Regular Article

HEMATOPOIESIS AND STEM CELLS

Pivotal role of Pten in the balance between proliferation and differentiation of hematopoietic stem cells in zebrafish

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Key Points

- Loss of the tumor suppressor, PTEN, results in enhanced blood stem cell proliferation and arrested differentiation, hallmarks of leukemia.
- *Pten* mutant zebrafish embryos display defective hematopoiesis and constitute an excellent tool to assess drug treatment.

Self-renewing hematopoietic stem/progenitor cells (HSPCs) produce blood cells of all lineages throughout life. Phosphatase and tensin homolog (PTEN), a tumor suppressor that antagonizes phosphatidylinositol 3-kinase (PI3K) signaling, is frequently mutated in hematologic malignancies such as bone marrow failure and leukemia. We set out to investigate whether Pten is required for hematopoiesis. Analysis of zebrafish mutants lacking functional Pten revealed that HSPCs colonized the caudal hematopoietic tissue normally. There, HSPCs hyperproliferated and engaged in all blood lineages. However, they failed to differentiate into mature blood cells. Hence, Pten mutant zebrafish embryos displayed hallmarks of leukemia in humans. Inhibition of PI3K signaling in mutants lacking functional Pten suppressed hyperproliferation and released the differentiation arrest. We conclude that Pten has an essential role in the balance between proliferation and differentiation of blood cells. (*Blood*. 2014;123(2):184-190)

Introduction

Understanding the development of blood stem cells has been a challenge for decades and still little is known about the ontogeny of hematopoietic stem/progenitor cells (HSPCs) that give rise to all blood lineages. All vertebrates possess 2 waves of hematopoiesis: the primitive wave and the definitive wave.[1-3] The primitive wave gives rise to primitive erythrocytes and macrophages, as well as megakaryocytes in mice[4] and neutrophils in zebrafish,[5] whereas the definitive wave or adult hematopoiesis generates all hematopoietic cell lineages. In mammals and zebrafish, HSPCs emerge from the ventral wall of the dorsal aorta in a conserved region known as the aorta-gonad-mesonephros (AGM).[6-8] After leaving the AGM, mammalian HSPCs transiently colonize the fetal liver before seeding the bone marrow. In zebrafish, HSPCs colonize the caudal hematopoietic tissue (CHT)[9] before seeding the definitive hematopoietic organs, the thymus, and head kidney. In the hematopoietic organs, HSPCs commit to progenitors before maturing and giving rise to all blood cell populations.

HSPCs are tightly regulated in terms of dormancy and self-renewal, proliferation, and differentiation. Disrupting this balance leads to pathological consequences such as bone marrow failure or hematologic malignancy. For example, T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic tumor arising from the malignant transformation of hematopoietic progenitors.[10] To date, several genes and underlying pathways have been linked to T-ALL. One of these genes is the tumor suppressor, phosphatase and tensin homolog (PTEN). Deletion mutations in PTEN appear in 5% to 10% of T-ALL cases, and about 17% of patients lack PTEN expression.[10,11] Recent work using a conditional knockout mouse model demonstrated that loss of Pten in bone marrow HSPCs causes expansion of the short-term population and declining of the long-term population of HSPCs. Mice with Pten-deficient bone marrow HSPCs develop myeloproliferative disorder (MPD) eventually.[12-15]

PTEN is a phosphatase specific for the D3 position of the second messenger, phosphatidylinositol (3,4,5)-triphosphate, which is formed by phosphatidylinositol 3-kinase (PI3K). Thus, PTEN is one of the few known lipid phosphatases counteracting the PI3K-Akt (also known as protein kinase B [PKB]) pathway.[15] Loss of Pten function is embryonic lethal in many organisms, including the mouse, *Caenorhabditis elegans*, and *Drosophila*.[16-18] The zebrafish genome encodes 2 *pten* genes with redundant function, designated *ptena* and *ptenb*.[19] Single mutants display no morphologic phenotype and are viable and fertile, but mutants that retain only a single wild-type copy of *pten* develop hemangiosarcomas during adulthood.[20] *Ptena*$^{-/-}$*ptenb*$^{-/-}$ mutants, hereafter referred to as *Pten* mutants, lack functional Ptena and Ptenb, are embryonic lethal at 5 to 6 days postfertilization (dpf), and display hyperplasia and dysplasia.[19]

We used zebrafish mutants lacking functional Pten to address hematopoiesis. Zebrafish embryos survive without circulating erythrocytes[21] and hence provide an excellent system to investigate the

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early stages of hematopoiesis, which have not been addressed in other systems before. In particular, we investigated whether homing and proliferation of definitive HSPCs in the CHT, and their maturation into differentiated blood cells are Pten dependent. Here, we report that progenitors of all blood lineages are formed in the CHT in *Pten* mutants, but their differentiation into mature blood cells is arrested. Finally, we show that inhibition of PI3K signaling once the HSPCs have colonized the CHT allowed their differentiation into definitive blood cells.

Methods

Zebrafish husbandry

Ptena$^{-/-}$*ptenb*$^{-/-}$[19] and *Tg(cd41:eGFP)*[22] were maintained, crossed, raised, and staged as described.[23,24]

Confocal, fluorescence, brightfield microscopy

Fluorescence images of transgenic embryos were acquired using a TCS-SPE confocal microscope (Leica) and processed with Image J (http://rsb.info.nih.gov/ij/). Embryos were anesthetized with tricaine[23] and mounted on a glass cover dish with 0.7% low-melting agarose and covered with standard E3 medium. Whole-mount images from in situ hybridizations and Sudan Black (SB)-stained embryos were taken using a Zeiss Axioplan microscope connected to a Leica DFC480 camera.

