Phosphatidylinositol-3 kinase signaling controls survival and stemness of hematopoietic stem and progenitor cells

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Abstract
Hematopoietic stem and progenitor cells (HSPCs) are multipotent cells giving rise to all blood lineages during life. HSPCs emerge from the ventral wall of the dorsal aorta (VDA) during a specific timespan in embryonic development through endothelial hematopoietic transition (EHT). We investigated the ontogeny of HSPCs in mutant zebrafish embryos lacking functional pten, an important tumor suppressor with a central role in cell signaling. Through in vivo live imaging, we discovered that in pten mutant embryos a proportion of the HSPCs died upon emergence from the VDA, an effect rescued by inhibition of phosphatidylinositol-3 kinase (PI3K). Surprisingly, inhibition of PI3K in wild-type embryos also induced HSPC death. Surviving HSPCs colonized the caudal hematopoietic tissue (CHT) normally and committed to all blood lineages. Single-cell RNA sequencing indicated that inhibition of PI3K enhanced survival of multipotent progenitors, whereas the number of HSPCs with more stem-like properties was reduced. At the end of the definitive wave, loss of Pten caused a shift to more restricted progenitors at the expense of HSPCs. We conclude that PI3K signaling tightly controls HSPCs survival and both up- and downregulation of PI3K signaling reduces stemness of HSPCs.

Introduction
Stem cells define a particular type of cells that maintain self-renewal capacity and may differentiate into multiple cell types at the same time. Hematopoietic stem and progenitor cells (HSPCs) are multipotent cells giving rise to all blood lineages during life [1–3]. In all vertebrates, an initial primitive wave of hematopoiesis occurs in the embryo, giving rise to primitive erythrocytes and myeloid cells. A definitive wave follows in which HSPCs are generated that will found multi-lineage hematopoiesis in developmentally successive hematopoietic organs up to adulthood. Our understanding of the emergence of HSPCs during the definitive wave is derived primarily from pioneer live in vivo imaging [4–6]. HSPCs emerge in a process whereby cells in the ventral wall of the dorsal aorta (VDA) undergo an endothelial to hematopoietic transition (EHT) [5] and then transiently colonize the fetal liver in mammals [7], or the caudal hematopoietic tissue (CHT) in zebrafish [8]. There, HSPCs expand and differentiate into all blood lineages and supply the developing embryos with mature blood cells. Subsequently, HSPCs migrate again to colonize the thymus and the bone marrow in

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mammals [7] or whole kidney marrow in fish [8] to produce blood cells in the adult.

HSPCs are tightly regulated in terms of dormancy, self-renewal, proliferation, and differentiation. Disrupting this balance can have pathological consequences such as bone marrow failure or hematologic malignancy. The tumor suppressor, PTEN, has an important role in hematologic malignancies, particularly T-lineage acute lymphoblastic leukemia (T-ALL). Deleterious mutations in PTEN appear in 5–10% of T-ALL cases and about 17% of patients lack PTEN expression in the hematopoietic lineage [9, 10]. PTEN counteracts phosphatidylinositol-3 kinase (PI3K) and hence acts upstream in the PI3K-Akt (also known as Protein kinase B) pathway [11]. Loss of PTEN function and hence acts upstream in the PI3K-Akt signaling pathway [11]. Loss of PTEN function results in hyperactivation of the PI3K-Akt signaling pathway. Clonal evolution of leukemia-propagating cells in zebrafish highlights the role of Akt signaling in the process [12]. Conditional deletion of Pten in mice in hematopoietic stem cells (HSCs) in adult bone marrow promotes HSC proliferation, resulting in depletion of long-term HSCs, indicating that Pten is essential for the maintenance of HSCs [13, 14].

The zebrafish genome encodes two pten genes with redundant function designated ptena and ptenb [15]. Single mutants display no morphological phenotype and are viable and fertile, but mutants that retain only a single wild-type copy of pten develop hemangiosarcomas during adulthood [16]. Ptena/− ptenb/− mutants lack functional Ptena and Ptenb, are embryonic lethal at 5–6 days postfertilization (dpf), and display hyperplasia and dysplasia [15]. We reported that double mutant zebrafish larvae lacking functional Pten display increased numbers of HSPCs in the CHT at 4–5 dpf. Whereas these HSPCs commit to different blood lineages, they fail to differentiate into mature blood cells. Inhibition of PI3K using LY294002, which compensates for the loss of Pten, restores differentiation of HSPCs into mature blood cells. Hence, loss of Pten enhances HSPCs proliferation and arrests differentiation [17].

