Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells

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Recent advances have highlighted extensive phenotypic and functional similarities between normal stem cells and cancer stem cells. This raises the question of whether disease therapies can be developed that eliminate cancer stem cells without eliminating normal stem cells. Here we address this issue by conditionally deleting the *Pten* tumour suppressor gene in adult haematopoietic cells. This led to myeloproliferative disease within days and transplantable leukaemias within weeks. *Pten* deletion also promoted haematopoietic stem cell (HSC) proliferation. However, this led to HSC depletion via a cell-autonomous mechanism, preventing these cells from stably reconstituting irradiated mice. In contrast to leukaemia-initiating cells, HSCs were therefore unable to maintain themselves without *Pten*. These effects were mostly mediated by mTOR as they were inhibited by rapamycin. Rapamycin not only depleted leukaemia-initiating cells but also restored normal HSC function. Mechanistic differences between normal stem cells and cancer stem cells can thus be targeted to deplete cancer stem cells without damaging normal stem cells.

Cancer stem cells have notable phenotypic and mechanistic similarities to normal stem cells in the same tissues[1–4]. Acute myeloid leukaemia (AML) is sustained by leukaemic stem cells that are also called leukaemia-initiating cells because they are defined by their ability to transfer disease on transplantation into irradiated mice[5–7]. Leukaemia-initiating cells express markers similar to normal HSCs[5,6] and depend on similar mechanisms to self-renew[8,9]. Brain cancer stem cells also express markers of normal neural stem cells and depend on similar pathways for their proliferation[4,10]. The Hedgehog, Wnt and Notch pathways that often promote cancer cell proliferation also promote normal stem cell self-renewal[1,2,11,12]. Conversely, tumour suppressors that inhibit cancer cell proliferation—such as p53, p16^INK4a and p19^ARF—also inhibit stem cell self-renewal[11,13,14]. Whether cancer stem cells arise from normal stem cells or other cells, their similarity to normal stem cells indicates that they inherit or acquire stem cell properties. This raises the question of whether it will be possible to identify therapies that eliminate cancer stem cells without eliminating normal stem cells in the same tissues.

We have addressed this issue by examining the effect of *Pten* deletion on leukaemia-initiating cells and normal HSCs. PTEN (for phosphatase and tensin homologue) is a phosphatase that negatively regulates signalling through the phosphatidylinositol-3-OH kinase (PI(3)K) pathway, inhibiting proliferation and survival[15,16]. *Pten* is commonly deleted or otherwise inactivated in diverse cancers[17], including haematopoietic malignancies[18–21]. Here we report that whereas *Pten* deletion causes the generation of transplantable leukaemia-initiating cells, it also causes the depletion of normal HSCs, thus identifying a mechanistic difference between the maintenance of normal stem cells and cancer stem cells.

Pten deletion leads to leukaemogenesis

Pten was conditionally deleted from 6-to-8-week-old *Pten^{fl/fl}*; *Mx-1-Cre* mice by administering seven doses of polyinosine-polycytidine (pIpC) over 14 days to induce Cre expression[22,23]. After 14 days, *Pten* seemed to be completely deleted from HSCs and other haematopoietic cells (Supplementary Fig. 1). We analysed *Pten^{fl/fl}*; *Mx-1-Cre* mice, as well as *Pten^{fl/+}*; *Mx-1-Cre* littermate control mice, five days after pIpC treatment. Almost all *Pten^{fl/fl}*; *Mx-1-Cre* mice (17 out of 19) developed myeloproliferative disease marked by a tenfold increase in spleen cellularity (Fig. 1c), complete histological effacement of the splenic architecture (Fig. 1b), reduced bone marrow cellularity (Fig. 1c), and increased blast cell frequency (Fig. 1d). The increased spleen cellularity was largely attributable to extramedullary haematopoiesis (Supplementary Fig. 2c, d) with a prominent expansion in the number of immature myeloid cells (Supplementary Fig. 2e–g; Supplementary Table 1). None out of 20 *Pten^{fl/+}*; *Mx-1-Cre* littermates showed these changes after pIpC treatment (Fig. 1c, a, d).

Within 4 to 6 weeks after pIpC treatment, most *Pten^{fl/fl}*; *Mx-1-Cre* mice progressed to frank leukaemia[24], including AML and acute lymphoblastic leukaemia (ALL), and died (for the criteria used to diagnose leukaemias, see Supplementary Table 2). AMLs were characterized by large numbers of chloroacetate-esterase-positive myeloid blasts in the spleen (Fig. 1e), and ALLs were characterized by large numbers of terminal deoxynucleotidyl transferase (TdT)-positive lymphoid blasts throughout the thymus, which was also enlarged and effaced (Fig. 1f). The bone marrow contained Mac-1^+Gr-1^{low}CD4^− myeloid blasts and CD4^+CD8^+CD3^+Mac-1^− lymphoid blasts (Fig. 1h, i; data not shown). Karyotypic analysis of myeloid blasts from four *Pten*-deleted mice with AML revealed

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marked aneuploidy and/or chromosomal translocations, suggesting leukaemogenesis was associated with additional mutations after *Pten* deletion (Supplementary Fig. 3). Common abnormalities across multiple karyotypes from each mouse suggested that the AMLs were clonal or oligoclonal (Supplementary Fig. 3).

HSCs proliferate after *Pten* deletion but become depleted

We examined the cell cycle status of whole bone marrow cells and Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells five days after completing pIpC administration. Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells are highly enriched for HSCs[25–27], representing only 0.005% of bone marrow cells. Nineteen out of 20 mice transplanted with 15 Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells from control mice showed long-

term multilineage reconstitution by the donor cells (Supplementary Table 3). There was no significant effect of *Pten* deletion on the cell cycle distribution of whole bone marrow cells (Fig. 2a, b). In contrast, there was a three-to-four-fold increase in the percentage of dividing Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells, and in the percentage that incorporated the nucleotide analogue BrdU (5-bromodeoxyuridine) over a 19-h period (Fig. 2c–f). These data suggest that *Pten* promotes quiescence in HSCs, and that in the absence of *Pten* HSCs are driven into cycle.