In situ hybridization

Whole-mount in situ hybridization was performed according to standard protocols.[25,26] Embryos stained with *rag1* were used to measure *rag1*$^{+}$ area with Image J software. Thirty-two hours postfertilization (hpf) embryos were genotyped after in situ hybridization according to Faucherre et al.[19]

Whole-mount immunohistochemistry

Immunohistochemistry was performed using anti–phosphorylated Histone 3 (pHis3) (Abcam)[19] with slight modifications. Briefly, 4 dpf embryos were fixed overnight in 2% paraformaldehyde and subsequently washed with phosphate-buffered saline (PBS) including 0.3% Triton X-100. Permeabilization was performed by incubating embryos for 5 minutes on ice in 2.5 mg/mL trypsin solution (Worthington Biochemical Corporation). Subsequently, embryos were rinsed 3 times for 5 minutes with PBS including 0.3% Triton X-100. Embryos were blocked for several hours with 0.1% bovine serum albumin (BSA), 2% lamb serum, 0.1% dimethylsulfoxide (DMSO) and 1% Triton-X100 in PBS and incubated with polyclonal anti-pHis3 antibody2 (1:1000 in blocking buffer) overnight. Embryos were washed with 0.3% Triton X-100 in PBS several times and incubated in blocking buffer for several hours. Secondary antibody (Alexa 568, 1:1000) was applied overnight and embryos were washed 10 × 10 minutes with 0.3% Triton X-100 in PBS and processed for imaging.

Quantification of GFP^{low} and GFP^{high} cells using *Tg (cd41:eGFP)*

Ptena$^{+/-}$*ptenb*$^{-/-}$ with *Tg(cd41:eGFP)* were crossed, offspring were mounted at 4 dpf, and the entire CHT was imaged by confocal microscopy. Green fluorescent protein (GFP)^{low}- and GFP^{high}-expressing cells were quantified using Volocity software.

SB staining

Five-dpf-old embryos were fixed and stained using SB as described.[5] Images of SB-stained embryos were taken and cells were counted using Image J software.

LY294002 treatment

Embryos were incubated with 4μM LY 294002 from 82 hpf onwards, fixed at 5 dpf, and processed for in situ hybridization or SB staining. SB^{+} cells were counted in the head, yolk sac, trunk, tail, and CHT using Image J software.

Results

Primitive wave of hematopoiesis is independent of Pten

To assess hematopoiesis systematically, we first analyzed the primitive wave of hematopoiesis. In situ hybridization was performed at 32 hpf using key markers that are associated with early erythrocytes (*gata-1*) and cells of the primitive myeloid lineage (*pu.1*, *l-plastin*, and *c-fms*).[2,5,9,27-29] Expression pattern analysis for *gata1*, *pu.1*, *l-plastin*, and *c-fms* revealed no differences between siblings and *Pten* mutant embryos (supplemental Figure 1, available on the *Blood* website), indicating that initiation of the primitive wave was not affected in *Pten* mutants.

Homing and hyperproliferation of HSPCs in *Pten* mutant embryos

To investigate definitive hematopoiesis in *Pten* mutants, we first determined expression of *c-myb* and *scl* that mark HSPCs[9,30] at 5 dpf. We detected expression of both genes in the CHT (Figure 1A-D brackets), indicating that homing of HSPCs was not dependent on Pten function. However, *Pten* mutants displayed more *c-myb*$^{+}$ and *scl*$^{+}$ cells, compared with wild-type embryos at 5 dpf (Figure 1A-D). We wondered whether the loss of function of Pten was associated with an elevated number of HSPCs in the CHT. To investigate this, we used the *Tg(cd41:eGFP)* line in which HSPCs express a low level of GFP (GFP^{low}) and thrombocytes a high level of GFP (GFP^{high}).[31] The number of GFP^{low+} HSPCs in the CHT was significantly increased in *Pten* mutants at 4 dpf, compared with siblings (Figure 1E-G arrowheads). Next, we investigated whether the elevated number of HSPCs was due to enhanced proliferation. Immunohistochemistry using pHis3-specific antibodies in the *Tg(cd41:eGFP)* line revealed an increased number of mitotic GFP^{low+} HSPCs in *Pten* mutant embryos compared with siblings at 4 dpf (Figure 1H-M). Predominantly GFP^{low+} HSPCs stained positive for pHis3 in *Pten* mutant embryos (Figure 1I,M). Our data demonstrate that Pten is not required for HSPCs to colonize the CHT, but loss of Pten led to enhanced proliferation of HSPCs.