The past decades have led to an increase in our knowledge of hematopoiesis, but we are still far from a complete understanding of how HSPCs are established. Likewise, the role of Pten in steady-state hematopoiesis has been studied, but its potential role in the ontogeny of HSPCs is not fully understood. We have addressed these questions in zebrafish larvae. We imaged the emergence of HSPCs from the VDA in vivo in ptena/− ptenb/− embryos and in PI3K inhibitor treated wild-type embryos, which showed surprisingly similar defects. Furthermore, we performed single-cell RNA sequencing (scRNA-seq) during the onset and at the end of the definitive wave. Our results indicate that elevated and reduced PI3K signaling had opposite effects on HSPCs at the end of the definitive wave.

### Results

#### The onset of the definitive wave of hematopoiesis is independent of Pten

The onset of the definitive wave starts at 32 h postfertilization (hpf) with the specification of endothelial cells that will become HSPCs in the floor of the dorsal aorta (DA) in the AGM region (Fig. 1a), a conserved process between mammals and zebrafish [4–6]. Runx1 expression from 32 hpf onwards and c-myb expression from 35 hpf onwards mark the hemogenic endothelium of the VDA and its HSPC progeny [8, 39]. We found that ptena/− ptenb/− mutant embryos expressed runx1 and c-myb along the VDA during the period that HSPCs emerge (between 30 and 44 hpf) just like their siblings (Fig. S1), indicating that loss of Pten does not affect the number of hemogenic endothelial cells.

#### Loss of Pten results in apoptosis of HSPCs during EHT in ptena/− ptenb/− mutant embryos

In zebrafish, endothelial cells from the VDA transform into HSPCs in a process called EHT [5]. Subsequently, HSPCs join the blood flow in the underlying posterior cardinal vein to transiently seed the CHT [4, 5, 8]. We monitored EHT events in the AGM by time-lapse confocal imaging of an area spanning two adjacent intersegmental vessels in the tg(kdrl:eGFP) transgenic background from 35 to 48 hpf (Fig. 1a, b). The vasculature of ptena/− ptenb/− mutants and siblings was indistinguishable at this stage [40]. The floor of the aorta in ptena/− ptenb/− mutant embryos displayed the characteristic contraction then bending of cells toward the subaortic space [5], indicating that the initiation of EHT was normal in ptena/− ptenb/− mutant embryos. However, half of the EHT events in ptena/− ptenb/− mutant embryos were abortive, in that 13 out of 24 HSPCs (54% in 9 embryos) failed to detach and disintegrated (Fig. 1c–h and Movie S1). In contrast, siblings or wild-type embryos did not display abortive EHT (n = 75 in total). Live imaging using acridine orange [30] revealed apoptotic cells in the DA of ptena/− ptenb/− mutant embryos, but not siblings (Fig. 1i–l). Activated caspase-3 immunostaining [31] confirmed apoptosis of kdrl:eGFP-positive cells at the VDA in ptena/− ptenb/− mutant embryos (Fig. 1m–p). Hence, about half of the HSPCs in ptena/− ptenb/− mutant embryos failed to complete EHT and instead underwent apoptosis.

#### The number of HSPCs that colonize the CHT is reduced in ptena/− ptenb/− mutant embryos

Following EHT, HSPCs transiently colonize the CHT [8]. We generated a tg(kdrl:Dendra2) transgenic line. The Dendra2 protein along the entire VDA was
Photoconverted green-to-red between 26 and 28 hpf, i.e. before the onset of EHT events (Fig. 2a). Photoconverted HSPCs in ptena−/− ptenb−/− mutant embryos colonized the CHT between 50 and 60 hpf, albeit less HSPCs were detected than in the CHT of siblings (Fig. 2b, c). We quantified the number of HSPCs that colonized the CHT at 48 hpf, i.e. by the peak of HSPC emergence from the VDA, using tg(cd41:eGFP) embryos, which express low GFP (GFPlow) in HSPCs [20, 41]. Consistent with the initial apoptosis of half of the EHT-derived HSPCs, 51% less GFPlow HSPCs were detected in the CHT of ptena−/− ptenb−/− mutant embryos at 48 hpf compared to siblings (Figs. 2d–f and S2). When injected with ptenb-mRNA at the one-cell stage, ptena−/− ptenb−/− mutant embryos did no longer show a significant loss of HSPCs compared to their siblings (Fig. 2g), indicating that the observed defects indeed were caused by loss of functional Pten.

