Regulation of murine normal and stress-induced erythropoiesis by Desert Hedgehog

Ching-in Lau, Susan V. Outram, José Ignacio Saldaña, Anna L. Furmanski, Johannes T. Dessens, and Tessa Crompton

Introduction

The function of Hedgehog signaling in hematopoiesis is controversial, with different experimental systems giving opposing results. Here we examined the role of Desert Hedgehog (Dhh) in the regulation of murine erythropoiesis. Dhh is one of 3 mammalian Hedgehog family proteins. Dhh is essential for testis development and Schwann cell function. We show, by analysis of Dhh-deficient mice, that Dhh negatively regulates multiple stages of erythrocyte differentiation. In Dhh-deficient bone marrow, the common myeloid progenitor (CMP) population was increased, but differentiation from CMP to granulocyte/macrophage progenitor was decreased, and the mature granulocyte population was decreased, compared with wild-type (WT). In contrast, differentiation from CMP to megakaryocyte/erythrocyte progenitor was increased, and the megakaryocyte/erythrocyte progenitor population was increased. In addition, we found that erythroblast populations were Dhh-responsive in vitro and ex vivo and that Dhh negatively regulated erythroblast differentiation. In Dhh-deficient spleen and bone marrow, BFU-Es and erythroblast populations were increased compared with WT. During recovery of hematopoiesis after irradiation, and under conditions of stress-induced erythropoiesis, erythrocyte differentiation was accelerated in both spleen and bone marrow of Dhh-deficient mice compared with WT. (Blood. 2012;119(20):4741-4751)
The objective of this study was to examine the requirement for Dhh in erythropoiesis in the spleen and BM. Erythrocytes, like other blood lineages, develop from HSCs through a sequence of well-defined intermediates. In mice, HSC have been defined by their absence of lineage-specific markers, and expression of Sca-1 and c-kit, and are therefore referred to as Lin−Sca-1+ c-kit+ (LSK) stem cells. LSKs give rise to several progenitor cells, including common myeloid progenitor cells (CMP), which in turn give rise to granulocyte/macrophage progenitor cells (GMP) and megakaryocyte/erythroid progenitor cells (MEP). The MEP then give rise to the erythroid lineage, first by differentiating into burst-forming unit cells (BFU-E), the first erythroid committed cells, and then colony-forming units (CFU-E).36 BFU-E and CFU-E cannot be identified by cell surface markers, so they are quantified by their ability to produce colonies in functional assays in vitro. These cells then develop through a series of erythroblast stages, which are defined by surface expression of CD71 and Ter119.37 Ter119medCD71hi (population I, proerythroblast) precursors differentiate to Ter119hiCD71hi (population II, basophilic erythroblast), which then down-regulate CD71 to become Ter119hiCD71med (population III, polychromatophilic erythroblast) and then Ter119hiCD71− (population IV, orthochromatic erythroblast) cells. At the later erythroblast stages, the nucleus progressively shrinks and is shed before the cells become mature erythrocytes.38 The developmental program from HSCs to mature erythrocyte is regulated by complex transcriptional networks and by environmental signals.39,40 In the adult, most erythropoiesis occurs in the BM, but under conditions of erythropoietic stress (anemia, hypoxia), the number of erythrocytes is increased, and this process of stress-induced erythropoiesis occurs predominantly in the spleen.35,41

Here, we show that Dhh functions as a negative regulator of normal and stress-induced erythropoiesis, at multiple stages of differentiation, in both the spleen and BM.

Methods

Mice

C57BL/6 mice (B&K Universal Ltd), Dhh+/− mice,14 a gift from Andrew McMahon (Harvard University), backcrossed onto C57BL/6 mice for more than 8 generations, were bred and maintained at University College London. In some experiments, mice were irradiated with 4 Gy from a 60Co γ-ray source, or anemia was induced by intraperitoneal injection of phenylhydrazine (60 mg/kg body weight; Sigma-Aldrich). All animal work was carried out under the United Kingdom Home Office regulations under the United Kingdom Home Office (Project License PPL 70/7088).