Consistent with this, the absolute number of Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells per *Pten*^*fl/fl*^; *Mx-1-Cre* mouse increased by approximately threefold within five days of pIpC treatment (Fig. 2g; see Methods for details). Notably, when *Pten*^*fl/fl*^; *Mx-1-Cre* mice were
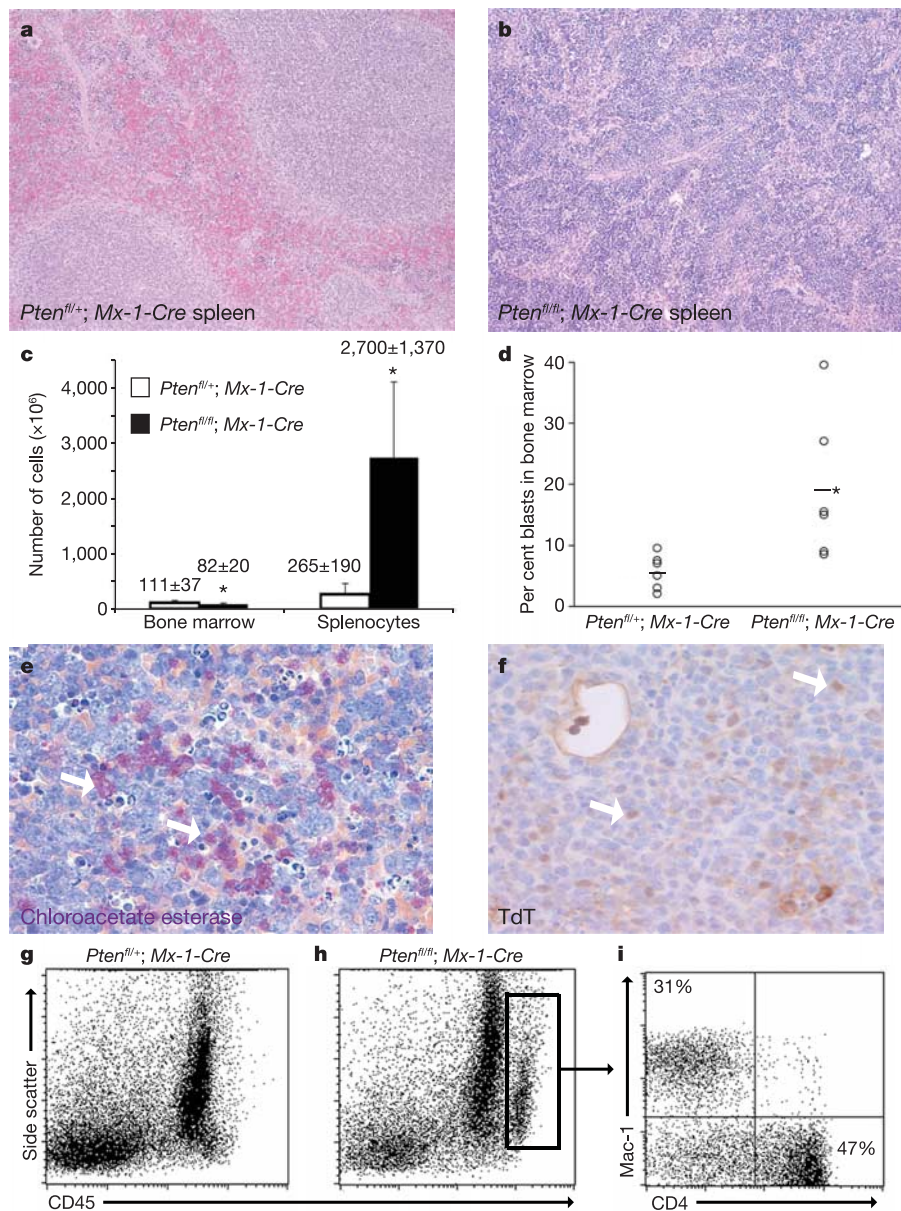

Figure 1 | *Pten* deletion from adult haematopoietic cells leads to myeloproliferative disease that progresses to AML and ALL. **a–c,** Within five days of *Pten* deletion, *Pten*^*fl/fl*^; *Mx-1-Cre* mice developed myeloproliferative disease marked by increased spleen cellularity (**c**), reduced bone marrow cellularity (**c**), and complete effacement of the splenic architecture (compare **b** with **a**) owing to myeloid-predominant extramedullary haematopoiesis (Supplementary Fig. 2). Values are mean ± s.d. *, $P < 0.05$. **d**, Blast cell frequency was significantly increased in *Pten*^*fl/fl*^; *Mx-1-Cre* mice, but five days after pIpC treatment only a minority

of these mice (2 out of 6) showed more than 20% blasts. Bars indicate mean values. *, $P < 0.01$. **e, f,** By six weeks after pIpC treatment, most *Pten*^*fl/fl*^; *Mx-1-Cre* mice had greater than 20% blast cells in the bone marrow, were acutely ill, and showed the pathological features of both AML (**e**; mononuclear blasts in the spleen; white arrows) and ALL (**f**; TdT-positive lymphoid blasts in the thymus; white arrows). **g–i,** The bone marrow of *Pten*^*fl/fl*^; *Mx-1-Cre* mice contained a blast cell population (box in **h**), with myeloid (**i**; Mac-1-positive) and lymphoid (**i**; CD4-positive) cells, not evident in control mice (**g**).

examined 24 to 39 days after pIpC treatment, the number of Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells declined significantly below that found in control mice (Fig. 2g). This suggested that HSCs transiently expanded in number after *Pten* deletion, but were unable to maintain themselves and subsequently became depleted.

To test the function of *Pten*-deficient HSCs, we transplanted 15 Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ donor cells from *Pten*$^{fl/fl}$; *Mx-1-Cre* mice or littermate controls (five days after pIpC) into irradiated recipient mice along with 200,000 recipient bone marrow cells (Fig. 2h). Whereas control cells gave high levels of long-term multilineage reconstitution in all five recipients, *Pten*-deficient cells initially gave multilineage reconstitution, but by eight weeks after transplantation none of the seven recipients remained multilineage-reconstituted. None of these recipients developed AML or ALL. Small numbers of *Pten*-deficient HSCs were thus capable of efficiently engrafting and undergoing multilineage differentiation but became depleted over time and gave only transient, rather than long-term, multilineage reconstitution. In four independent experiments, a total of 23 mice that were injected with 15 Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells from *Pten*$^{fl/fl}$; *Mx-1-Cre* donors did not develop leukaemia. Eighteen of these mice showed transient multilineage reconstitution whereas only two remained long-term multilineage-reconstituted, and levels of reconstitution declined continuously in

both of these mice (Supplementary Table 3). The fact that donor chimaerism consistently declined over time, even in mice that did not develop leukaemia, demonstrates that the depletion of *Pten*-deficient HSCs is not caused by leukaemogenesis.