Fig. 1 A population of HSPCs fails to complete EHT and undergoes apoptosis in ptena−/− ptenb−/− mutant embryos. a, b Brightfield image of a wild-type or ptena−/− ptenb−/− mutant zebrafish embryo at 35 hpf. The area from which HSPCs originate is indicated with a yellow box. A close up is indicated with a white box. c–h Four-dimensional imaging of tg(kdrl:eGFP) wild-type or ptena−/− ptenb−/− mutant embryos between 35 and 48 hpf. Still frames from Movie S1. Arrowheads: HSPCs undergoing EHT; asterisk: disintegrating HSPCs. Confocal image z-stacks (2 µm step size, with ×40 objective and ×2 zoom; anterior to the left; maximum projections of a representative embryo; time in hh: mm. i–l Acridine orange staining. Arrows and circles: HSPCs in VDA of 40–45 hpf embryos. Asterisks: apoptotic HSPCs. Scale bar: 50 µm. Representative embryos are shown and the number of embryos that showed this pattern/total number of embryos is indicated. DA dorsal aorta, PCV posterior cardinal vein. m–p Confocal images of apoptotic endothelial cells in the VDA of fixed wild-type or ptena−/− ptenb−/− mutant zebrafish embryos. In green: tg(kdrl:eGFP); in red: anti-activated caspase-3 immunohistochemistry staining. Apoptotic cells are indicated with an asterisk. Representative embryos are shown and the number of embryos displaying this particular pattern/total number of embryos is indicated in the bottom right. Anterior to the left; 2 µm step size; maximum projections; scale bar: 100 µm.
**PI3K inhibition rescues EHT events in ptena<sup>−/−</sup> ptenb<sup>−/−</sup> mutant embryos but is detrimental for HSPCs in wild-type embryos**

To address whether apoptosis of half of the EHT-derived HSPCs was due to enhanced PI3K signaling, embryos were treated with the PI3K inhibitor LY294002 from the onset of EHT (32 hpf) onwards. Inhibition of PI3K prevented apoptosis of HSPCs in ptena<sup>−/−</sup> ptenb<sup>−/−</sup> mutant embryos, in that none of the HSPCs that we imaged disintegrated (Table 1) (Fisher’s exact test, *p* = 0.0013) (Fig. 3a–c). Surprisingly, in wild-type and sibling embryos that were treated with LY294002 in parallel with the ptena<sup>−/−</sup> ptenb<sup>−/−</sup> mutant embryos, disintegrating HSPCs in the VDA were observed (*n* = 6) (Fig. 3d–g and Movie S2) (Fisher’s exact test, *p* = 0.021).

Consistent with abortive EHT events upon LY294002 treatment, significantly less GFP<sup>low</sup> HSPCs in *tg(cd41:eGFP)* siblings (*n* = 25) (e) and ptena<sup>−/−</sup> ptenb<sup>−/−</sup> mutants (*n* = 12) (f) is expressed as average number of cells in siblings (*n* = 41) or ptena<sup>−/−</sup> ptenb<sup>−/−</sup> mutants after injection with synthetic ptenb-mRNA (*n* = 15). Error bars indicate standard error or the mean (SEM). Shapiro–Wilk test for normal distribution and Welch’s two-tailed *t*-test were used for statistical analysis; ***p* < 0.001. Representative embryos are shown and the number of embryos that showed this pattern/total number of embryos is indicated.