Flow cytometry, histology, staining, and antibodies

BM was isolated from femur. Cell suspensions from spleen and BM were prepared, stained and analyzed as described.17,32 using directly conjugated antibodies from BD Biosciences PharMingen and eBioscience. Data are representative of more than 3 experiments. Statistical analysis was unpaired Student’s t test (equal or unequal variance depending on data). For some experiments, BM cells and splenocytes were isolated and sorted using MoFlo XDP Sorter (Beckman Coulter) to obtain populations of Ter119hiCD71hi (population II, basophilic erythroblast) cells. At the later erythroblast stages, the nucleus progressively shrinks and is shed before the cells become mature erythrocytes.38 The developmental program from HSCs to mature erythrocyte is regulated by complex transcriptional networks and by environmental signals.39,40 In the adult, most erythropoiesis occurs in the BM, but under conditions of erythropoietic stress (anemia, hypoxia), the number of erythrocytes is increased, and this process of stress-induced erythropoiesis occurs predominantly in the spleen.35,41

For histology, spleens were fixed in phosphate-buffered formalin (10% volume/volume), paraffin-embedded, and sectioned for H&E staining, by standard protocols. Pictures were photographed by Leica DFC320 digital camera (Leica Microsystems) with Leica DML Microscope (Leica Microsystems), and acquired by software Leica Qwin vLite3.2.1 (Leica Microsystems).

Hematopoietic colony assay

Colony-forming assays were performed using methocult methylcellulose-based medium (StemCell Technologies). A total of 2×104 BM cells and 2×105 spleen cells were plated in 1 mL of methylcellulose medium (M3334 and M3434) in a 35-mm culture dish (StemCell Technologies). Cultures were incubated at 37°C in 5% CO2. BMU-E on methylcellulose medium M3434 was counted after 7 days.

Cell culture and purification

BM cells and splenocytes were isolated and cultured at a concentration of 5×106 cells/mL in AIM-V medium at 37°C and 5% CO2. Cells were harvested at 18 hours and Ter119+ erythroblast populations purified by magnetic bead separation using the EasySep Biotin positive selection kit (StemCell Technologies) according to the manufacturer’s instructions.

Genotyping and PCR analysis

Animals were genotyped by PCR. DNA extraction and PCR analysis were as described,17 using approximately 0.5 μg genomic DNA as template. Primers are as follows: For mutated Dhh allele, Dhhneo forward, GCCATGTCGGGATGCGTG; reverse, CCAGGAAGACGACGACGT- GCGTG; and wild-type (WT), Dhh forward, ATCCACGTACGTTG- CAAAGC, and reverse, GTTCAGAGAAGCAGGAC.

Quantitative RT-PCR

RNA extraction and cDNA synthesis were as described.33 One primer for each pair was designed to span exon-exon boundaries to avoid amplification of genomic DNA. The following primers were used: Gapdh forward, CCTGGAAACCTGTGCAATG; reverse, AGAGTGGGATGTC- TGCTTGAATC; Smo forward, TTCTTCAACAGGCTGAT; reverse, CGTATGGCTTCTATGAG; and Ptch forward, TGCTTCTCAGTTCTCACT; reverse, CCACAACTTGTTGTTGG. HPRT and Glu1 primers were as described.43
Results

Dhh and components of the Hh signaling pathway are expressed in adult spleen and bone marrow

Given that the involvement of Hh proteins in erythropoiesis is controversial, we investigated the possible function of Dhh in erythropoiesis. We therefore first considered whether Dhh is expressed in the adult spleen and BM, comparing expression between WT and Dhh\(^{-/-}\) spleen and bone marrow. We found Dhh transcription, by quantitative RT-PCR, in WT spleen, but not Dhh\(^{-/-}\) spleen, and we were also able to detect Dhh transcription in WT BM (Figure 1A). This is consistent with recent reports that Dhh is expressed in stromal cells in the BM, and by nonhematopoietic cells of the spleen stroma.\(^{35,44}\)

