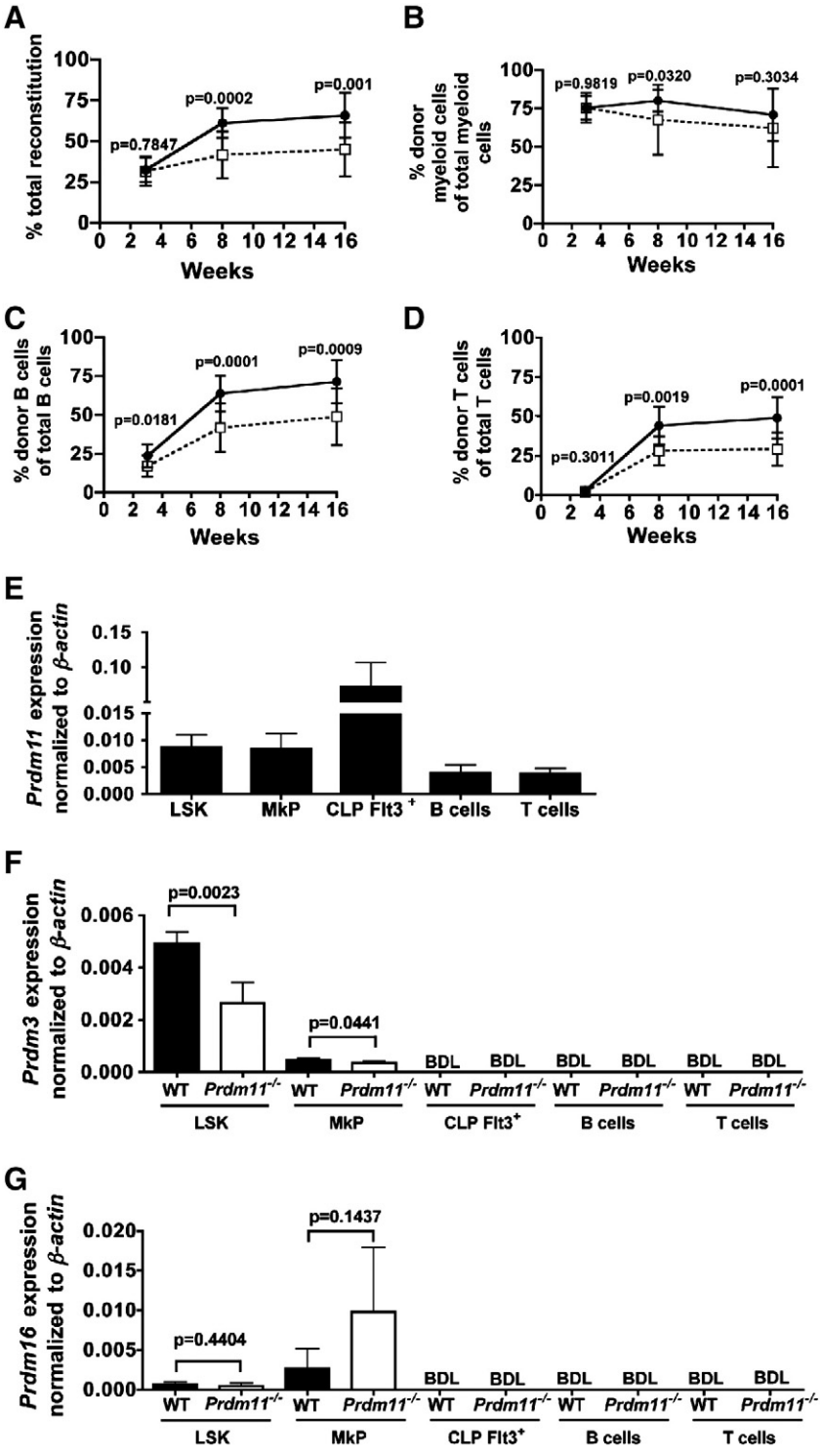


Figure 3 PRDM11 deficient mice regenerate the hematopoietic system post-bone marrow transplantation with a mild defect in lymphopoiesis. Whole BM from one-year-old PRDM11 deficient (CD45.2; white square, black dashed line) or WT control mice (CD45.2; black dot and line) was intravenously transplanted in 1:1 ratio (10^6:10^6 cells) with WT competitor cells (CD45.1) into lethally irradiated WT recipient mice (CD45.1). PB was analyzed by FACS at 3, 8 and 16 weeks post-BMT and investigated for test cell derived CD45.2$^+$ A) % total reconstitution, B) % myeloid cells, C) % B cells, and D) % T cells. Four *Prdm11*$^{-/-}$ donor mice were assayed in 19 WT recipient mice and 4 WT donors were assayed in 12 WT recipient mice. qRT-PCR of indicated FACS sorted sub-populations from WT and *Prdm11*$^{-/-}$ mice for mRNA expression of E) *Prdm11*, F) *Prdm3* and G) *Prdm16* normalized to *β-actin*. Four individual mice per genotype were investigated. Error bars indicate standard deviation. BDL = below detection level.

of WT controls albeit with a marginal drop in *Prdm11*$^{-/-}$ derived myeloid cells at 8 weeks (Fig. 3B). In addition, we also investigated the engraftment and regeneration capacity of HSCs from 10–14 week-old *Prdm11*$^{-/-}$ donors by competitive (Supplemental Fig. 2) and non-competitive (Supplemental Fig. 3) BMT, but found no difference in reconstitution capacity in comparison with WT controls. In summary, our data indicate that the HSCs from one-year-old *Prdm11*$^{-/-}$ mice can regenerate the hematopoietic system post-BMT, but that they are mildly impaired in their ability to fully reconstitute the lymphoid lineage. It is tempting to speculate that the impairment in lymphoid differentiation happens downstream of the phenotypically intact CLP Flt3^{+} compartment. Taken together, in striking contrast to PRDM3 and PRDM16, our data suggest that PRDM11 is dispensable for regeneration of the hematopoietic system following transplantation.