Arrest of differentiation in all blood cell lineages in *Pten* mutants

What are the consequences of lack of Pten function for the various definitive blood lineages generated from the HSPCs? We first investigated the lymphoid lineage. *Pten* mutants contained less *rag1*$^{+}$ cells in the nascent thymus, compared with siblings (Figure 2A-D). Quantification of the *rag1*$^{+}$ area in wild-type and *Pten* mutant embryos indicated a significant reduction in the *rag1*$^{+}$ area in *Pten* mutants (Figure 2E). To assess whether reduced expression of *rag1* was a consequence of reduced numbers of lymphoid progenitors, *ikaros* expression was evaluated. Surprisingly, *ikaros* expression was detected in the mutant thymus at the same level as in wild-type (Figure 2F-G inset). In addition, ectopic *ikaros* expression was detected in *Pten* mutants (Figure 2G) and *ikaros* expression was strongly enhanced in the CHT of *Pten* mutants (Figure 2H-I). The

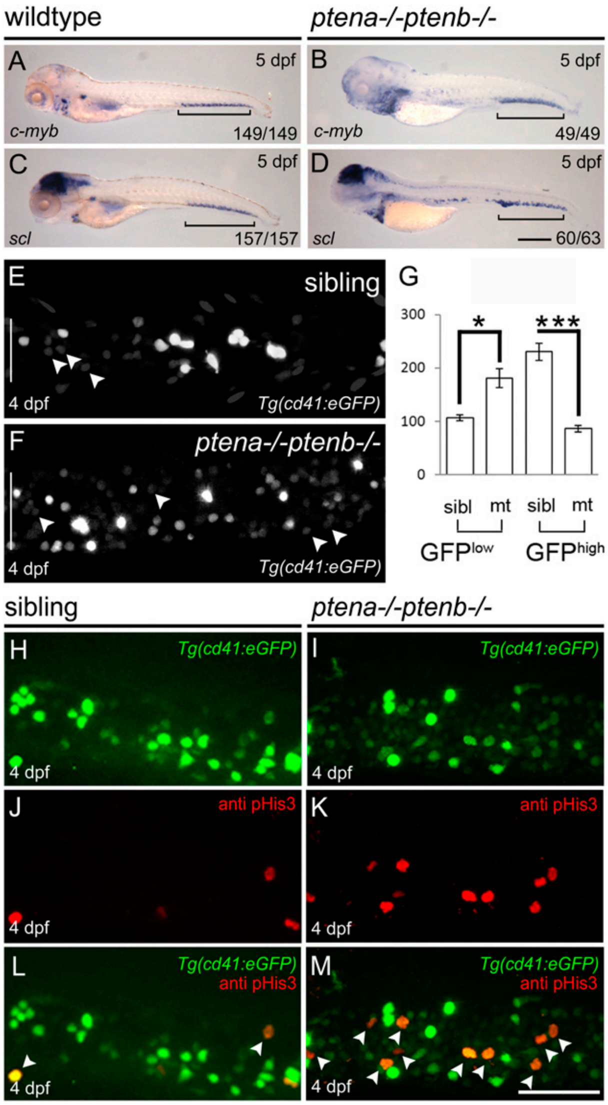

Figure 1. Enhanced proliferation of HSPCs in the CHT of *Pten* mutants. *C-myb* (A-B) and *scl* expression (C-D) were examined by in situ hybridization at 5 dpf in wild-type and *Pten* mutant (*ptena*$^{-/-}$*ptenb*$^{-/-}$) embryos as indicated. Representative embryos are depicted with anterior to the left. Brackets indicate the CHT; scale bar (200 μm) in panel D is representative for panels A-D. (E-G) The CHTs of sibling and *Pten* mutant embryos in the *Tg(cd41:eGFP)* background were imaged by confocal microscopy with a ×40 objective. Maximum projections of z-planes (step size, 2 μm) are shown (E-F). The number of HSPCs (GFP^{low}) (indicated by arrowheads) and thrombocytes (GFP^{high}) were counted in the entire CHT (height of CHT is depicted by a white bar in panels E and F) at 4 dpf using Volocity (siblings, n = 14; *ptena*$^{-/-}$ *ptenb*$^{-/-}$, n = 10). (G) Results are expressed as average number of cells per CHT and error bars indicate SEM. Normal distribution of data points was assessed with the Shapiro-Wilk test. Statistical comparisons of groups were performed by the 2-tailed *t* test, respectively, with the Mann-Whitney *U* test. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs control. (H-M) Cell proliferation was assessed in the CHT of 4-dpf-old *Pten* mutant *Tg (cd41:eGFP)* embryos and siblings by immunohistochemistry using pHis3-specific antibodies. Representative *Tg(cd41:eGFP)* (H-I), pHis3 (J-K), and merged images are shown (L-M). GFP and pHis3 double-positive cells are indicated with arrowheads in panels L and M. Scale bar, 50 μm.

reduced number of *rag1*$^{+}$ thymocytes and elevated number of *ikaros*$^{+}$ lymphoid progenitors indicate that in *Pten* mutants, most lymphoid progenitors are arrested at a specific stage between *ikaros* and *rag1* expression.

The *Tg(cd41:eGFP)* line revealed that the number of GFP^{high} thrombocytes in the CHT was significantly reduced by 4 dpf in the CHT (Figure 1E-F), suggesting that thrombocytic differentiation was impaired in *Pten* mutants.