**PI3K inhibition in wild-type embryos results in HSPCs that engage in all blood lineages**

In situ hybridization was performed using a panel of blood progenitor markers. LY294002 treatment reduced expression of the HSPC marker *c-myb* at 4 dpf (Fig. 4a, b).
The lineage markers *globin* (erythroid lineage), *ikaros* (lymphoid lineage), and *l-plastin* (pan-leukocytic, including myeloid lineage) were expressed, but reduced in LY294002-treated larvae compared to controls (Fig. 4c–h). LY294002-treated HSPCs also committed to the thrombocytic lineage as demonstrated by GFP<sup>high</sup> cells in *tg*(cd41: eGFP) embryos at 5 dpf [20] (Fig. 4i, j). The number of GFP<sup>low</sup> cells in the definitive hematopoietic organs, thymus, and kidney of 8 and 12 dpf *tg*(cd41:eGFP) larvae was reduced in response to LY294002 (Fig. 4k–n). These results show that reduced PI3K signaling did not block specification of particular blood lineages, but that the reduction in HSPC numbers affected founding of the definitive hematopoietic organs by HSPCs.

**Singecell RNA sequencing reveals two types of HSPCs, one of which is preferentially lost upon inhibition of the PI3K-pathway**

To investigate transcriptomic changes in HSPCs between LY294002-treated embryos and their controls during EHT, we performed scRNA-seq. Transgenic *tg*(kdrl:mCherry-CAAX/cd41:eGFP) embryos were treated with LY294002 and AGM regions were isolated by dissection at 36hpf. The AGM regions of ~2000 control embryos were pooled and AGM regions were isolated by dissection at 36hpf. The embryos were treated with LY294002 [34]. Isolation of 3219 cells in total, i.e. <1 kdrl<sup>+/cd41<sup>low</sup> mCherry<sup>/GFP<sup>low</sup></sup> HSPC per embryo, was in line with earlier reports (3 HSPCs per embryo per hour [5, 42]). After FACS filtering, 2512 cells remained. RaceID3 [43] was used for differential gene expression analysis and clustering of the cells (Fig. 5). The resulting t-SNE map highlighted particular cell types, in line with recent scRNA-seq studies of hematopoietic organs of zebrafish [44–51], which expressed validated hematopoietic lineage markers (Table S1). Cells in clusters 2 and 4 expressed HSPC-related genes, such as *gata2b, gfi1aa, meis1b, myb, and pmp22b*, consistent with expression in mammalian HSCs and zebrafish HSPCs [45–49, 51] (Fig. 5a, b). RaceID3 subdivided the main HSPC cluster into two, HSPCs I and HSPCs II. Expression of ENSDARG00000080337_ACO24175.4 and *tmed1b* was higher in the HSPCs II cluster (cl2) than the HSPCs I cluster (cl4) (Figs. 5h and S3) and the expression of several other genes was also significantly different between these clusters (Fig. S4). Cells in cluster 5 expressed endothelial transcripts, that are known to be involved in the EHT-process (*cdh5* [52, 53], *adgrg1* [44, 54]), indicating an EHT progenitor lineage (Figs. 5c and S3). Signature genes *mpx, lyz, marco,* and *mtap4* were expressed in clusters 1 and 3 [55], indicating a myeloid/neutrophil- and myeloid/monocyte-progenitor lineage, respectively (Figs. 5d, e and S3). All markers that were used to identify clusters are listed in Table S2 and the distribution of expression of selected markers is depicted in Fig. S5.

Cells from LY294002-treated embryos had an uneven distribution over all clusters. In HSPCs II and myeloid/neutrophil progenitors, cells from LY294002-treated embryos were underrepresented compared to control embryos (Fisher’s exact test, *p* < 0.001). In HSPCs I, EHT- and myeloid/monocyte progenitors, cells from LY294002-treated embryos were overrepresented (Fisher’s exact test, *p* < 0.001) (Fig. 5f, i). These data indicate that LY294002 treatment led predominantly to loss of cells from HSPCs I cluster, which is consistent with the imaging data where half of the HSPCs fail to complete EHT (Fig. 3d–g). The loss of HSPCs I in response to PI3K inhibition is accompanied by an increase in HSPCs II and myeloid/neutrophil progenitors.

