LETTERS

PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention

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Haematopoietic stem cells (HSCs) must achieve a balance between quiescence and activation that fulfils immediate demands for haematopoiesis without compromising long-term stem cell maintenance, yet little is known about the molecular events governing this balance[1–3]. Phosphatase and tensin homologue (PTEN) functions as a negative regulator of the phosphatidylinositol-3-OH kinase (PI(3)K)–Akt pathway, which has crucial roles in cell proliferation, survival, differentiation and migration[4,5]. Here we show that inactivation of PTEN in bone marrow HSCs causes their short-term expansion, but long-term decline, primarily owing to an enhanced level of HSC activation. PTEN-deficient HSCs engraft normally in recipient mice, but have an impaired ability to sustain haematopoietic reconstitution, reflecting the dysregulation of their cell cycle and decreased retention in the bone marrow niche. Mice with PTEN-mutant bone marrow also have an increased representation of myeloid and T-lymphoid lineages and develop myeloproliferative disorder (MPD)[6]. Notably, the cell populations that expand in PTEN mutants match those that become dominant in the acute myeloid/lymphoid leukaemia that develops in the later stages of MPD. Thus, PTEN has essential roles in restricting the activation of HSCs, in lineage fate determination, and in the prevention of leukaemogenesis.

The *Pten* tumour suppressor gene is commonly mutated in malignancy, including leukaemias that feature dysregulated haematopoiesis[4,5]. To ablate PTEN function in adult HSCs, we crossed a polyinosine-polycytidine (pIpC)-inducible *Mx-1-Cre* recombinase mouse line[7,8] with a conditional PTEN-mutant (*Pten*$^{fl/fl}$) mouse line[9], and induced Cre expression with pIpC on postnatal day 21, 23 and 25 (Supplementary Fig. 1a). Times measured from the final pIpC injection are expressed as days post-induction (DPI). Our study focuses on *Mx-1-Cre*$^{+}$;*Pten*$^{fl/fl}$ animals, hereafter referred to as 'PTEN mutants'. Control animals were pIpC-treated *Mx-1-Cre*$^{-}$;*Pten*$^{fl/fl}$ mice, which are phenotypically equivalent to animals with a wild-type *Pten* allele (Supplementary Fig. 1b).

At 30 DPI, we found that PTEN-mutant mice had fewer HSCs than controls, as measured by the absolute number of Lin^{-}Sca-1^{+}c-Kit^{+} cells (referred to as 'LSK' cells) in the bone marrow using flow cytometry. LSK cells are a heterogeneous population that includes long-term HSCs (LT-HSCs; LSK Flk-2^{-}) and short-term HSCs (ST-HSCs; LSK Flk-2^{+}), which are capable of sustaining haematopoiesis for many months, or for a few weeks, respectively[10]. The decrease in PTEN-mutant HSCs stemmed from a decline in the LT-HSC population, whereas the ST-HSC population was unchanged (Fig. 1a). Thus, PTEN is required for LT-HSC maintenance.

PTEN controls cell cycle entry and progression through the inhibition of PI(3)K–Akt activity[4,5], so we examined the cell cycle profiles of the HSCs—as determined by RNA versus DNA content in LSK cells. PTEN mutants showed a 2–3-fold decrease in the proportion of HSCs in the G0 phase and a concomitant increase in the proportion of HSCs in the S + G2M phases (Fig. 1b). Consistent with this result, staining for the proliferation antigen Ki67 revealed that the proportion of cycling HSCs is aberrantly high in PTEN mutants (Supplementary Fig. 1c).