To assess erythropoiesis, we used in situ hybridization and found elevated numbers of *globin*$^{+}$ and *gata1*$^{+}$ early erythroid progenitors in the CHT of *Pten* mutants (Figure 3A-D). Further differentiation of these early erythroid progenitors could not be addressed, for in fish, erythroid differentiation normally occurs very gradually over several days within the circulation.[21,32] The death of PTEN mutants by 5 to 6 dpf[19] only allows us to detect the definitive committed (*gata1*$^{+}$, *globin*$^{+}$) early erythroid progenitors, that are mostly not yet circulating by that time and hence are located in the CHT.[33]

Next, expression of myeloid cell markers was assessed. Clearly more *l-plastin*$^{+}$, *mpo*$^{+}$ (neutrophil lineage), and *c-fms*$^{+}$ (macrophage lineage) cells dispersed in peripheral tissues were detected in *Pten* mutant than in wild-type embryos at 5 dpf (Figure 3E-J). SB stains the granules of neutrophils[5] and was used to quantify mature neutrophils in *Pten* mutant embryos and siblings. SB^{+} cells in the head and yolk sac region are predominantly derived from the primitive wave, whereas SB^{+} cells in the trunk, tail, and CHT are mainly definitive wave-derived.[5] The number of SB^{+} neutrophils was counted in the different areas of 6 wild-type and 7 Pten mutant embryos as illustrated in supplemental Figure 2. The number of SB^{+} cells from the primitive wave was enhanced more than twofold in *Pten* mutants (Figure 3K,L,O), whereas the number of

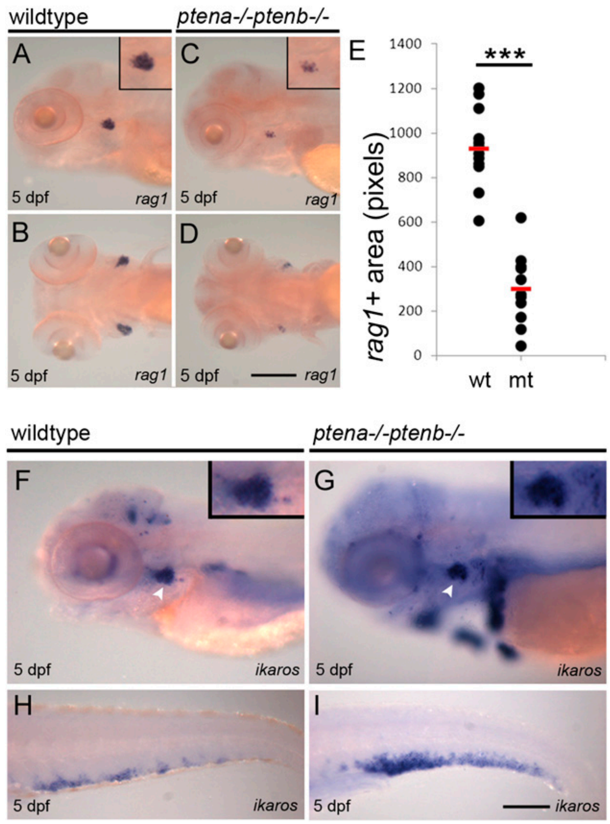

Figure 2. Differentiation arrest of lymphoid progenitors in *Pten* mutants. *Rag1* is expressed in lymphocytes and highlights the thymus. Representative lateral and dorsal views of wild-type (A-B) and *Pten* mutant embryos (C-D) are shown; close-ups of the thymus in the insets. (E) *Rag1*$^{+}$ area in wild-type and *Pten* mutant embryos at 5 dpf was quantified and is depicted in scatter plot; wild-type, n = 14; *Pten* mutant (mt), n = 11. The average *rag1*$^{+}$ area is highlighted with a red bar and is significantly reduced in *Pten* mutants ($P < .001$, 2-tailed *t* test). (F-I) *Ikaros* was used as a marker for lymphoid progenitors in 5-dpf wild-type (F,H) and *Pten* mutant embryos (G,I) in the thymus and CHT, respectively; thymus is indicated with arrowhead. Representative embryos are depicted with anterior to the left. Scale bars in panels D and I represent 200 μm. wt, wild type.