**More HSPCs upon inhibition of PI3K and less HSPCs in pten mutants**

The CHTs of ~100 control and 100 LY294002-treated embryos were processed for scRNA-seq. Of the 928 cd41<sup>low</sup>
cells that were analyzed, 684 remained after filtering. RaceID3 separated the cells in distinct clusters (Fig. 6a). Cells in cluster 2 expressed erythrocyte progenitor-related genes (hbbe2, alas2, and cahz) (Fig. 6b). Cluster 3 is characterized by cells expressing genes related to thrombocyte/erythrocyte progenitors (gata1a, klf1 [51, 55, 56]) (Fig. 6c). Cells in cluster 1 express genes indicative of HSPCs, including c-myb (Fig. 6d). Cluster 4 represents early myeloid progenitors, as runx3, pu.1 (also known as spi1b), and cebp [55] are highly expressed (Fig. 6e). Cluster 5 is characterized by neutrophil progenitor-related gene expression (mpx) (Fig. 6f). Analysis of the distribution of hematopoietic cells, using a Fisher’s exact test indicated that the thrombocyte/erythrocyte progenitor cells were
Fig. 4 HSPCs of LY294002-treated embryos engage in all blood lineages, but show impaired colonization of definitive hematopoietic organs. 

a–h Control and LY294002-treated (from 32–60 hpf) embryos were fixed at 4 dpf. Markers for definitive blood lineages were assessed by in situ hybridization in the CHT: c-myb (HSPCs; a, b), globin (erythrocyte lineage; c, d), ikaros (lymphocyte lineage; e, f), l-plastin (leukocytes; g, h). Representative embryos are shown, with anterior to the left. The number of embryos that showed a particular pattern/total number of embryos is indicated in the bottom right corner of each panel. i, j GFP<sup>high</sup> thrombocytes were imaged at 5 dpf in tg(cd41:eGFP) embryos. Scale bar: 100 µm. k–n High-resolution imaging at 12 dpf of kidney (k, l, dorsal view) (control, n = 6; LY294002-treated, n = 8; 4 µm step size) and thymus (m, n, lateral view) (control, n = 6; LY294002-treated, n = 7; 2 µm step size). Anterior to the left; maximum projections of representative larvae. Scale bar: 100 µm.
underrepresented in the LY294002-treated embryos (p < 0.01) and HSPCs were significantly overrepresented (p < 0.001) (Fig. 6g, h). These results indicate a significant shift toward HSPCs at the expense of the thrombocyte/erythrocyte progenitor cluster in response to LY294002 treatment.

Likewise, we assessed transcriptomic differences by scRNA-seq in HSPCs from the CHT between ptena−/−
\textit{pten}^−/− mutant embryos and their siblings at 5 dpf. Approximately 100 \textit{ptena}^−/−/\textit{ptenb}^−/− mutant embryos and siblings were selected based on phenotype [15], which yielded 614 \textit{cd41}^{low} cells after filtering. RaceID3 indicated that clusters emerged representing the same hematopoietic lineages as described for the wild-type and \textit{LY294002}-treated data (cf. Figs. 6 and 7). Analysis of the distribution of hematopoietic cells from \textit{pten} mutants and their siblings over the five clusters indicated that the erythrocyte- and neutrophil progenitor cells were overrepresented in the pten

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6}
\caption{SeRNA-seq seq reveals a shift toward HSPCs in \textit{LY294002}-treated 5 dpf old embryos. CHTs of control and \textit{LY294002}-treated embryos (5 dpf, ~100 embryos each) were dissected, pooled, dissociated, FACS sorted and submitted to SORT-seq. \textbf{a} Visualization of k-medoid clustering and cell-to-cell distances using t-SNEs. Each dot represents a single cell. Colors and numbers indicate cluster and correspond to colors in \textbf{h}. In total, 684 cells are shown. \textbf{b–f} t-SNEs maps highlighting the expression of marker genes for each of the different cell types found. Transcript counts are given in a linear scale. \textbf{b} Erythrocyte progenitors, \textbf{c} Thrombocyte/erythrocyte progenitors, \textbf{d} HSPCs, \textbf{e} Myeloid progenitors, \textbf{f} Neutrophil progenitors. \textbf{g} t-SNE map highlighting the distribution of \textit{LY294002}-treated embryos and their controls (\textbf{h}). The percentage of cells from \textit{LY294002}-treated embryos and their controls in the different clusters. Fisher’s exact test with multiple testing correction (Fdr) were used for statistical analysis. **\textit{p} < 0.01, ***\textit{p} < 0.001.

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mutant ($p < 0.001$ and $p < 0.05$) and that HSPCs were significantly underrepresented ($p < 0.001$) (Figs. 7g, h and S6). These results indicate a significant shift in $pten^{-/-} ptenb^{-/-}$ mutant embryos toward erythrocyte progenitor and neutrophil progenitors at the expense of HSPCs.