We performed similar experiments with whole bone marrow cells. Whereas all recipients of control bone marrow cells showed long-term multilineage reconstitution (Fig. 2i) with high levels of donor cells (Fig. 2j), the percentage of recipients that were multilineage-reconstituted by *Pten*$^{fl/fl}$; *Mx-1-Cre* cells, and the levels of reconstitution in these recipients, declined over time. The results with whole bone marrow rule out the possibility that *Pten*-deficient HSCs simply changed their surface-marker phenotype. Moreover, only 3 out of 10 recipients of *Pten*$^{fl/fl}$; *Mx-1-Cre* bone marrow cells developed AML or ALL, further demonstrating that the loss of HSC activity was not secondary to leukaemogenesis.

Pten acts cell-autonomously to maintain HSCs

The possibility remained that a few days of exposure to the myelo-proliferative disease in donor mice might have irreversibly damaged the *Pten*-deficient HSCs before transplantation. To test whether the depletion of HSCs in *Pten*$^{fl/fl}$; *Mx-1-Cre* mice was an indirect consequence of neoplasms/altered haematopoietic environment, or whether *Pten* is required cell-autonomously for the maintenance of
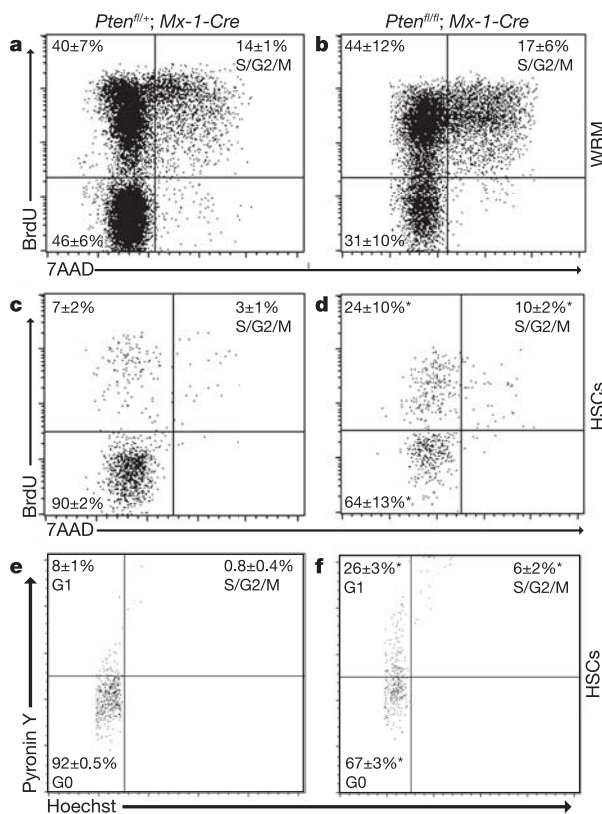

Figure 2 | **HSCs proliferate after *Pten* deletion, transiently expanding in number before becoming depleted. a**, **b**, No significant difference in the cell cycle status of whole bone marrow (WBM) cells from *Pten*$^{fl/fl}$; *Mx-1-Cre* mice (**b**) and littermate controls (**a**) was observed five days after pIpC treatment. BrdU was administered for 19 h to mark cells that entered S phase, and 7-amino-actinomycin D (7-AAD) indicated DNA content. Values are mean ± s.d. of three experiments. **c**, **d**, Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ HSCs from *Pten*$^{fl/fl}$; *Mx-1-Cre* mice (**d**) included significantly (*, $P < 0.05$) more dividing cells relative to controls (**c**). Values are mean ± s.d. of three experiments. **e**, **f**, Significantly (*, $P < 0.01$) fewer Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells from *Pten*$^{fl/fl}$; *Mx-1-Cre* mice (**f**) were in G0 compared with controls (**e**) on the basis of Hoechst/Pyronin Y staining of DNA/RNA content. Values are mean ± s.d. of three experiments. **g**, Total number of Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells in *Pten*$^{fl/fl}$; *Mx-1-Cre* mice

was significantly increased relative to littermate controls five days after pIpC treatment, but significantly decreased relative to controls 24 to 39 days after pIpC administration. Values are mean ± s.d. of six experiments. **h**, Fifteen donor Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells from *Pten*$^{fl/fl}$; *Mx-1-Cre* mice or controls were transplanted into irradiated recipients along with 200,000 recipient bone marrow cells. Whereas control cells gave long-term multilineage reconstitution in all recipients ($n = 5$), *Pten*-deficient cells gave only transient multilineage reconstitution ($n = 7$; *, $P < 0.05$). **i**, **j**, Similar results were observed when 300,000 bone marrow cells from *Pten*$^{fl/fl}$; *Mx-1-Cre* mice or controls were transplanted into irradiated recipients ($n = 10$ and 4, respectively; *, $P < 0.05$) along with 300,000 recipient bone marrow cells. In **h** and **j**, error bars represent standard deviation.

HSCs, we transplanted $Pten^{fl/fl}$; Mx-1-Cre bone marrow cells or control bone marrow cells (both CD45.2$^+$) into recipient mice (CD45.1$^+$) along with half as many recipient bone marrow cells (Fig. 3a). Six weeks after transplantation, when these mice showed stable chimaerism, $Pten$ was deleted and the relative frequencies of donor and recipient HSCs were monitored over time. As expected, two days after pIpC treatment donor cells accounted for roughly two-thirds of HSCs in the bone marrow irrespective of whether they were from control (Fig. 3b) or $Pten$-deficient (Fig. 3c) mice.
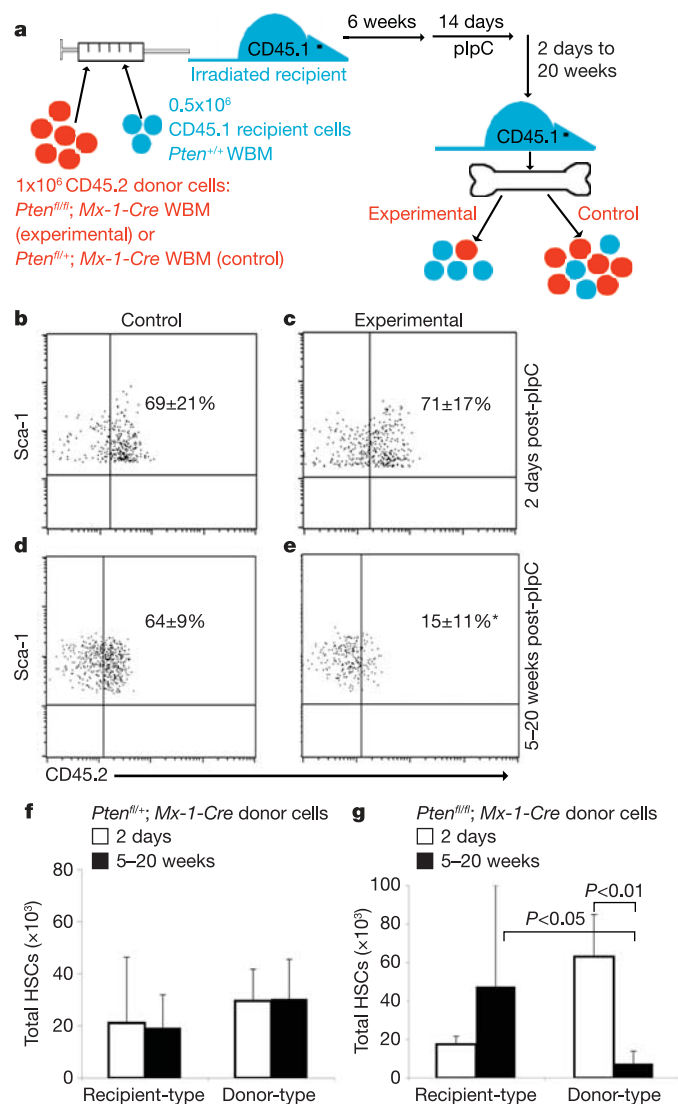