As Dhh is expressed in tissues where erythropoiesis occurs, we tested whether developing erythrocyte-lineage cells express components of the Hh signaling pathway. We stained the 4 erythrocyte-committed erythroblast populations, defined by Ter119 and CD71 expression, with antibodies directed against the Hh-signal transduction molecule Smo and the cell surface Hh-receptor Ptc (Figure 1B). In cells isolated from both spleen and BM, we found highest Smo expression on the most immature erythroblast population I (Ter119\(^{med}\)CD71\(^{hi}\)), with gradual reduction in cell surface expression in each subsequent population, so that population IV did not express detectable cell-surface Smo. In contrast, cell surface expression of Ptc was detectable on all erythroblast populations, consistent with its potential function as an Hh-sequestering protein.

To assess expression of the Hh target genes/signaling genes in erythroblast populations, we sorted populations I to III from BM
expression peaks at a later stage.2,31 Earliest progenitors express highest levels of Smo, but observed in the later population III. This pattern of expression is Hh-responsive target gene I and III. Interestingly, we found highest expression of the population I, and reduced but detectable expression in populations II and III, with highest expression in population I, and reduced but detectable expression in populations II and III. Interestingly, we found highest expression of the Hh-responsive target gene Gli1 in the later population III. The fact that Smo and Ptc1 expression are highest in population I suggests that these cells transduce the strongest Hh signal. Gli1 is not required to initiate the Hh signal but is up-regulated in response to it, consistent with the fact that the highest level Gli1 expression was observed in the later population III. This pattern of expression is similar to that observed during thymocyte development, where the earliest progenitors express highest levels of Smo, but Gli1 expression peaks at a later stage.2,31

We did not detect Dhh expression in erythroblast populations I to III from either spleen or BM (data not shown), and a previous study has located Dhh protein expression to spleen stroma by immunohistochemistry.35 To confirm this, we prepared RNA from splenic rudiment (stroma) and carried out quantitative RT-PCR. Transcription of Dhh was detected in WT spleen stroma, but not stroma from Dhh−/− spleen (Figure 1D).

**Erythroblasts are Dhh-responsive in vitro and ex vivo**

To verify that erythroblasts are capable of transducing a Dhh signal, we treated WT BM for 18 hours with recombinant (r) Dhh, neutralizing anti-Hh mAb 5E1, both treatments together, or isotype control mAb. After culture, cells were stained with anti-CD71 and anti-Ter119 to confirm that the short treatment had not changed the relative distribution of the subsets (top panel dot plots), and with PI to confirm that survival/cell cycle status was not affected (middle panel, histograms) in erythroblasts. Erythroblasts II to IV were purified from each culture by magnetic bead purification for Ter119+ cells, and RNA prepared for quantitative RT-PCR analysis of Gli1 expression (bar chart). (B) Erythroblast population II was sorted from magnetic bead lymphocyte-depleted cells from BM (left dot plot) and spleen (right dot plot). Gates used for sorting are shown. Bar chart represents Gli1 expression, measured by quantitative RT-PCR, on RNA prepared from population II sorted from WT (filled bars) and Dhh−/− (open bars) BM and spleen.

and spleen and performed quantitative RT-PCR for analysis of transcription of Ptc1, Smo, and Gli1 (Figure 1C). We did not carry out this analysis on population IV because the RNA isolated from these cells was of poor quality, as the nucleus is shrinking and will be shed. Transcription of Smo was highest in population I, and down-regulated in populations II and III, in cells sorted from both BM and spleen. Transcription of Ptc1, the cell surface receptor for Hh proteins, mirrored that of Smo, with highest expression in population I, and reduced but detectable expression in populations II and III. Interestingly, we found highest expression of the Hh-responsive target gene Gli1 in the later population III. The fact that Smo and Ptc1 expression are highest in population I suggests that these cells transduce the strongest Hh signal. Gli1 is not required to initiate the Hh signal but is up-regulated in response to it, consistent with the fact that the highest level Gli1 expression was observed in the later population III. This pattern of expression is similar to that observed during thymocyte development, where the earliest progenitors express highest levels of Smo, but Gli1 expression peaks at a later stage.2,31