Next, we examined the relationship between PTEN expression and cell cycle status. PTEN is phosphorylated at its carboxy terminus by casein kinase II, producing a phospho-PTEN (p-PTEN) form that is unable to be recruited to the plasma membrane to antagonize PI(3)K–Akt activity[11]. *In vivo* p-PTEN is indicative of cells in which Akt is in an active state[12], so we distinguished between p-PTEN and

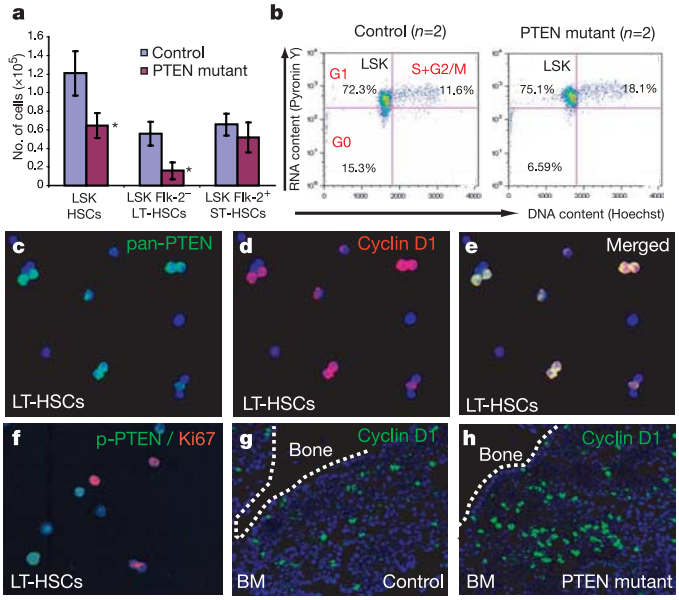

Figure 1 | PTEN governs the number and activation state of HSCs and PTEN expression is associated with cyclin D1. a, Absolute number of HSCs (LSK), LT-HSCs (LSK Flk-2^{-}) and ST-HSCs (LSK Flk-2^{+}) in control and PTEN-mutant bone marrow at 30 DPI. *, $P < 0.05$. **b**, Comparison of LSK cells in G0, G1 or S+G2/M phases between control animals and PTEN mutants. **c**–**e**, Sorted LSK Flk-2^{-} preparations co-stained with antibodies that recognize pan-PTEN (green; **c**, **e**) and cyclin D1 (red; **d**, **e**), and counterstained with 4,6-diamidino-2-phenylindole (DAPI; blue). Concurrence of two signals is shown in the merged view (**e**). **f**, Co-staining with antibodies that recognize p-PTEN (green) and Ki67 (red), indicating that most p-PTEN^{+} cells are Ki67^{-}. **g**, **h**, Bone marrow (BM) sections from control (**g**) and PTEN-mutant (**h**) mice stained with anti-cyclin D1 antibody (green). Error bars in **a** represent s.d.

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the non-phosphorylated form (non-p-PTEN) by staining with one antibody that recognizes both forms of PTEN (pan-PTEN), and another antibody that recognizes only p-PTEN. Performing immunofluorescence in sorted LT-HSCs, we observed a strong association between the pan-PTEN signal and the presence of cyclin D1. The pan-PTEN signal was found almost exclusively (98% of the time) in cells that were also cyclin D1 positive (cyclin $D1^+$), and most (82%) cyclin $D1^+$ cells were also pan-$PTEN^+$ (Fig. 1c–e; Supplementary Fig. 1d). Cyclin D1, a known target of the PI(3)K–Akt pathway[4], maintains cells at the G1 phase in preparation for the G1/S-phase transition, so these data indicate that PTEN is regulated through the cell cycle and is predominantly expressed in cells at G1. In contrast, only around half of the cyclin $D1^+$ cells were found to be p-$PTEN^+$ (data not shown), although p-PTEN was rarely present in cycling ($Ki67^+$) HSCs (Fig. 1f). We also observed that PTEN deficiency markedly increased the number of cyclin $D1^+$ cells in bone marrow sections (Fig. 1g, h), although this probably reflects the combined impact of the mutation on HSCs and on multiple lineages of their progeny.