Figure 3 | $Pten$ is required cell-autonomously for HSC maintenance.
a, Irradiated (CD45.1) recipients were transplanted with a 2:1 ratio of donor (CD45.2) $Pten^{fl/fl}$; Mx-1-Cre bone marrow cells (experimental treatment), or control bone marrow cells (control treatment), to recipient bone marrow cells. Six weeks after transplantation, $Pten$ was deleted.
b, c, Two days after pIpC treatment, donor cells accounted for 69 to 71% of Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ HSCs in recipient mice as expected, whether the mice had been transplanted with control ($Pten^{fl/+}$; Mx-1-Cre) donor cells (**b**) or $Pten^{fl/fl}$; Mx-1-Cre donor cells (**c**). Values are mean ± s.d. of three mice per treatment. **d, e**, Subsequently, 5 to 20 weeks after pIpC treatment, control cells still accounted for 64% of bone marrow Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ HSCs (**d**), but $Pten^{fl/fl}$; Mx-1-Cre donor cells accounted for only 15% of Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ HSCs (**e**). Values are mean ± s.d. of ten mice per treatment. *, $P < 0.05$. **f, g**, Control HSCs were stable over time (**f**), whereas $Pten^{fl/fl}$; Mx-1-Cre donor HSCs became depleted relative to recipient (wild-type) HSCs in the same mice (**g**). Values are mean ± s.d.

Consistent with a cell-autonomous requirement for $Pten$ in HSCs, the number of $Pten$-deficient HSCs declined over time and the number of wild-type recipient HSCs in the same mice increased. By 5 to 20 weeks after pIpC administration, control $Pten^{fl/+}$; Mx-1-Cre donor cells still accounted for 64% of bone marrow Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells (Fig. 3d), but $Pten^{fl/fl}$; Mx-1-Cre donor cells accounted for only 15% of bone marrow Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells (Fig. 3e). The total number of control $Pten^{fl/+}$; Mx-1-Cre donor HSCs was stable over time (Fig. 3f). In contrast, $Pten^{fl/fl}$; Mx-1-Cre donor HSCs initially dominated the HSC pool, but by 5 to 20 weeks after pIpC treatment recipient HSCs outnumbered $Pten$-deficient donor HSCs by 6.4-fold (Fig. 3g). To confirm functionally the depletion of donor HSCs, we transplanted 1×10^6 bone marrow cells, or 100 Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells, into irradiated mice. All recipients of bone marrow cells from control mice (from Fig. 3d) showed long-term multilineage reconstitution by donor cells (Supplementary Table 4). However, recipients of $Pten$-deficient donor (from Fig. 3e) bone marrow cells or Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells never achieved multilineage donor cell reconstitution (Supplementary Table 4). As $Pten$-deficient HSCs were depleted and control HSCs were expanded within the same mice, these data confirm that $Pten$ is required cell-autonomously for the maintenance of HSCs.

$Pten$ deletion seemed to deplete HSCs by inhibiting self-renewal rather than by promoting cell death. We did not detect any increase in cell death, as assessed by staining for annexin V or activated caspase-3, in either whole bone marrow cells or Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells from $Pten^{fl/fl}$; Mx-1-Cre mice four weeks after pIpC treatment (Supplementary Fig. 4). Moreover, approximately 90% of single Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells from either $Pten$-deleted mice or control mice formed colonies in methylcellulose irrespective of whether they were isolated five days or four weeks after pIpC treatment (Supplementary Fig. 5). If HSCs were destined to undergo cell death or rapid terminal differentiation after $Pten$ deletion, then Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells from $Pten$-deleted mice should have formed fewer colonies in methylcellulose. Together with the ability of $Pten$-deficient HSCs to efficiently engraft and transiently reconstitute irradiated mice (Fig. 2), these data suggest that HSCs show less self-renewal potential after $Pten$ deletion.

$Pten$-deficient leukaemias are transplantable

Leukaemia-initiating cells are defined by their ability to transfer disease upon transplantation into irradiated mice[5–7]. These cells are rare among unfractionated leukaemia cells but are highly enriched among cells that express HSC markers[5–7]. To test whether the neoplasms in $Pten^{fl/fl}$; Mx-1-Cre mice were transplantable, we transplanted bone marrow cells, splenocytes, Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells[25–27], Mac-1$^+$CD4$^-$CD45^{hi} myeloid blasts, Mac-1$^+$B220$^-$CD3$^-$ myeloid cells, CD4$^+$Mac-1$^-$CD45^{hi} lymphoid blasts, or CD3$^+$Mac-1$^-$/B220$^+$Mac-1$^-$ lymphoid cells from five independent $Pten^{fl/fl}$; Mx-1-Cre donors (which were euthanized owing to illness) into irradiated recipients. Virtually every recipient of 5×10^5 to 2×10^6 $Pten^{fl/fl}$; Mx-1-Cre donor bone marrow cells (Fig. 4a) or splenocytes (Fig. 4c) died within four weeks of transplantation with ALL and/or AML. Only a minority of the recipients of 3×10^5 $Pten^{fl/fl}$; Mx-1-Cre donor bone marrow cells died, with 2 out of 14 developing AML and 2 out of 14 developing ALL (Fig. 4b). By limiting dilution statistics[28], this suggests that approximately 1 out of every 600,000 bone marrow cells (0.00017%) used in these experiments were capable of initiating AML or ALL. Recipients of control bone marrow cells ($n = 20$), splenocytes ($n = 7$) or HSCs ($n = 17$) from $Pten^{fl/+}$; Mx-1-Cre mice never developed leukaemia (data not shown).