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we analyzed the cultures for CD71 and Ter119 expression, and cell cycle/survival status. Viability was good, and we found no difference between the conditions in relative composition of the erythroblast subsets, cell cycle, or survival status after culture (Figure 2A), confirming that it was reasonable to carry out the Gli1 expression studies on the Ter119+ population. Treatment with rDhh increased expression of the Hh-target gene Gli1, whereas neutralization of Hh by 5E1 treatment down-regulated Gli1 transcription. The addition of rDhh to 5E1 treatment partially recovered Gli1 transcription, confirming specificity of the reagents, whereas treatment with an isotype-control antibody did not significantly affect Gli1 transcription (Figure 2A).

To assess the impact of Dhh on Hh signal transduction in erythroblasts ex vivo, we sorted erythroblast population II from WT and Dhh−/− BM and spleen and again measured transcription of the Hh-target gene Gli1 (Figure 2B). Expression of Gli1 was approximately 6-fold higher in WT erythroblasts compared with their Dhh−/− counterparts in spleen, and approximately 2.25-fold higher in WT erythroblasts compared with Dhh−/− in BM, indicating that Dhh signal transduction is active in developing WT erythroblasts and accounts for most Hh-dependent transcription in these cells. The fact that some Gli1 expression was detectable in the Dhh−/− cells indicated that erythroblasts were also transducing signals from either Ihh or Shh in both BM and spleen, hence the remaining Gli1 expression.

Taken together, these experiments show that developing erythroblasts undergo active Dhh signaling during their differentiation in the spleen and BM.

**Abnormal erythropoiesis in Dhh−/− mice**

Given that Dhh is expressed at sites of erythropoiesis and differentiating cells of the erythroid lineage transduce Hh signals, we asked if Dhh plays a role in the regulation of erythropoiesis, by analysis of Dhh−/− mice. We found that the spleen of Dhh−/− mice...
was larger than that of WT littermates (Figure 3A). There was no significant difference in total red blood cell (RBC) counts in Dhh−/− blood compared with WT, but the number of reticulocytes in the blood was significantly increased (Figure 3B-C). In the spleen, there was an increase in each population of erythroblasts (populations I to IV; Figure 3D). In the BM, we found a statistically significant increase in the proportions of erythroblast populations III and IV (Figure 3D). The increase in reticulocytes in the Dhh−/− blood and in erythroblast populations in the Dhh−/− BM and spleen indicated that Dhh is a negative regulator of erythropoiesis. There are several possible mechanisms that might contribute to, or account for, the increased erythropoiesis in Dhh−/− mice. First, the absence of Dhh might increase the proliferation of the erythroblast subsets. Second, it might influence the frequency of the precursors that give rise to them. Third, the absence of Dhh might increase the rate of erythroblast differentiation between the different stages to produce more reticulocytes.

To distinguish between these possibilities, we first assessed the cell-cycle status of the erythroblast populations by PI staining of Ter119+ cells isolated from Dhh−/− and WT spleen and BM. There was no significant difference in the proportion of cells in S + G2/M between Dhh−/− and WT in either spleen or BM (Figure 3E). These experiments indicated that Dhh does not act as a negative regulator of proliferation of erythroblast cells but more likely regulates an earlier precursor and/or the rate of differentiation along the erythrocyte lineage.