HSCs are normally located in bone marrow niches[3,7,13,14]. As the PTEN–Akt pathway can regulate cell migration[4], we asked whether the lack of PTEN affects the retention of HSCs in the bone marrow. We used flow cytometry at 2, 6, 9, 12 and 30 DPI to examine HSC (LSK cell) numbers in the bone marrow, peripheral blood and spleen. In addition, at the 30-day time point we used quantitative colony-forming unit (CFU) assays (see Supplementary Methods) to functionally assess the numbers of either HSCs (by measuring CFU-spleen, or CFU-S) or multiple myeloid progenitors (measuring CFU in culture, or CFU-C). We found that PTEN deficiency led to a modest increase in the percentage of bone marrow HSCs at 2 DPI, but a decline from 6 DPI onwards (Fig. 2a). These changes were accompanied by dramatic increases in the HSC population in the peripheral blood and spleen (for example, a 10-fold increase on day 30 in the spleen; Fig. 2a, middle and lower panels; Supplementary Fig. 2a, b, d). Thus, PTEN mutation leads to mobilization of HSCs from the bone marrow into the peripheral blood and spleen. CFU-S assays confirmed this result. PTEN mutant-derived bone marrow contained fewer CFU-S than control bone marrow; PTEN-mutant spleen cells formed CFU-S, whereas those derived from control mice did not (Fig. 2b; Supplementary Fig. 2c). HSC mobilization can result in extramedullary (outside the bone marrow) haematopoiesis when the bone marrow is stressed. Indeed, we found that in PTEN-mutant mice progenitor cells were present ectopically in the peripheral blood and spleen. The number of peripheral blood- or spleen-derived CFU-C was substantially increased in the PTEN mutants compared with controls, whereas bone marrow-derived CFU-C remained unchanged (Fig. 2c; Supplementary Fig. 2e).

Exploring the mechanism of HSC mobilization[15], we found that PTEN deficiency did not affect the ability of HSCs to adhere to fibronectin, collagen or laminin (Supplementary Fig. 3c), nor was there any significant alteration in migration speed (data not shown) or migrating cell number (Supplementary Fig. 3b) when PTEN-mutant cells were exposed to stromal cell-derived factor 1 (SDF-1; also known as CXCL12). Moreover, in PTEN mutants there was no change in the expression of either CXCR4 (an SDF-1 receptor) or $\alpha_{4/5}$ integrin, both of which are important for HSC homing and migration[15–17] (Supplementary Fig. 3a).

We then performed *in vivo* homing and lodging assays, in which fluorescently labelled bone marrow is transplanted into wild-type hosts and assessed for the ability to home to or lodge within the bone marrow (primarily a property of HSCs). We found that PTEN deficiency did not affect homing to the bone marrow of irradiated hosts (a treatment that destroys resident HSCs, resulting in vacant niches and elevated levels of SDF-1) (Fig. 2d). In contrast, when transplanted into non-irradiated hosts (that is, having intact bone marrow with few vacant niches and baseline SDF-1 levels), only half as many mutant as control cells lodged in the bone marrow at 6-hours post-transplantation, and this difference was greater still by 18-hours post-transplantation (Fig. 2e). Thus, PTEN HSCs are capable of reaching and residing in the bone marrow when conditions are conducive to homing, but they do not perform as well as normal HSCs when there are few vacant niches and competing wild-type host HSCs are present.