**Discussion**

We used zebrafish mutant embryos lacking functional Pten to investigate how loss of Pten affects the ontogeny of hematopoiesis. Characterization of zebrafish $ptena^{-/-}$

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**Fig. 7** ScRNA-seq reveals a shift toward more differentiated cell types in 5 dpf old $ptena^{-/-} ptenb^{-/-}$ mutant embryos. CHTs of control and $ptena^{-/-} ptenb^{-/-}$ mutant embryos (5 dpf, ~100 embryos each) were dissected, pooled, dissociated, FACS sorted and submitted to SORT-seq. a Visualization of k-medoid clustering and cell-to-cells distances using t-SNEs. Each dot represents a single cell. Colors and numbers indicate cluster and correspond to colors in h. In total, 614 cells are shown. b-f t-SNEs maps highlighting the expression of marker genes for each of the different cell types found. Transcript counts are given in a linear scale. b Erythrocyte progenitors, c Thrombocyte/erythrocyte progenitor, d HSPCs, e Myeloid progenitors, f Neutrophil progenitors. g t-SNE map highlighting the distribution of $ptena^{-/-} ptenb^{-/-}$ mutant embryos and their siblings. h The percentages of cells from $ptena^{-/-} ptenb^{-/-}$ mutant embryos and their siblings in the different clusters. Fisher’s exact test with multiple testing correction (Fdr) were used for statistical analysis. *$p < 0.05$, ***$p < 0.001$. 
pten\(^{-/-}\) mutant embryos led to the unexpected finding that half of the HSPCs undergo apoptosis upon emergence from the VDA during EHT at the onset of the definitive wave (Fig. 1). Loss of function of Pten is usually linked to enhanced cell survival, such as for instance in Pten knock-out mice [57]. We reported that \(\gamma\)-irradiation reduces apoptosis in pten\(^{-/-}\)-ptenb\(^{-/-}\) mutant embryos [15]. Apoptosis of zebrafish HSPCs has been reported before, in that grechetto mutants display decreasing numbers of HSPCs due to apoptosis [52]. Runx1 knockdown also induced abortive EHT events due to apoptosis [5]. Runx1 expression was not affected in the VDA of ptena\(^{-/-}\)-ptenb\(^{-/-}\) mutants (Fig. S1), suggesting that the mechanism underlying EHT defects in ptena\(^{-/-}\)-ptenb\(^{-/-}\) mutant embryos and Runx1 morphants are distinct. Apoptosis of HSPCs in pten mutants is due to enhanced PI3K-mediated signaling, because treatment with a PI3K inhibitor rescued apoptosis of HSPCs. Surprisingly, treatment of wild-type embryos with the PI3K inhibitor induced death of half of the HSPCs upon emergence from the VDA as well (Fig. 3). These results suggest that upon emergence from the VDA, HSPCs require a moderate level of PI3K signaling, as hyperactivation of PI3K signaling in Pten mutants may indicate that the HSPCs II cells that survive PI3K inhibition are less stem cell-like and more progenitor-like, poised to differentiate.

In response to LY294002 treatment, the number of cd41\(^{low}\) HSPCs was reduced in the CHT at 4 dpf and in the definitive hematopoietic organs at 8 and 12 dpf (Figs. 3 and 4). scRNA-seq of putative HSPCs (cd41\(^{low}\), kdrl\(^{+}\)) cells at the end of the definitive wave (5 dpf) indicated initiation of differentiation in different blood lineages (Fig. 6), consistent with in situ hybridization (Fig. 4). Yet, inhibition of PI3K arrested differentiation, i.e. increased HSPC fate, predominantly at the expense of thrombocyte/erythrocyte progenitor fate (Fig. 6). Overall, it is evident that there is a significant reduction in hematopoietic cell number (Figs. 4 and 6), which may be caused by preferential loss of HSPCs with more stem cell-like properties (Fig. 5).

ScRNA-seq at the end of the definitive wave showed a significant increase in erythrocyte- and neutrophil progenitors in ptena\(^{-/-}\)-ptenb\(^{-/-}\) mutant embryos (Figs. 7 and S6), consistent with earlier in vivo data [17]. However, we reported an overall increase in HSPCs, due to hyperproliferation, whereas here, we observed a decrease in HSPCs in the scRNA-seq data. An explanation for this apparent discrepancy is that the hyperproliferating HPSCs we observed earlier [17] actually have initiated differentiation already and are scored as erythrocyte and neutrophil progenitors by scRNA-seq.