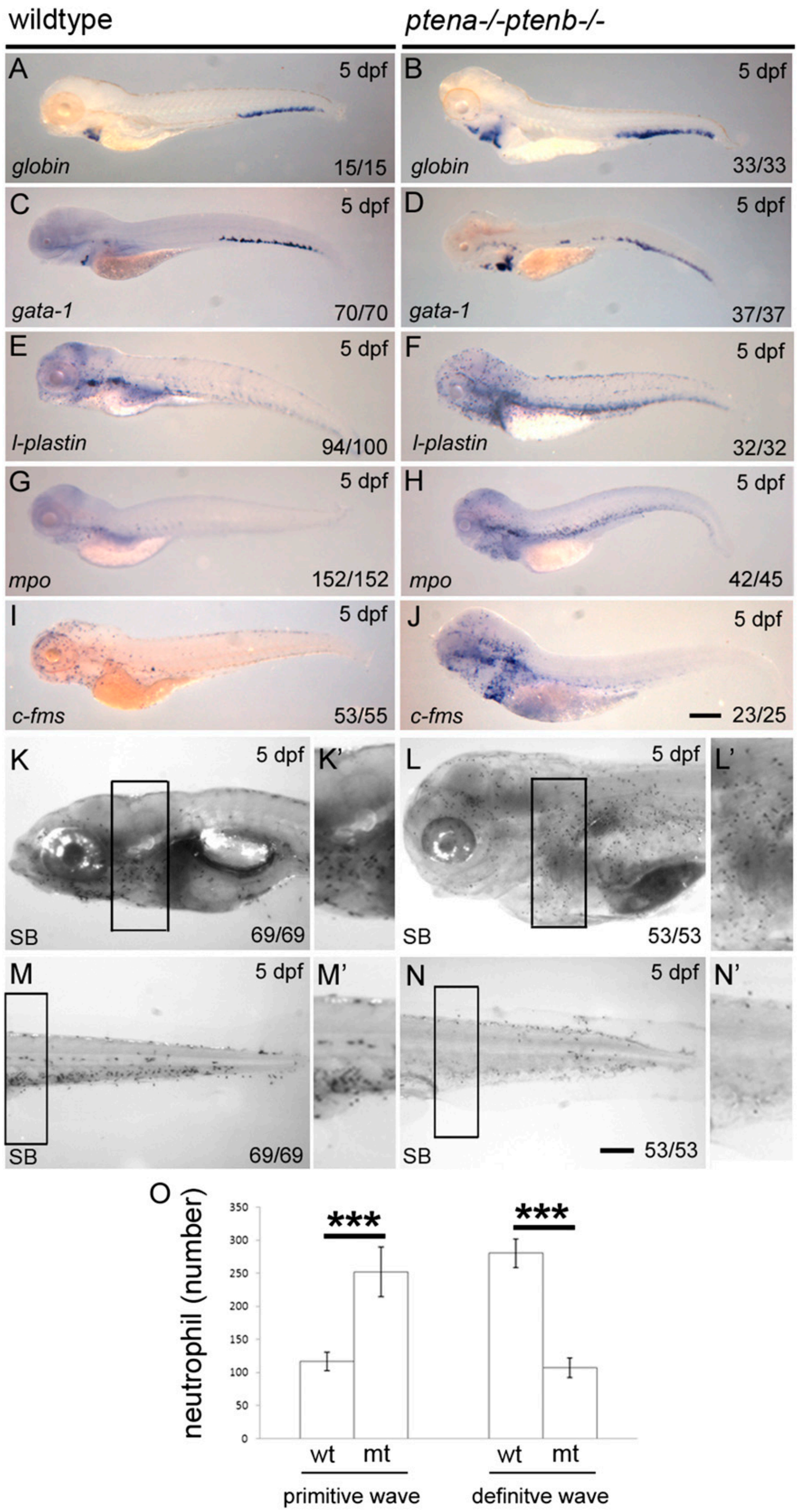

Figure 3. Enhanced numbers of progenitors and arrest of neutrophil differentiation in *Pten* mutants. A panel of in situ hybridization markers for blood lineages was used on 5 dpf wild-type and *Pten* mutant ($ptena^{-/-}ptenb^{-/-}$) embryos: (A-B) *Globin* and (C-D) *gata1*, both indicative of erythroblasts; (E-F) *l-plastin*, marking lymphocytes, macrophages, and neutrophils; (G-H) *mpo*, indicating neutrophils; (I-J) *c-fms*, indicative of macrophages. (K-N) At 5 dpf, SB staining was used to assess the number of mature neutrophils in various parts of the embryo. Panels K′-N′ represent magnifications of the boxed areas in the corresponding panels. (O) SB^+ cells were counted in the head and yolk sac (mainly the primitive wave) and in the trunk, tail, and CHT region (mainly the definitive wave) as indicated in supplemental Figure 2. The results are expressed as average number of cells per embryo with the error bars indicating SEM; wild type, n = 6, $ptena^{-/-}ptenb^{-/-}$ (n = 7). Normal distribution of data points was assessed with the Shapiro-Wilk test. Statistical comparison of groups was performed by the 2-tailed *t* test. $*P < .05$, $**P < .01$, $***P < .001$ as indicated. Representative embryos are depicted; scale bars in panel J (200 μm) and N (100 μm) are representative for panels A-J, respectively, panels K-N. wt, wild type; mt, *Pten* mutant.

SB^+ cells from the definitive wave was reduced more than twofold (Figure 3M-O). The elevated number of mpo^+ cells in the CHT, combined with reduced number of SB^+ cells in the trunk, tail, and CHT, suggests an arrest in differentiation of neutrophils in the absence of Pten function. To verify whether cell death might cause the lack of differentiated cells, we performed acridine orange staining. *Pten* mutant embryos did not exhibit enhanced apoptosis in the CHT (supplemental Figure 3), suggesting that the absence of differentiated cells is rather due to perturbed maturation. These results demonstrate that in the absence of Pten all blood lineages are specified, yet differentiation is arrested, suggesting a requirement for Pten in terminal blood cell differentiation.