HSCs that mobilize to the spleen are more proliferative than HSCs

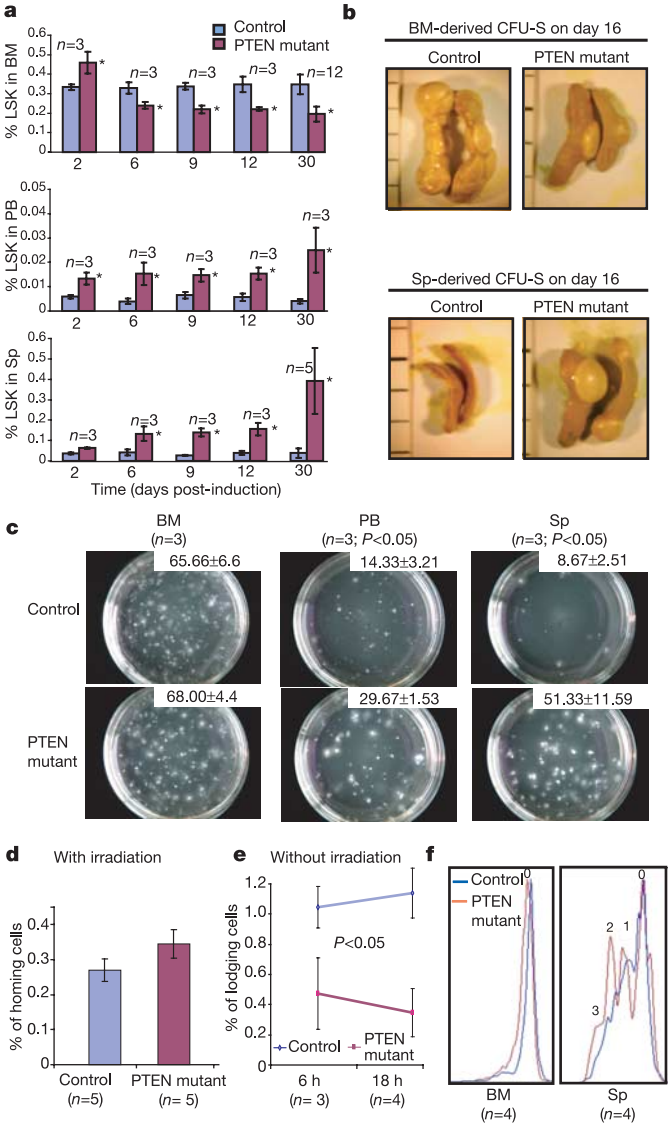

Figure 2 | Deletion of PTEN results in HSC expansion and mobilization and impaired bone marrow lodging. a, Flow cytometry analysis of LSK cells on the indicated days post-induction in control and PTEN-mutant bone marrow (BM; upper panel), peripheral blood (PB; middle panel) and spleen (Sp; lower panel). *, $P < 0.05$. **b**, Comparison of CFU-S on day 16 post-transplantation for control and PTEN-mutant bone marrow (upper panels) and spleen (lower panels). **c**, Comparison of *in vitro* CFU-C (a combination of CFU-E (erythroid), CFU-GM (granulocyte, macrophage) and CFU-GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte)) in control and PTEN-mutant bone marrow (left panels), peripheral blood (middle panels) and Sp (right panels) counted on day 12. **d**, Analysis of bone marrow homing ability in control and PTEN-mutant bone marrow in an irradiated host mouse. **e**, **f**, Analysis of the bone marrow lodging ability of control and PTEN-mutant bone marrow in a non-irradiated host (**e**) and the corresponding proliferation profiles of lodged cells assessed by the dilution of their fluorescent label (**f**). Peaks in **f** represent cell populations that underwent the number of cell divisions (0, 1, 2 or 3) as shown. Error bars in **a**, **d**, **e** represent s.d.

retained in the bone marrow[18]. If the ability to lodge in the bone marrow is linked to cell cycle status, the reduced lodging of PTEN-mutant cells may reflect their increased likelihood of being in an activated state. Indeed, our lodging assays showed a strong association between proliferation rate and lodging location (Fig. 2f). Irrespective of PTEN status, cells that lodged in the bone marrow were predominantly quiescent (no cell division) up to the 18-hour time point. In contrast, of the cells that were lodged in the spleen at the same time point, 60% of the PTEN mutants versus 45% of the controls ($P = 0.0042$) had undergone one, two or three cell divisions (compare peaks of the blue and red curves, Fig. 2f).

During haematopoietic cell development, HSCs progressively give rise to different progenitor cells, which, in turn, generate the various mature lineages[10,19,20] (see also Fig. 3a). Thus, we determined the impact of PTEN deficiency on the haematopoietic lineage at 30 DPI, and found that CLP (common lymphoid progenitor) numbers were significantly lower in PTEN-mutant bone marrow compared with control bone marrow, whereas the CMP (common myeloid progenitor), GMP (granulocyte, monocyte progenitor) and MkEP (megakaryocyte, erythroid progenitor) populations remained unchanged (Fig. 3b). Although the number of Gr-1$^+$Mac-1$^+$ myeloid cells was increased, the number of B lymphocytes (B220$^+$) was substantially decreased (Fig. 3c), the latter change involving both the loss of CLPs