Conditional knock-out of Pten in HSCs in mouse adult bone marrow drives HSCs into the cell cycle, resulting in transient expansion of the spleen and eventually in depletion of HSCs in the bone marrow. These conditional PTEN-deficient mice die of a myeloproliferative disorder that resembles acute myeloid/lymphoid leukemia, indicating that PTEN is required for maintenance of HSCs [13, 14]. It is noteworthy that there are differences between the conditional mouse models and the zebrafish model we used. In the mouse, Pten is deleted in adult bone marrow cells, well after HSCs have formed, whereas in zebrafish, Pten is
systemically deleted and therefore effective prior to the emergence of HSPCs. Studies in mice showed that regardless of cell state, HSCs and multipotent progenitors had a lower protein synthesis rate than more restricted hematopoietic progenitors. Loss of PTEN in HSCs caused depletion of HSCs, due to a higher rate of protein synthesis [59], which is consistent with our observation that loss of Pten in zebrafish caused HSPCs to hyperproliferate and become less stem cell like.

Long-term HSCs are quiescent, whereas short-term HSCs proliferate more [2]. It would be tempting to speculate that the HSPCs that undergo apoptosis upon loss of Pten or upon PI3K inhibition are involved in long-term colonization of definitive hematopoietic organs. The surviving HSPCs in pten mutants at the onset of the definitive wave would then represent multipotent progenitors that only have limited potential for self-renewal. Investigating the regulatory network underlying the surviving and disintegrating HSPCs will further expand our understanding of short- and long-lived HSPCs and will eventually contribute to the development of efficient stem cell-based therapies [60, 61].

Methods

Zebrafish husbandry

Ptena+/−ptenb+/−, ptena−/−, ptenb−/− [15], Tg(kdrl:eGFP) [18], Tg(kdrl:mCherry-CAAX) [19], and Tg(cd41:eGFP) [20] were maintained according to FELASA guidelines, crossed, raised, and staged as described [21–23]. Pten mutant fish (embryos) were genotyped by sequencing [15]. The tg(kdrl:Dendra2) line was derived by Tol2-mediated transgenesis [24] of a construct containing the ~7.0 kb kdrl-promoter (a kind gift from D. Stainier), driving the expression of Dendra2 [25]. From 24hpf onwards, all embryos were grown in PTU-containing medium to block pigmentation.

LY294002 treatment

Embryos were treated with 5 µM LY294002 (Calbiochem, San Diego, CA, USA) or DMSO control in the dark. For early treatment, embryos were incubated with LY294002 from 32 hpf onwards and mounted after 4 h for time-lapse confocal imaging. For late treatment and to investigate thymus and kidney colonization, embryos were treated with 5 µM LY294002 from 32 to 60 hpf and imaged.

Constructs, mRNA synthesis, and microinjections

The Ptenb-mCherry fusion construct in the vector pCS2+ was obtained as described in refs. [15, 26] and linearized with NotI. To synthesize 5′ capped sense mRNA, the mMessage mMachine SP6 kit (Ambion) was used. mRNA injections were performed at the one-cell stage using a total of 300 pg of mRNA.

Confocal, fluorescence, brightfield microscopy, and time-lapse imaging

Fluorescence images of transgenic embryos were acquired using TCS-SPE and time-lapse imaging using TCS-SP2 as described [27] and processed with ImageJ [28]. For all live imaging embryos were anesthetized with tricaine [21], mounted on a glass cover dish with 0.7% low melting agarose and covered with standard E3 medium. Whole mount bright field images were taken with a Leica DC 300F stereomicroscope.

In situ hybridization

Whole mount in situ hybridization was performed according to standard protocols [29] and images were taken using a Zeiss Axioplan microscope connected to a Leica DFC480 camera.

Acridine orange staining and whole mount immunohistochemistry

Embryos were incubated with 5 µg/ml acridine orange [30] for 20 min between 35 and 40 hpf and subsequently washed with standard E3 medium. Embryos were then imaged as described above. Immunohistochemical labeling performed using fixed (40 hpf) embryos to detect apoptosis using an activated caspase-3-specific antibody (BD Pharmingen) [31]. After confocal images were collected embryos were genotyped.