To test whether leukaemia-initiating cells co-purify with HSCs, we transplanted 10 to 15 Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells from $Pten^{fl/fl}$; Mx-1-Cre donors into 33 irradiated recipients, five of which died from AML within four weeks (Fig. 4d). This suggests that 1 out

of every 81 cells in this population (1.2%) were capable of initiating AML[28]—a considerable enrichment compared with whole bone marrow, although the vast majority of cells that co-purified with HSCs did not transfer disease.

Half of the recipients of 15,000 to 25,000 myeloid blasts died from AML within four weeks of transplantation (Fig. 4e), suggesting that 1 out of every 36,000 (0.003%) myeloid blast cells initiated AML. Therefore, AML-initiating cells were enriched among blast cells when compared with whole bone marrow, but not nearly as enriched as among cells that expressed HSC markers. Recipients of bulk myeloid cells, lymphoid blasts and bulk lymphoid cells also developed ALL and/or AML, although leukaemia-initiating cells were not as enriched within these populations (Fig. 4f–h). Thus, a variety of cell populations contained leukaemia-initiating cells.

Rapamycin depletes leukaemia-initiating cells

The observation that *Pten* deletion leads to the depletion of normal HSCs but promotes the generation of leukaemia-initiating cells provided a rare distinction between the mechanisms that regulate the maintenance of normal stem cells compared with leukaemia-initiating cells. The PI(3)K pathway is highly branched, but activates the mammalian target of rapamycin (mTOR) among other downstream effectors[29,30]. mTOR kinase activity is inhibited by the drug rapamycin[31,32], and human AMLs and ALLs have been shown to respond to rapamycin[33–35]. Therefore, we administered rapamycin to *Pten*^{fl/fl};*Mx-1-Cre* mice to test whether it depleted leukaemia-initiating cells or rescued normal HSC function.

Pten^{fl/fl};*Mx-1-Cre* mice became overtly ill after pIpC treatment as they developed leukaemias, exhibiting lethargy, ruffling of fur, and hunched posture (Fig. 5a). All three such mice in this experiment died within 3 to 4 weeks of pIpC treatment from AML and ALL (Fig. 5c). In contrast, three mice that were maintained on daily injections of rapamycin (4 mg per kg of body weight) remained healthy and active four weeks after pIpC treatment (Fig. 5b). These rapamycin-treated mice did not show any histological evidence of neoplasm, as the spleens had normal architecture with only focal areas of erythroid-predominant haematopoiesis (Fig. 5c). Daily injections of rapamycin for seven days after pIpC treatment also prevented the decrease in bone marrow cellularity (Fig. 5d) and the increase in spleen cellularity (Fig. 5e) observed in *Pten*^{fl/fl};*Mx-1-Cre* mice, without significantly affecting these parameters in control mice. Hence, mice maintained on rapamycin immediately after *Pten* deletion did not develop signs of haematopoietic malignancy.

To determine whether rapamycin eliminated leukaemia-initiating cells, we treated *Pten*-deleted mice with vehicle or rapamycin for six weeks and then transplanted graded doses of whole bone marrow cells into irradiated mice (which no longer received rapamycin). Recipients of bone marrow cells from vehicle-treated mice all died in a dose-dependent manner within 20 to 31 days of transplantation (Fig. 5f). In contrast, recipients of bone marrow cells from rapamycin-treated mice remained healthy and never showed signs of leukaemia, irrespective of the dose of cells transplanted (Fig. 5f). This demonstrates that rapamycin inhibits the generation or maintenance of leukaemia-initiating cells.

To test whether rapamycin was effective against established leukaemias, mice that had been transplanted with *Pten*^{fl/fl};*Mx-1-Cre* bone marrow cells were treated with daily injections of vehicle or rapamycin, beginning 15 weeks after pIpC administration. Although all three vehicle-treated mice died from ALL and/or AML within five weeks, all three rapamycin-treated mice remained overtly healthy (Supplementary Table 5). Almost all recipients of bone marrow cells from a vehicle-treated mouse died in a dose-dependent manner (Fig. 5g). In contrast, most recipients of bone marrow cells from rapamycin-treated mice survived (Fig. 5g). Thus, rapamycin reduced the frequency of leukaemia-initiating cells even when treatment was initiated after the onset of frank leukaemia. The two rapamycin-treated mice that were not sacrificed to provide a source of cells for

transplantation were treated with daily injections of rapamycin for 15 weeks. Although these mice seemed overtly healthy, with normal-sized spleens and thymuses, one showed histological evidence of myeloproliferative disease and the other showed signs of AML and ALL.

Rapamycin treatment was also initiated after the transplantation of 2×10^6 bone marrow cells from a *Pten*-deficient mouse with AML and ALL into irradiated recipients. Vehicle-treated recipients all died
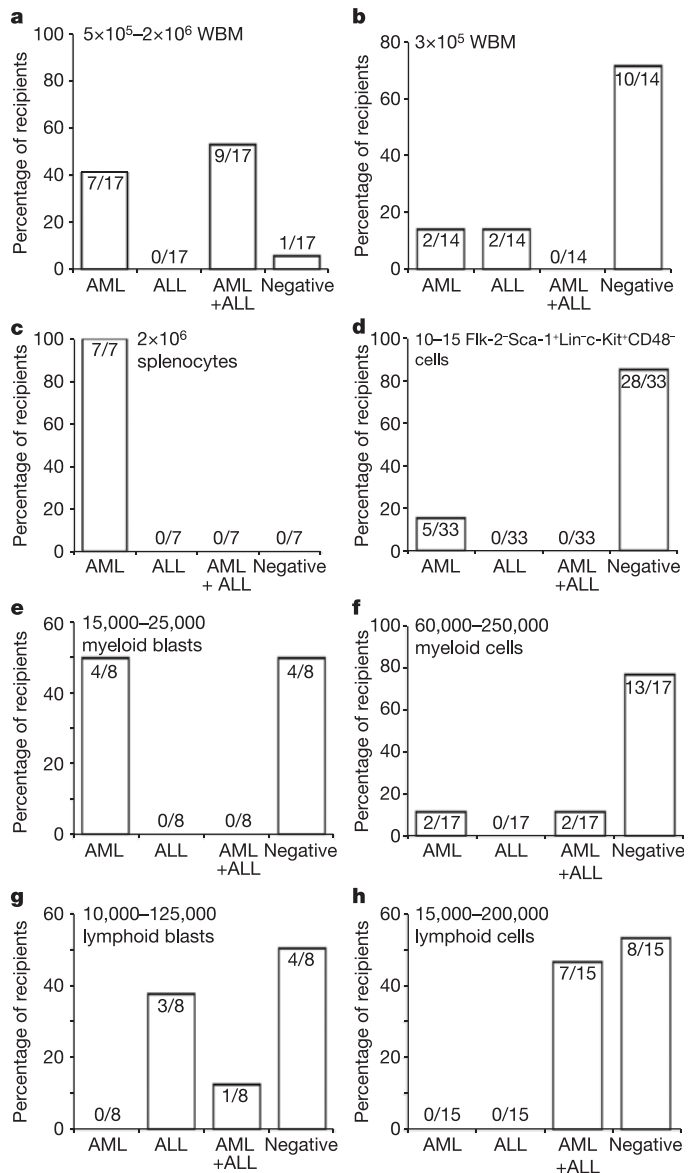