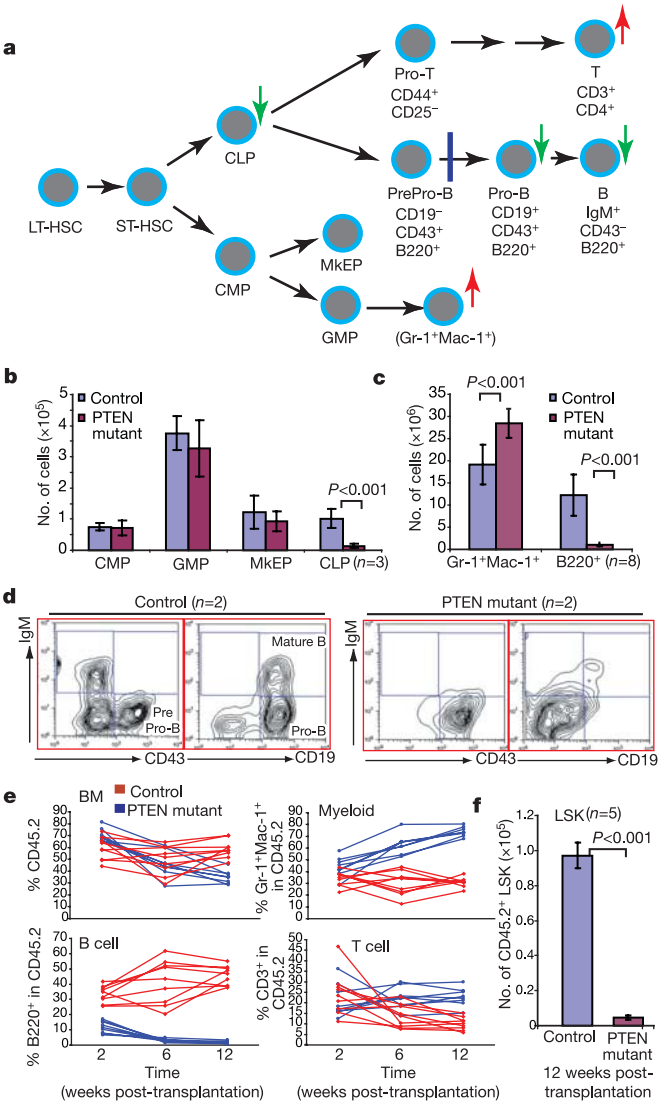

Figure 3 | Lineage analysis and competitive repopulation assay. **a**, Haematopoietic lineage tree showing the impact of PTEN deficiency. Increased and decreased cell populations are marked by red and green arrows, respectively. The blue vertical bar indicates a point of developmental blockade. See the text for definitions of the cell populations. **b**, **c**, Absolute numbers of CLP, CMP, GMP and MkEP cells (**b**) and Gr-1$^+$Mac-1$^+$ myeloid and B220$^+$ B-lineage populations (**c**) in control and PTEN-mutant bone marrow at 30 DPI. **d**, Analysis of B-lineage development, profiling sorted B220$^+$ cell populations from controls and PTEN mutants. **e**, **f**, Competitive repopulation analysis of control and PTEN-mutant bone marrow. Recipient mice were transplanted with 2×10^5 rescue bone marrow cells together with 1×10^6 PTEN-mutant bone marrow or 2×10^5 control bone marrow cells. Analyses of the donor (CD45.2$^+$) contribution to different lineages in the peripheral blood (**e**) and to HSCs in the bone marrow (**f**). Error bars in **b**, **c**, **f** represent s.d.