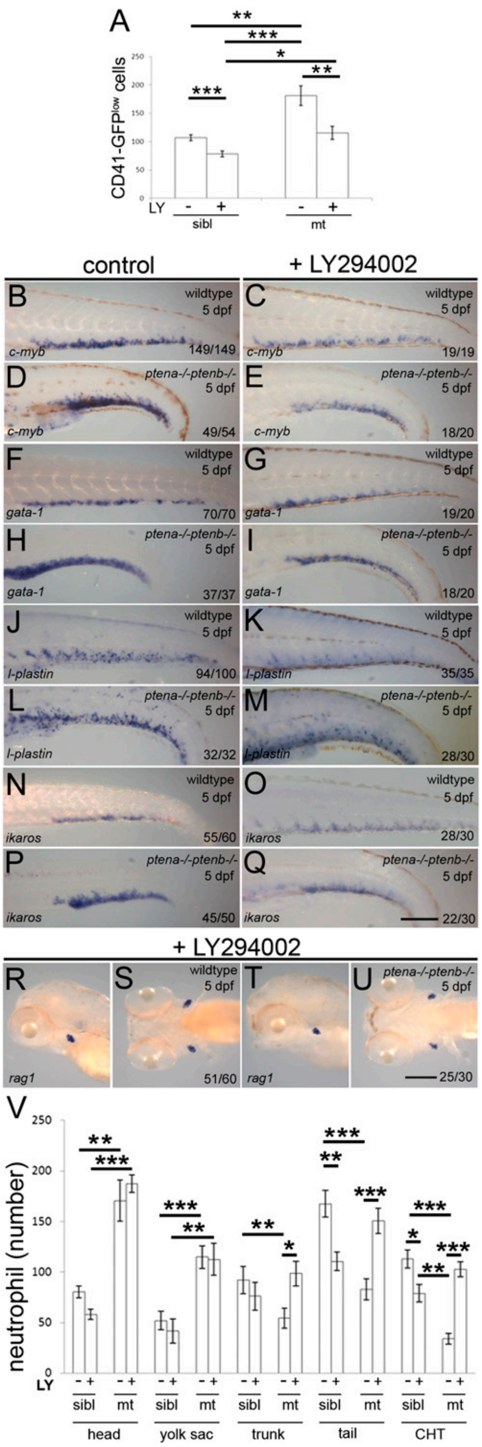

Figure 4. Inhibition of PI3K signaling suppressed enhanced proliferation and released differentiation arrest in *Pten* mutants. Embryos were treated with 4μM LY294002 or DMSO (control) from 82 hpf onward. (A) The CHTs of sibling (sibl) and *Pten* mutant (mt) embryos in *Tg(cd41:eGFP)* background were imaged. The number of HSPCs (GFP^{low}) were counted in the whole CHT at 4 dpf (sibling, n = 14; sibling LY294002 treated, n = 10; *ptena*$^{-/-}$ *ptenb*$^{-/-}$, n = 10; *ptena*$^{-/-}$ *ptenb*$^{-/-}$ LY294002 treated, n = 9). Results are expressed as the average number of cells per CHT and error bars indicate SEM. Normal distribution of data points was assessed with the Shapiro-Wilk test. Statistical comparisons of groups were performed by the 2-tailed *t* test, respectively, with the Mann-Whitney *U* test. **P* < .05, ***P* < .01, ****P* < .001 vs control. (B-U) In situ hybridization markers for blood lineages were used on 5-dpf wild-type and *Pten* mutant (*ptena*$^{-/-}$ *ptenb*$^{-/-}$) embryos: (B-E) *c-myb* is expressed in HSPCs; (F-I) *gata-1*, indicative of erythroblasts; (J-M) *l-plastin*, marking lymphocytes, macrophages, and neutrophils; (N-Q) *ikaros*, indicating lymphoblasts and (R-U) *rag1*, expressed in lymphocytes. Representative embryos are depicted; scale bars in panels

Figure 4 (continued) Q and U (200 μm) are representative for panels B-U. (V) At 5 dpf, SB staining was used to assess the number of neutrophils in various parts of the embryo. SB$^+$ cells in control (−, n = 10) and LY294002-treated (+, n = 16) siblings (sibl) and control (−, n = 11) and LY294002-treated (+, n = 17) *Pten* mutant (mt) embryos were counted in various parts of the embryo as indicated in supplemental Figure 2 and the results are expressed as average number of cells per embryo with the error bars indicating SEM. Normal distribution of data points was assessed with the Shapiro-Wilk test. Statistical comparison of groups was performed by the 2-tailed *t* test. **P* < .05, ***P* < .01, ****P* < .001 as indicated.

Arrested differentiation in *Pten* mutants is reversed by inhibition of PI3K signaling

Our data suggest that proliferation of definitive blood cell progenitors was enhanced, while concomitantly differentiation was arrested in *Pten* mutant embryos. To address directly whether the lack of Pten function was causative, we treated *Pten* mutant embryos with the PI3K inhibitor, LY294002, from 82 hpf onwards, that is, after the HSPCs had colonized the CHT. *Pten* mutant embryos displayed enhanced numbers of HSPCs at 5 dpf, compared with siblings (Figure 4A), consistent with our data at 4 dpf (Figure 1E-G). Treatment with 4μM LY294002 from 82 hpf onwards significantly decreased the number of CD41-GFP^{low} cells in *Pten* mutants and siblings as well (Figure 4A). Expression of the progenitor marker, *c-myb*, which was enhanced in the *Pten* mutants at 5 dpf, was also diminished by treatment with LY294002 from 82 hpf (Figure 4B-E) in both mutant and wild-type embryos, consistent with the CD41-GFP^{low} data. These data suggest that inhibition of PI3K signaling after colonization of the CHT suppressed proliferation of HSPCs.

Next, we addressed whether the blood cell lineage commitment was affected by treatment with LY294002 from 82 hpf onward. Expression of *gata-1*, *l-plastin*, and *ikaros* were determined for the erythroid, myeloid, and lymphoid lineages, respectively. While 5 dpf *Pten* mutant embryos exhibited elevated expression of *gata-1*, *l-plastin*, and *ikaros* in the CHT compared with wild-type embryos (Figure 4F-Q), treatment with LY294002 from 82 hpf onward suppressed this upregulation of *gata-1*, *l-plastin*, and *ikaros* in *Pten* mutant embryos to levels that were comparable to wild type (Figure 4F-Q). These results indicate that LY294002-induced inhibition of elevated PI3K activity in *Pten* mutants leads to suppression of the enhanced numbers of blood cell progenitors committed to the erythroid, myeloid, and lymphoid lineages.