Figure 4 | AML- and ALL-initiating cells are rare in *Pten*-deleted mice, but are transplantable, are contained within multiple distinct populations, and are highly enriched among cells that express HSC markers.
a–h, Whole bone marrow (WBM) cells (**a**, **b**), whole splenocytes (**c**), Flk-2^{−}Sca-1^{+}Lin^{−}c-Kit^{+}CD48^{−} HSCs[25,26] (**d**), CD45^{hi}Mac-1^{+}CD4^{−} myeloid blasts (**e**), Mac-1^{+}B220^{−}CD3^{−} myeloid cells (**f**), CD45^{hi}CD4^{+}Mac-1^{−} lymphoid blasts (**g**), or CD3^{+}Mac-1^{−}/B220^{+}Mac-1^{−} lymphoid cells (**h**) were transplanted into irradiated recipients from 3 to 5 independent *Pten*^{fl/fl};*Mx-1-Cre* donor mice with leukaemia. Donor (CD45.2^{+}) cells were competed against 200,000 recipient (CD45.1 +) bone marrow cells for radioprotection. Bars indicate the proportion of recipients that died with AML, ALL, or AML and ALL, or which survived with no signs of neoplasm ('Negative'). Recipients of *Pten*^{fl/+};*Mx-1-Cre* control bone marrow cells ($n = 20$), splenocytes ($n = 7$) or HSCs ($n = 17$) never developed leukaemia (data not shown).

within 25 days of transplantation (Fig. 5h). In contrast, rapamycin-treated recipients died 40 to 60 days after transplantation (Fig. 5h). When initiated after the onset of leukaemia, rapamycin was effective in prolonging the life of mice, but not in curing the leukaemias.

Rapamycin also inhibited the survival and proliferation of clonogenic leukaemia cells in culture. Freshly isolated or cultured myeloid blast cells from $Pten^{fl/fl}$; Mx-1-Cre mice with AML were plated into methylcellulose. Rapamycin significantly reduced the percentage of blast cells that formed colonies, as well as colony size, in a dose-dependent manner (Supplementary Fig. 6a–e). Rapamycin also significantly reduced the percentage of myeloid blasts in S phase of the cell cycle, and increased the percentage of cells expressing activated caspase-3 (Supplementary Fig. 6f, g).

Rapamycin rescues *Pten*-deficient HSCs

Rapamycin also restored the capacity of $Pten$-deficient HSCs to provide long-term multilineage reconstitution to irradiated mice. Daily injections of rapamycin for seven days after pIpC administration did not affect the overall rate of proliferation in bone marrow, but did normalize the cell cycle distribution of Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells in $Pten^{fl/fl}$; Mx-1-Cre mice without affecting the proliferation of HSCs from control littermates (Fig. 6a). Rapamycin also eliminated the HSC expansion observed seven days after pIpC treatment (Fig. 6b), and the HSC depletion observed after four weeks in $Pten^{fl/fl}$; Mx-1-Cre mice (Fig. 6c), without affecting HSC numbers in control mice. Most notably, rapamycin restored the potential of Flk-2$^-$Sca-1$^+$Lin$^-$c-Kit$^+$CD48$^-$ cells isolated from
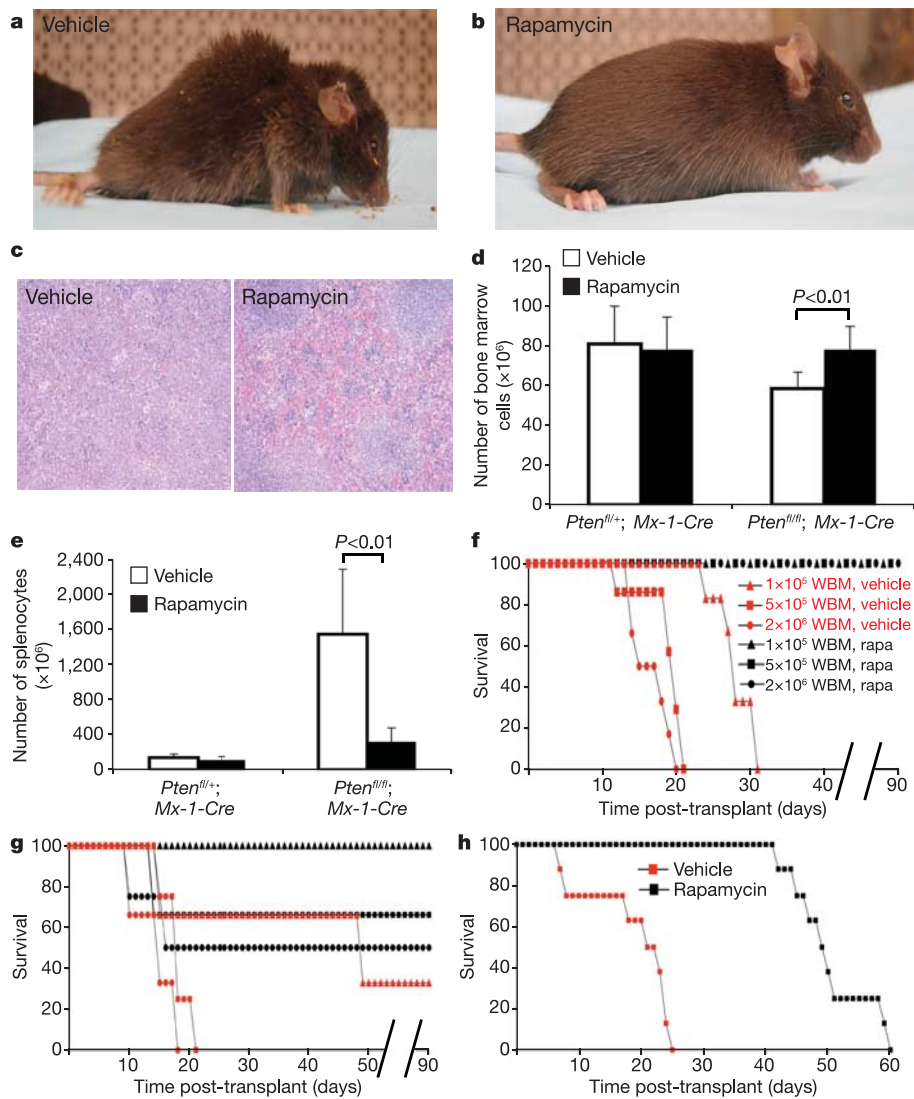