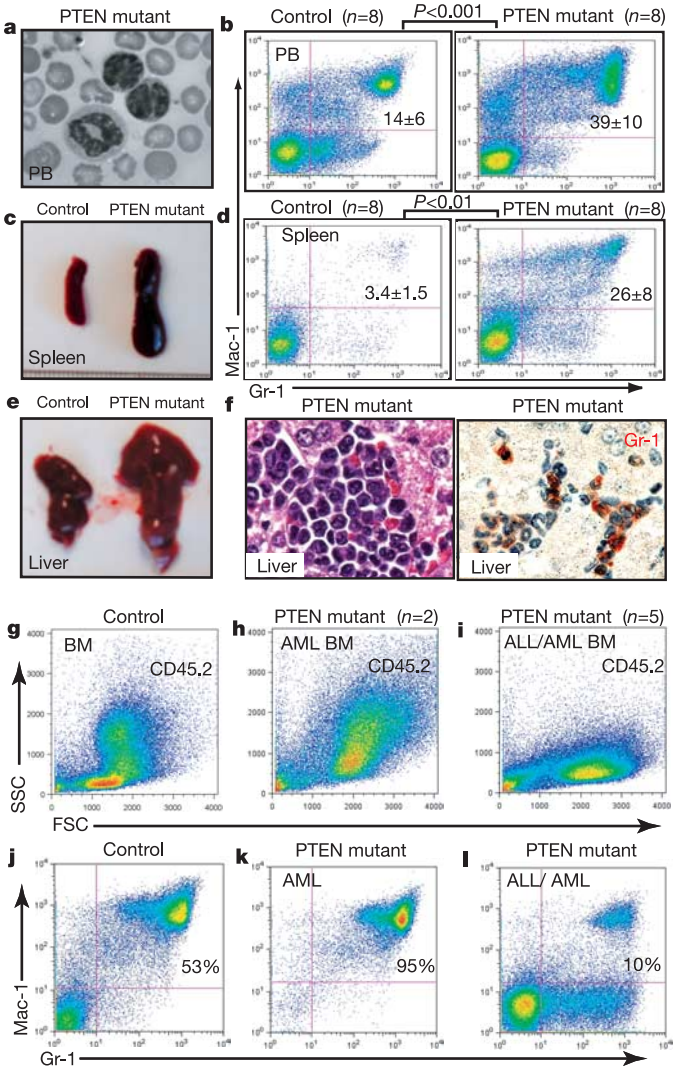

Figure 4 | Loss of PTEN results in myeloproliferative disorder (MPD) and leukaemia. **a**–**f**, Occurrence of MPD in PTEN-mutant mice as evidenced by an increased number of proliferative myeloid progenitors found in the peripheral blood (**a**, **b**), spleen (**c**, **d**) and liver (**e**, **f**), where myeloid cell infiltration in PTEN mutants is shown by Gr-1 staining. All tissues were studied at 30 DPI. **g**–**l**, Leukaemia developed from transplantation of PTEN-mutant bone marrow. Flow cytometry analyses of bone marrow from lethally irradiated mice transplanted with control bone marrow (**g**, **j**) or PTEN-mutant bone marrow (**h**, **i**, **k**, **l**). Relative heterogeneity (**g**; control) or homogeneity (**h**, **i**; PTEN-mutant) of CD45.2$^+$ donor cell types in the bone marrow, revealed by separating cells according to their light-scattering properties (SSC, side scatter; FSC, forward scatter). Further characterization of the bone marrow according to Gr-1 and Mac-1 marker expression in CD45.2$^+$ donor cells from control (**j**) or PTEN-mutant (**k**, **l**) bone marrow.

and a developmental block of cells in the prepro-B-lymphoid ($CD19^-CD43^+$) stage (Fig. 3d). Similar results were obtained from competitive repopulation assays (described below), with a decrease in the B lineage but increases in the myeloid and T lineages (Fig. 3e).