Finally, we addressed whether arrested differentiation in *Pten* mutant embryos is caused by elevated PI3K signaling. *Rag1* expression that is greatly reduced in *Pten* mutant embryos at 5 dpf (Figure 2A-D) was fully restored after treatment with LY294002 (Figure 4R-U). Furthermore, quantification of SB$^+$ mature neutrophils at 5 dpf following late LY294002 treatment demonstrated a dramatic increase in the number of definitive wave-derived mature neutrophils in the CHT, trunk, and tail of *Pten* mutants, but not siblings (Figure 4V). In contrast, LY294002 treatment did not significantly affect the number of primitive wave-derived SB$^+$ cells in the head and yolk sac of *Pten* mutants and siblings (Figure 4V). Taken together, these data indicate that inhibition of PI3K signaling in *Pten* mutant embryos suppressed the enhanced proliferation of HSPCs in the CHT and at the same time released the blood cell differentiation arrest.

Discussion

PTEN is a lipid phosphatase that counteracts PI3K activity and is one of the most frequently mutated tumor suppressor genes in a wide range of cancer types, including leukemia.[12-15,34,35] PI3K signaling regulates hematopoietic stem cell (HSC) function. For instance, deletion of the downstream factors, Akt1 and Akt2, increases HSC quiescence,[36] whereas activation of PI3K signaling promotes HSC proliferation and depletion.[12,13] In the mouse embryo, HSPCs emerge from the dorsal aorta at embryonic day 10.5 (E10.5) and transiently colonize the fetal liver before seeding the organs of adult hematopoiesis.[8,37-39] Similarly, zebrafish HSPCs emerge from the ventral wall of the dorsal aorta and transiently colonize the CHT, which is homologous to the fetal liver in mouse, before colonization of the adult organs of hematopoiesis.[9] We used zebrafish mutant embryos lacking functional Pten to investigate whether loss of Pten affects homing of HSPCs to the CHT and further we addressed whether Pten is required for blood lineage specification and differentiation.

Our data revealed that Pten is not required for colonization of the CHT by HSPCs. Surprisingly, quantification of CD41^{low}-positive HSPCs and analysis of *c-myb* and *scl* expression patterns revealed an increased number of HSPCs in the CHT during the course of development. PTEN is a tumor suppressor and loss of PTEN function is associated with enhanced cell proliferation. Reminiscent of studies in the mouse,[12,13] we found increased numbers of mitotic cells in the CHT and conclude that in the absence of Pten, HSPCs colonize the niche and hyperproliferate.

PTEN is frequently mutated in leukemia resulting in replacement of normal bone marrow cells with leukemic cells. Acute myeloid leukemia patients suffer from a drop in erythrocytes, platelets, and normal white blood cells. Our investigation revealed that HSPCs lacking functional Pten engage in all blood lineages, indicating that lineage specification is a Pten-independent process. However, definitive differentiation of the thromboid, myeloid, lymphoid, and possibly other blood lineages is arrested in *Pten* mutants. We observed elevated numbers of mitotic HSPCs in the CHT of *Pten* mutant embryos, but no obvious differences in apoptosis between wild-type and *Pten* mutant embryos, indicating that enhanced proliferation caused the observed elevated number of HSPCs in mutant embryos. It is well established that proliferation and differentiation of stem cells, including HSCs, are inversely correlated.[40] Our results are consistent with this notion. Yet, the potential of HSPCs to commit to distinct blood lineages appears not to be affected in *Pten* mutants, in that we observed progenitors of all the main lineages. Moreover, inhibition of PI3K after the HSPCs had colonized the CHT, released the arrest of differentiation, and resulted in the production of definitively differentiated blood cells, including SB$^+$ neutrophils and *rag1*$^+$ thymocytes, indicating that these cells can produce mature blood cells.

The role of Pten in mouse HSCs has been addressed by conditional knockout of Pten using the Mx-1 promoter driving Cre recombinase in adult bone marrow cells, respectively, the VE-cadherin promoter in fetal liver.[12,13,41] HSCs are driven into the cell cycle in the absence of Pten, resulting in mobilization of HSCs from the bone marrow or fetal liver and transient expansion of the spleen, leading to depletion of HSCs. These conditional Pten-deficient mice die of a MPD that resembles acute myeloid/lymphoid leukemia.[12,13,41] Our observations that HSPCs hyperproliferate in the CHT of Pten mutant zebrafish embryos are consistent with the expansion of bone marrow HSCs in conditional mouse models.

Here, we report that zebrafish mutant embryos lacking functional Pten develop multiple hematopoietic abnormalities during development. In particular, we show that whereas Pten is not required for HSPCs to colonize the CHT/niche, loss of Pten results in enhanced proliferation of HSPCs. Additionally, enhanced PI3K signaling in response to loss of Pten does not favor a certain blood lineage in that all progenitors are defined. However, differentiation into mature blood cells is impaired in *Pten* mutants, which is reversed by antagonizing PI3K signaling by treatment with LY294002. We conclude that the zebrafish *Pten* mutants are a powerful tool to further study hematopoietic abnormalities/hematologic malignancies and demonstrate that zebrafish mutants are a suitable model for drug screening.

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Authorship

Contribution: S.C. and J.d.H. designed experiments with input from K.K. and P.H.; S.C. and R.K. performed experiments; and S.C. and J.d.H. wrote the manuscript with input from P.H. and K.K.