Photoconversion

Fluorescent tracing of VDA-derived HSPCs colonizing the CHT was done using the tg(kdrl:Dendra2) line as described before [32, 33] with a Leica SP5 confocal microscope with a ×20 dry objective. At 28 hpf an area of ~40 × 750 nm around the VDA, parallel to the yolk sac extension was photoconverted. The 405 nm UV laser intensity and exposure time were optimized for strong Dendra2-conversion without cell damage. After photoconversion embryos were transferred to E3 medium and at 50–60 hpf their CHT areas were imaged on a Leica SPE Live confocal microscope using a ×20 dry objective. To exclude bleed-through of Dendra-green, red channel detection was set stringently (630–680 nm).

Quantification of GFPlow progenitor cells using tg (cd41:eGFP)

GFPlow and GFPhigh expressing cells were quantified in the CHT at 48 hpf, 50 hpf or 4 dpf using confocal imaging and...
Volatility and Imaris software. Ptena/−/ptenb/−/ mutants on tg(cd41:eGFP) background were crossed and offspring was mounted at 48 hpf. Wild-type tg(cd41:eGFP) embryos were treated with 5 µM LY294002 as described above and mounted and imaged at 50 hpf or 4 dpf. All GFPlow expressing cells were counted in the entire CHT.

Flow cytometry

The aorta-gonad-mesonephros (AGM) of ~4000 36 hpf and 400 CHTs of 5 dpf old tg(kdrl:mCherry/cd41:eGFP) embryos were dissected and collected in Leibovitz medium. After washing with phosphate-buffered saline, the AGMs were deyolked using calcium-free Ringer’s solution (116 mM NaCl, 2.9 mM KCl and 5 mM HEPES) and then AGMs and CHTs were dissociated in TrypLE Express (Gibco) for 45 min at 32 °C. The resulting cell suspension was washed in phosphate-buffered saline and passed through a 40-µm filter after resuspension in phosphate-buffered saline supplemented with 2 mM ethylenediamine-tetraacetic acid, 2% fetal calf serum, and 0.5 µg/ml 4′,6-diamidino-2-phenylindole (DAPI), to exclude dead cells. Cells with kdrl- and cd41low-positive signal were subjected to fluorescence-activated cell sorting (FACS) using a BD FACSAriaII and BD FACSFusion.

ScRNA-seq with SORT-seq

ScRNA-seq was performed by Single-Cell Discoveries BV (Utrecht, the Netherlands), according to an adapted version of the SORT-seq protocol [34, 35], with adapted primers described in ref. [36].

Data analysis

During sequencing, Read1 used for identification of the Illumina library barcode, cell barcode, and UMI. Read2 was used to map to the reference transcriptome of Zv9 Danio rerio. Data were demultiplexed as described [37]. Single-cell transcriptomics analysis was done using the RaceID algorithm, following an adapted version of the RaceID manual (https://cran.r-project.org/web/packages/RaceID/vignettes/RaceID.html). Cells that had <1500 UMIs and genes that were detected in <5 UMIs in five cells were discarded. The number of initial clusters was set at 5. Differential gene expression analysis was done as described in ref. [34] with an adapted version of the DESeq2 algorithm [38].

Data availability

For original data, please contact j.denhertog@hubrecht.eu. scRNA data are available at GEO under accession number GSE166900.

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Author contributions

SB-F, SC, and JdH designed experiments with input from KK and PH; SB-F and SC performed the experiments; BP and SSM generated the Tg(kdrl:Dendra) line and helped perform the photoconversion experiments; KK and JdH supervised the work; SB-F, SC, PH, KK, and JdH wrote the manuscript.

Compliance with ethical standards

Conflict of interest

The authors declare no competing interests.

Ethical approval

All animal experiments described in this manuscript were approved by the local animal experiments committee (Hubrecht Institute: Koninklijke Nederlandse Academie van Wetenschappen-Dierexpressment commissie protocol H1180701 and University Montpellier: Direction Sanitaire et Vétérinaire de l’Hérault and Comité d’Ethique pour l’Expérimentation Animale under reference CEEA-LR-13007) and performed according to local guidelines and policies in compliance with national and European law.

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