To test the reconstitution ability of PTEN-deficient HSCs, we performed competitive repopulation assays that compare the contribution of marked ($CD45.2^+$) donor cells to that of recipient cells ($CD45.1^+$) following transplantation into lethally irradiated mice. The contribution (per cent $CD45.2^+$) of donor-derived cells, analysed in the peripheral blood at the indicated times post-transplantation, progressively decreased for PTEN-mutant donors but not for control donors (Fig. 3e). Analysis of the bone marrow at the 12-week time point revealed that—despite using five times more mutant cells—the proportion of donor-derived LSK cells in the group transplanted with PTEN-mutant cells was decreased to one-eighth that of the control-transplanted group (Fig. 3f). Thus, the general decline observed in the contribution of PTEN-mutant cells to the bone marrow reflects a depletion of the PTEN-deficient HSCs. Similar results were obtained from transplanted spleen cells (Supplementary Fig. 4), indicating that the HSCs aberrantly located in the spleen of PTEN-mutant donor animals were functionally similar to those found in the bone marrow.

Loss of PTEN function also results in myeloproliferative disorder (MPD), which, in humans, will (in many cases) transform into leukaemia at later stages[6]. PTEN mutants at around 30 DPI showed increased populations of proliferative monocytes and granulocytes in the peripheral blood (Fig. 4a, b), spleen (Fig. 4c, d) and multiple tissues including liver (Fig. 4e, f). These are all features characteristic of MPD[6]. In our PTEN mutants, the MPD leads to the death of the animals at around 30–40 DPI, so we performed bone marrow transplants to extend the time over which the impact of PTEN loss could be studied. Mice transplanted with PTEN-mutant bone marrow cells (which were combined with normal rescue bone marrow cells necessary to sustain the recipient) showed the same features of MPD that were observed in primary (donor) PTEN-mutant mice, but also subsequently developed severe leukaemia or lymphoma and died within 3–4 months. Mice that received control bone marrow showed a normal heterogeneity of cell types (Fig. 4g, j), whereas the profiles obtained from recipients of PTEN-mutant bone marrow (Fig. 4h, i, k, l) were more homogeneous, reflecting a blast crisis stage of acute myeloid leukaemia (AML; 2 out of 7 cases) (Fig. 4h, k) or acute myeloid and lymphoid leukaemia (AML/ALL; 5 out of 7 cases) (Fig. 4i, l). Two of the five AML/ALL mice progressed further and developed T lymphoma (data not shown). In mice with AML, more than 80% of the bone marrow cells expressed the myeloid Gr-1/Mac-1 markers (Fig. 4k). Mice that developed T-lymphoid leukaemia showed one of two phenotypes. In three out of five cases, the bone marrow was dominated by $CD3^+/CD4^+$T lymphocytes (>80%; Supplementary Fig. 5a). In the other two cases, over 80% of bone marrow cells were $CD44^+/CD25^-$, indicative of an enriched population of pro-T cells (Supplementary Fig. 5b), and only 10–20% of cells were myeloid (Fig. 4l).

In adults, homeostasis between the quiescent and activated states of stem cells is essential to balance stem cell maintenance with ongoing tissue regeneration[1,3]. This study has shown that inactivation of PTEN deregulates HSCs, reducing the proportion that are quiescent (in G0), increasing the proportion that actively cycle, and compromising the ability to maintain sufficient LT-HSCs. We suggest that PTEN is a critical component of a molecular switch governing the forward (G0–G1) or backward (G1–G0) transitions between the quiescent and activated states in LT-HSCs—a model that is consistent with the impact of PTEN loss on HSCs and with our observation that PTEN in LT-HSCs is primarily associated with the cyclin $D1^+$ population. In the absence of PTEN, cell cycle entry (the forward transition) may occur more readily, as recently suggested for neural stem cells[21], or the switch may become 'forward only' and prevent LT-HSCs in G1 from returning to a quiescent state. The distinct populations of LT-HSCs containing p-PTEN and non-p-PTEN raises the possibility that PTEN phosphorylation may serve as a sensor for this switch. Depending on environmental cues, the activated (G1) LT-HSCs may progress further through the cell cycle—a process controlled, in part, by PI(3)K–Akt activity. A potential downstream target at the G1-to-S transition is Myc activity, which can be controlled by Akt through phosphorylation of GSK3β (ref. 22). PI(3)K–Akt also counters the cell cycle inhibitors p21 and p27 (ref. 4), so it is notable that the constitutive activation of HSCs we obtained by inactivation of PTEN resembles the result of deleting p21 in this population[23]. Equally, leukaemia in animals carrying the Akt mutation can be partially rescued by treatment with rapamycin, an inhibitor of the serine–threonine kinase mTOR (mammalian target of rapamycin)[24]. The forward and reverse transitions involve changes not only in cell cycle status but also in niche interaction properties[25], so the PTEN–Akt pathway, which affects both of these components, may help coordinate the two. The precise molecular basis of the aberrant mobilization of HSCs that have a PTEN mutation to the peripheral systems remains to be determined, but PTEN-mutant cells appear to be deficient in their retention ability in bone marrow niches.