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References

1. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell.* 2008;132(4):631-644.
2. Amatruda JF, Zon LI. Dissecting hematopoiesis and disease using the zebrafish. *Dev Biol.* 1999;216(1):1-15.
3. Galloway JL, Zon LI. Ontogeny of hematopoiesis: examining the emergence of hematopoietic cells in the vertebrate embryo. *Curr Top Dev Biol.* 2003;53:139-158.
4. Tober J, Koniski A, McGrath KE, et al. The megakaryocyte lineage originates from hemangioblast precursors and is an integral component both of primitive and of definitive hematopoiesis. *Blood.* 2007;109(4):1433-1441.
5. Le Guyader D, Redd MJ, Colucci-Guyon E, et al. Origins and unconventional behavior of neutrophils in developing zebrafish. *Blood.* 2008;111(1):132-141.
6. Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature.* 2010;464(7285):112-115.
7. Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature.* 2010;464(7285):108-111.
8. Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature.* 2010;464(7285):116-120.
9. Murayama E, Kissa K, Zapata A, et al. Tracing hematopoietic precursor migration to successive hematopoietic organs during zebrafish development. *Immunity.* 2006;25(6):963-975.
10. Van Vlierberghe P, Ferrando A. The molecular basis of T cell acute lymphoblastic leukemia. *J Clin Invest.* 2012;122(10):3398-3406.

11. Palomero T, Sulis ML, Cortina M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med.* 2007; 13(10):1203-1210.
12. Zhang J, Grindley JC, Yin T, et al. PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention. *Nature.* 2006;441(7092):518-522.
13. Yilmaz OH, Valdez R, Theisen BK, et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. *Nature.* 2006; 441(7092):475-482.
14. Gutierrez A, Sanda T, Grebliunaite R, et al. High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood.* 2009;114(3):647-650.
15. Song MS, Salmena L, Pandolfi PP. The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol.* 2012;13(5):283-296.
16. Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. *Nat Genet.* 1998;19(4):348-355.
17. Goberdhan DC, Paricio N, Goodman EC, Mlodzik M, Wilson C. Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes Dev.* 1999;13(24):3244-3258.
18. Mihaylova VT, Borland CZ, Manjarrez L, Stern MJ, Sun H. The PTEN tumor suppressor homolog in Caenorhabditis elegans regulates longevity and dauer formation in an insulin receptor-like signaling pathway. *Proc Natl Acad Sci U S A.* 1999;96(13):7427-7432.
19. Faucherre A, Taylor GS, Overvoorde J, Dixon JE, Hertog J. Zebrafish pten genes have overlapping and non-redundant functions in tumorigenesis and embryonic development. *Oncogene.* 2008; 27(8):1079-1086.
20. Choorapoikayil S, Kuiper RV, de Bruin A, den Hertog J. Haploinsufficiency of the genes encoding the tumor suppressor Pten predisposes zebrafish to hemangiosarcoma. *Dis Model Mech.* 2012;5(2):241-247.
21. Davidson AJ, Zon LI. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. *Oncogene.* 2004;23(43):7233-7246.
22. Lin HF, Traver D, Zhu H, et al. Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood.* 2005;106(12): 3803-3810.
23. Westerfield M. The zebrafish book. A guide for the laboratory use of zebrafish (Danio Rerio). Eugene, OR: University of Oregon Press; 2004.
24. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn.* 1995;203(3):253-310.
25. Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc.* 2008;3(1):59-69.
26. Schulte-Merker S. Looking at embryos. In: Nüsslein-Vernhard C, Dahm R, eds. Zebrafish: a practical approach. New York, NY: Oxford University Press; 2002:41-43.
27. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. *Blood.* 2001; 98(10):3087-3096.
28. Detrich HW III, Kieran MW, Chan FY, et al. Intraembryonic hematopoietic cell migration during vertebrate development. *Proc Natl Acad Sci U S A.* 1995;92(23):10713-10717.
29. Herbomel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. *Dev Biol.* 2001;238(2):274-288.
30. Gering M, Patient R. Hedgehog signaling is required for adult blood stem cell formation in zebrafish embryos. *Dev Cell.* 2005;8(3):389-400.
31. Kissa K, Murayama E, Zapata A, et al. Live imaging of emerging hematopoietic stem cells and early thymus colonization. *Blood.* 2008;111(3): 1147-1156.
32. Qian F, Zhen F, Xu J, Huang M, Li W, Wen Z. Distinct functions for different scl isoforms in zebrafish primitive and definitive hematopoiesis. *PLoS Biol.* 2007;5(5):e132.
33. Jin H, Sood R, Xu J, et al. Definitive hematopoietic stem/progenitor cells manifest distinct differentiation output in the zebrafish VDA and PBI. *Development.* 2009;136(4):647-654.
34. Maser RS, Choudhury B, Campbell PJ, et al. Chromosomally unstable mouse tumours have genomic alterations similar to diverse human cancers. *Nature.* 2007;447(7147):966-971.
35. Fukuda R, Hayashi A, Utsunomiya A, et al. Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). *Proc Natl Acad Sci U S A.* 2005;102(42):15213-15218.
36. Juntilla MM, Patil VD, Calamito M, Joshi RP, Birnbaum MJ, Koretzky GA. AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. *Blood.* 2010; 115(20):4030-4038.
37. de Bruijn MF, Speck NA, Peeters MC, Dzierzak E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J.* 2000;19(11):2465-2474.
38. Kumaravelu P, Hook L, Morrison AM, et al. Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development.* 2002;129(21):4891-4899.
39. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell.* 1996;86(6):897-906.
40. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature.* 2001;414(6859):105-111.
41. Guo W, Lasky JL, Chang CJ, et al. Multi-genetic events collaboratively contribute to Pten-null leukaemia stem-cell formation. *Nature.* 2008; 453(7194):529-533.

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