Figure 5 | Rapamycin depletes leukaemia-initiating cells. a, b, Four weeks after pIpC treatment, $Pten^{fl/fl}$; Mx-1-Cre mice exhibited lethargy, ruffled fur, hunched posture, and required euthanization (**a**; $n = 3$), but if treated daily with 4 mg per kg rapamycin remained healthy and active (**b**; $n = 3$). **c**, Rapamycin-treated mice had normal spleen architecture (right panel), whereas vehicle-treated littermates developed AML and ALL (left panel). **d, e,** Rapamycin prevented the decrease in bone marrow cellularity (**d**) and the increase in spleen cellularity (**e**) in $Pten^{fl/fl}$; Mx-1-Cre mice, but did not affect these parameters in controls. Values are mean ± s.d. of 6 to 7 mice per treatment. **f**, Mice transplanted with 1×10^6 $Pten^{fl/fl}$; Mx-1-Cre donor (CD45.2) bone marrow cells along with 0.5×10^6 control (CD45.1) bone marrow cells were treated with vehicle or rapamycin (4 mg per kg per day) for six weeks, starting immediately after pIpC treatment. Graded doses of

whole bone marrow (WBM) cells from these mice were transplanted into lethally irradiated recipients along with 2×10^5 recipient (CD45.1) bone marrow cells. Recipients of bone marrow from vehicle-treated mice died with leukaemia, whereas recipients of marrow from rapamycin-treated mice remained healthy. **g**, Similar donor mice were treated for five weeks with vehicle or rapamycin (4 mg per kg per day) beginning 15 weeks after pIpC treatment. Nearly all recipients of bone marrow from vehicle-treated mice died of leukaemia in a dose-dependent manner, whereas recipients of cells from rapamycin-treated mice usually survived. **h**, Finally, 2×10^6 donor (CD45.2) bone marrow cells from a $Pten^{fl/fl}$; Mx-1-Cre mouse with AML and ALL were transplanted into sublethally irradiated recipients. The recipients were treated with vehicle or rapamycin (0.4 mg per kg per day). Panels **f** and **h** show one representative experiment out of three.

$Pten^{fl/fl}$; $Mx-1-Cre$ mice to provide long-term multilineage reconstitution seven days after pIpC treatment (Fig. 6d, e). This confirms that Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells from $Pten^{fl/fl}$; $Mx-1-Cre$ mice are HSCs, and that rapamycin restores normal function to these cells.

The mechanism responsible for the depletion of $Pten$-deficient HSCs remains to be elucidated. One possibility is that persistent activation of the PI(3)K pathway following the loss of $Pten$ leads to reduced HSC self-renewal via a gradual increase in the rate at which HSCs exit the stem cell pool. Another possibility is that $Pten$ deficiency induces the gradual senescence of HSCs. Conditional deletion of $Pten$ leads to a p53-dependent senescence of prostate cells[36]. Leukaemias might acquire secondary mutations that inactivate the senescence response.

Deletion of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ also leads to HSC proliferation followed by a slow depletion[37]. Like PTEN, p21[CIP1] also regulates HSC quiescence. This raises the possibility that compounds that promote stem cell quiescence might consistently have different effects on normal stem cells and cancer stem cells.

Conditional $Pten$ deletion in the fetal central nervous system increases the self-renewal and frequency of neural stem cells[38]. This is the opposite of what we observed, and may represent a general difference between fetal and adult stem cells rather than a difference between tissues. The balance of proto-oncogenes and tumour suppressor genes that regulate stem cell self-renewal changes between embryonic, fetal and adult life as the organogenic demand decreases and the risk of cancer increases[11,13,39].

These data demonstrate that it is possible to identify—and to target therapeutically—pathways that have distinct effects on normal stem cells and cancer stem cells within the same tissue. This is an important finding, because it has been proposed that oncogenic mutations confer self-renewal potential by activating pathways used by normal stem cells, irrespective of whether the mutations occur in stem cells or other cells[1,2,12]. By comparing the mechanisms that regulate the maintenance of normal stem cells versus cancer stem cells, it should be possible to design new therapies and to improve existing therapies.

METHODS

Mice. Mice were housed in the Unit for Laboratory Animal Medicine at the University of Michigan. $Pten^{fl/+}$ and $Mx-1-Cre$ mice were backcrossed for eight and six generations, respectively, onto a C57BL/Ka-CD45.2:Thy-1.1 background. Recipients in reconstitution assays were adult C57BL/Ka-CD45.1:Thy-1.2 mice.

Flow cytometry and isolation of HSCs. Bone marrow cells were flushed from the long bones (tibias and femurs) with Hank's buffered salt solution without calcium or magnesium, supplemented with 2% heat-inactivated calf serum (HBSS⁺; GIBCO). Cells were triturated and filtered through nylon screen (45 µm; Sefar America) to obtain a single-cell suspension. Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ HSCs and Thy-1.1^low Sca-1⁺Mac-1^low CD4^low B220⁻ multipotent progenitors (MPPs) were isolated as previously described[25,26,40]. For isolation of Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ HSCs, whole bone marrow cells were incubated with unconjugated monoclonal antibodies to lineage (Lin) markers including B220 (6B2), CD3 (KT31.1), CD4 (GK1.5), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5), Mac-1 (M1/70) and Ter119. After washing, cells were resuspended in anti-rat IgG conjugated to phycoerythrin (PE; Jackson ImmunoResearch). Cells were then stained with directly conjugated antibodies to Sca-1 (Ly6A/E-APC), c-Kit (2B8-biotin), Flk-2 (A2F10-PE; eBioscience) and CD48 (HM48-1-FITC; BD Pharmingen). To identify CD45.2⁺ HSCs, antibodies to CD45.2 (104-FITC; BD Pharmingen) and CD48 (HM48-1-PE; eBioscience) were used. HSCs were often pre-enriched by selecting c-Kit⁺ cells using paramagnetic microbeads and autoMACS (Miltenyi Biotec). To identify leukaemic blast cells, anti-CD45 (30-F11-APC; eBioscience) was used.