PTEN also has a role in controlling haematopoietic lineage fate, as evidenced by the decline in CLP and B-lineage numbers and the increase in the myeloid and T lineages that was observed in PTEN mutants. PTEN deficiency could distort multiple lineages if PTEN governs lineage choice in the lymphoid primed multipotent progenitor (LMPP) that is proposed to give rise to the T lineage, CLPs and GMPs[26–28] (see the model in Supplementary Fig. 5c). Most likely, however, PTEN acts at multiple stages as implied by lineage-specific PTEN inactivation studies[29,30]. Inactivation of PTEN results in transplantable MPD and acute myeloid/lymphoid leukaemias that express markers of the myeloid and T lineages. These are strikingly similar to the expanded cell populations observed in transplanted mice with bone marrow cells carrying a PTEN mutation, indicative of an intrinsic link between lineage regulation and leukaemogenesis. This may be because constitutive PI(3)K–Akt activity distorts lineage commitment and provides a growth advantage for certain progenitor cells over other lineages, resulting in expanded cell pools in which a second genetic mutation may occur. Progenitor pool expansion in leukaemia contrasts with the behaviour of PTEN-deficient HSCs, which undergo uncontrolled proliferation but eventually become depleted or exhausted. Exploring this difference between leukaemic cells and HSCs should provide insight into self-renewal mechanisms, and could identify potential therapeutic targets for eliminating leukaemic stem cells without adversely affecting normal stem cells.

METHODS

Additional details are provided in Supplementary Methods.

PTEN-mutant mice. $Pten^{fl/fl}$ mice[9] were crossed with $Pten^{fl/+}$ animals carrying an *Mx-1-Cre* transgene[8] to generate litters containing $Mx\text{-}1\text{-}Cre^+; Pten^{fl/fl}$, $Mx\text{-}1\text{-}Cre^+; Pten^{fl/+}$, $Pten^{fl/fl}$ and $Pten^{fl/+}$ mice. pIpC injection at weaning induced PTEN deletion.

Flow cytometry. The isolation and preparation of bone marrow, spleen and peripheral blood cells, and the method for subsequent flow cytometry assays, have been described previously[7].

Immunofluorescence studies. The immunostaining procedures used in this study have been described previously[7].

Colony-forming unit assays. See Supplementary Methods.

Cell migration and adhesion assays. Adhesion molecules were analysed by flow cytometry as described previously[17]. For details on the cell migration assays, see Supplementary Methods.

Cell proliferation and cell cycle analysis. Cell proliferation and cell cycle profiles were assessed by flow cytometry on the basis of Ki67, pyronin Y and Hoechst 33342 staining.

Competitive engraftment assay and leukaemia phenotypes. Bone marrow cells from control and PTEN-mutant mice ($CD45.2^+$ background) were each mixed with recipient bone marrow cells ($CD45.1^+$ background) and transplanted into lethally irradiated recipient mice. At different time points post-transplantation, the recipient mice were analysed for the reconstitution ability of donor cells

using different lineage markers. Tissues from the recipient mice that developed acute leukaemia were collected for further cell-surface marker staining.

Homing and lodging assays. Bone marrow cells were obtained from control and PTEN-mutant mice and labelled with carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) (Invitrogen) as per the manufacturer's instructions. The homing and lodging assays involved transplanting labelled bone marrow cells into lethally irradiated and non-irradiated mice, respectively. At different time points, recipient-derived bone marrow and spleen cells were evaluated by flow cytometry for donor cells labelled with CFDA.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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