We next speculated that compensatory up-regulation of the expression of other PRDM family members such as PRDM3 and PRDM16 could potentially compensate for the loss of PRDM11. We therefore tested the expression of *Prdm3* and *Prdm16* in selected hematopoietic subsets but did not find any evidence for transcriptional up-regulation of any of these transcripts in the *Prdm11*$^{-/-}$ animals (Figs. 3F–G). Whereas these findings clearly demonstrate that the mild phenotype in the *Prdm11*$^{-/-}$ mice is not due to a compensatory increase in the expression of *Prdm3* and *Prdm16* they do not exclude the possibility that the PRDM family members may exert redundant functions in HSPCs. In line with this we note that the lymphoid cells CLP Flt3^{+} B and T cells (as opposed to the other cell types tested) are completely devoid of both *Prdm3* and *Prdm16* expression, but do express *Prdm11*, which potentially may explain the mild lymphoid phenotype observed in *Prdm11*$^{-/-}$ animals (Figs. 3E–G).

PRDM11 is dispensable for megakaryocyte development

From the HemaExplorer web server we found *Prdm11* to be expressed in MkP cells (Bagger et al., 2012; Bagger et al.,2013). This was interesting since we observed a significant downregulation of phenotypically defined MkPs in the *Prdm11*$^{-/-}$ mice (Fig. 1E) that was further supported by the mild proliferative increase observed in the same population (Fig. 2A). This prompted us to further examine the role of PRDM11 in megakaryocyte (Mk) development. To do so we took advantage of the MegaCult cell culture system to test the *in vitro* CFU-Mk potential of whole BM cells from WT and *Prdm11*$^{-/-}$ mice, but found no difference in either CFU-Mk or in a mix with granulocytes/macrophages CFU-GM/CFU-Mk mix colonies (Fig. 4A). To further explore the Mk cell dependency on PRDM11 we analyzed PB from both WT and *Prdm11*$^{-/-}$ mice for platelet numbers and found no deviation in the capacity of the Mks to produce platelets in the absence of PRDM11. Taken together, we find very little evidence for PRDM11 being involved in the development of Mks or in their ability to form platelets.

Conclusions

Prdm11 is highly expressed in the earliest HSPCs and is downregulated following maturation. However, PRDM11 is dispensable for the maintenance of the HSPCs during steady-state hematopoiesis in one-year-old mice and is not directly involved in maintaining the balance between proliferation and quiescence. In addition, upon BMT PRDM11 deficient cells were able to fully regenerate the hematopoietic system long-term with only a minor drop in lymphoid output suggesting that PRDM11 is not involved in HSC engraftment and self-renewal. In conclusion, PRDM11 is largely dispensable for maintenance and regeneration of the hematopoietic system.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2013.07.009.

Acknowledgments

This work was supported by an Alfred Benzon fellowship to LAT and through a center grant from the Novo Nordisk Foundation (The Novo Nordisk Foundation Section for Stem Cell Biology in Human Disease). LAT and BTP designed the research, analyzed the data and wrote the manuscript. LAT, NBV and CC performed the experiments. CKF, KTJ and AHL generated and provided *Prdm11*$^{-/-}$ mice. The authors thank Ib Jarle Christensen for valuable statistical guidance, Anna Fossum for cell sorting and Janus Schou-Jakobsen and Robert Mansson for advice on qRT-PCR.

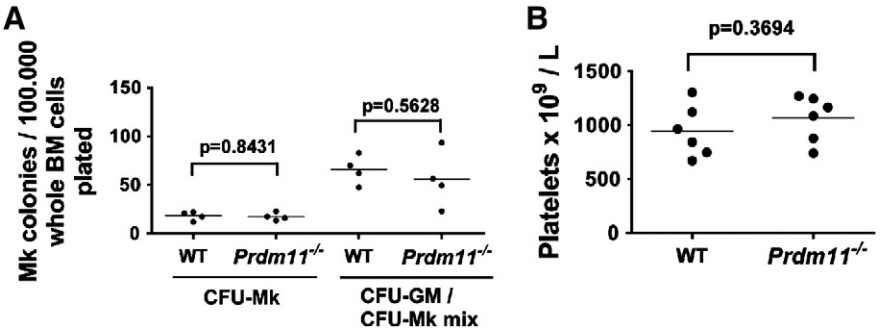


Figure 4 PRDM11 is dispensable for *in vitro* megakaryocyte formation and *in vivo* platelet production. A) *In vitro* megakaryocyte potential in WT and *Prdm11*$^{-/-}$ whole BM cells. Data show mean number of CFU-Mk and combined CFU-Mk/CFU-GM mix colonies. n =4. B) PB was sampled from WT and *Prdm11*$^{-/-}$ mice and analyzed for number of platelets. n = 6. Horizontal bar indicates mean.

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