The total number of Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells per mouse was calculated based on the frequency of this population in the bone marrow and spleen, the cellularity of the spleen and long bones, and by assuming that 15% of all bone marrow is within the long bones[41]. The blood and other tissues do not contribute significantly to the overall size of the HSC pool.

Long-term competitive reconstitution assays. Adult recipient mice were irradiated with an Orthovoltage X-ray source delivering 300 rad min⁻¹. Recipient mice received two doses of 540 rads each, delivered 3 h apart. For sublethal irradiation, mice were administered one dose of 800 rads. Donor (CD45.2⁺) HSCs were sorted and then re-sorted (for purity) into individual wells of a 96-well plate containing 200,000 CD45.1⁺ whole bone marrow cells in HBSS⁺. The contents of individual wells were injected into the retro-orbital venous sinus of irradiated CD45.1⁺ recipients. For at least 16 weeks after transplantation, blood was obtained from the tail veins of recipient mice, subjected to ammonium chloride/potassium bicarbonate red-cell lysis, and stained with directly conjugated antibodies to CD45.2 (104-FITC), B220 (6B2), Mac-1 (M1/70), CD3 (KT31.1) and Gr-1 (8C5) to assess donor cell engraftment. Cell cycle analysis was conducted as described in Supplementary Methods.

Administration of pIpC and rapamycin. As described previously[42], polyinosine-polycytidine (pIpC; Sigma) was resuspended in Dulbecco's-PBS at 2 mg ml⁻¹ and passed through a 0.22-µm filter. Mice received 25 µg of pIpC per gram of body mass every other day for two weeks. Rapamycin (Calbiochem and LC Laboratories) was administered by intraperitoneal injection at the indicated
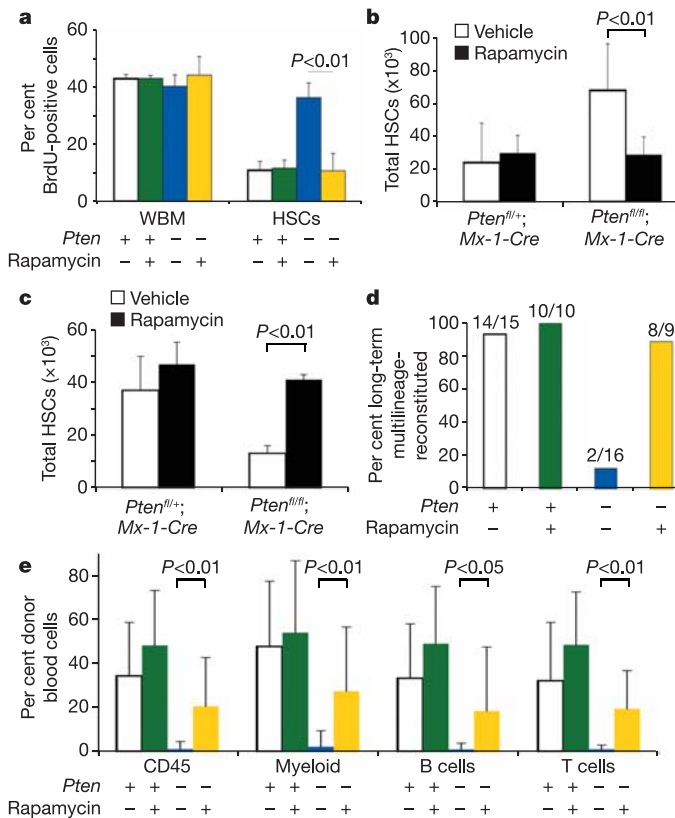

Figure 6 | Rapamycin rescues normal HSC function after $Pten$ deletion. a, Seven days of rapamycin (4 mg per kg per day) did not affect proliferation in whole bone marrow (WBM; 19-h pulse with BrdU), but did eliminate the increase in Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ HSC proliferation in $Pten^{fl/fl}$; $Mx-1-Cre$ mice without affecting proliferation in controls. Values are mean ± s.d. of three experiments. **b**, Rapamycin eliminated the HSC expansion observed seven days after pIpC treatment in $Pten^{fl/fl}$; $Mx-1-Cre$ mice without affecting HSCs in $Pten^{fl/+}$; $Mx-1-Cre$ controls. Values are mean ± s.d. of 6 to 7 mice per treatment. **c**, Rapamycin eliminated the depletion of HSCs observed 3 to 4 weeks after pIpC treatment in $Pten^{fl/fl}$; $Mx-1-Cre$ mice without affecting controls. Values are mean ± s.d. of three mice per treatment. **d, e**, Rapamycin restored the long-term (>16 weeks after transplantation) multilineage reconstitution potential of Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ cells from $Pten^{fl/fl}$; $Mx-1-Cre$ mice (**d**). Fifteen Flk-2⁻Sca-1⁺Lin⁻c-Kit⁺CD48⁻ donor HSCs from $Pten^{fl/fl}$; $Mx-1-Cre$ or control mice that had been treated with rapamycin (4 mg per kg per day) or vehicle for seven days after pIpC treatment were transplanted along with 200,000 recipient bone marrow cells into irradiated recipients. Recipients received vehicle or rapamycin (0.4 mg per kg per day) starting within two weeks of transplantation. The frequency of donor white blood (CD45.2⁺) cells, myeloid (Mac-1⁺) cells, B (B220⁺) cells and T (CD3⁺) cells was determined 16 to 18 weeks after transplantation (**e**). Values are mean ± s.d. of three experiments.

doses. It was reconstituted in absolute ethanol at $10\,\text{mg}\,\text{ml}^{-1}$ or $1\,\text{mg}\,\text{ml}^{-1}$ and diluted in 5% Tween-80 (Sigma) and 5% PEG-400 (Hampton Research) before injection. The final volume of all injections was $200\,\mu\text{l}$.

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