We therefore tested the ability of progenitors from Dhh−/− and WT spleen and BM to differentiate along the erythroid lineage in vitro, by assessment of their ability to form BFU. We found a statistically significant increase in BFU-Es in both BM and spleen from Dhh−/− compared with WT (Figure 4A). These data show that Dhh is a negative regulator of erythropoiesis, so we examined its influence on earlier hematopoietic populations. We found no difference in the relative proportions of
LSK stem cells, but the population of Sca-1^-c-kit^+ progenitors was increased in Dhh^-/- BM compared with WT (Figure 4B). When we gated on the progenitor population and subdivided it by expression of CD34 and FcγRII/III, we found that the proportion of the earlier CMP was increased in Dhh^-/- compared with WT. There was also a statistically significant increase in the proportion of MEP in the Dhh^-/- BM compared with WT and a concomitant decrease in the proportion of GMP. Thus, the ratio of MEP to GMP was statistically increased in the Dhh^-/- compared with WT (P = .05).}

**Figure 4. Dhh influences early erythropoiesis and progenitor differentiation.** (A) Scatter plot represents BFU assays, carried out on BM and spleen, from WT (filled squares) and Dhh^-/- (open squares). The mean for each group is indicated with a line. The increase in mean number of colonies in Dhh^-/- compared with WT is statistically significant for both BM (P = .05) and spleen (P = .032). (B) Dot plots represent analysis of LSK stem cells and Sca-1^-c-kit^+ progenitors in WT (left) and Dhh^-/- (right) BM, staining with antibodies against Sca-1 and c-kit, after exclusion of Lin^- cells. The bold region represents the gating used to analyze the Sca-1^-c-kit^+ progenitor population. Contour plots represent the subdivision of the progenitor population from WT (left) and Dhh^-/- (right), staining against CD34 and FcγRII/III, into CMP, GMP, and MEP. The percentage of progenitors in each subset and the regions used for their definition are shown. Top bar chart represents the mean percentage of Lin^- cells that are LSK (Sca-1^-c-kit^+), and progenitor (Sca-1^-c-kit^+) in WT (filled bars) and Dhh^-/- (open bars) BM. The increase in the mean percentage of progenitors in the Dhh^-/- compared with WT is statistically significant (P = .019). Middle bar chart represents the percentage of the progenitor population that is CMP, GMP, or MEP, as defined by CD34 and FcγRII/III expression, in WT (filled bars) and Dhh^-/- (open bars). The difference in mean proportion is statistically significant between WT and Dhh^-/- for CMP (P = .05), GMP (P = .0006), and MEP (P = .005). Bottom bar chart represents the mean progenitor ratio in WT (filled bars) and Dhh^-/- (open bars). The difference in mean ratios between WT and Dhh^-/- is statistically significant for MEP/GMP (P = .009) and CMP/GMP (P = .024), but not for CMP/MEP. The cartoon represents a summary of the effects of Dhh deficiency at this stage of differentiation. Absence of Dhh, reduced differentiation from CMP to GMP, and the GMP population are reduced. The CMP population is increased, and differentiation to MEP is favored. (C) Dot plot represents staining against Gr-1 and Mac-1 on BM cells from WT (left) and Dhh^-/- (right). The percentage of cells in each quadrant is shown. Bar chart represents the mean percentage of BM cells that are macrophages (Mac-1^-Gr-1^+) and granulocytes (Mac-1^-Gr-1^+) in WT (filled bars) and Dhh^-/- (open bars). The reduction in the mean percentage of granulocytes in Dhh^-/- compared with WT is statistically significant (P = .04).
Dhh is a negative regulator of stress-induced erythropoiesis in the spleen

Under conditions of erythropoietic stress, the production of erythrocytes is increased, and the major site of erythropoiesis moves to the spleen. To investigate the function of Dhh specifically in stress-induced erythropoiesis in the spleen, we treated Dhh−/− and WT mice with phenylhydrazine (PHZ) to cause anemia, and followed the kinetics of recovery of RBC counts and reticulocytes in the blood (Figure 6A) and erythroblast populations in the spleen and BM (Figure 6B). The Dhh−/− mice recovered more quickly than WT, and RBC counts had returned to normal in both by day 14 (Figure 6). Five days after treatment, the Dhh−/− spleen was larger than WT (Figure 6A). Erythropoiesis was strongly induced in both Dhh−/− and WT spleen, with an increase in erythroblast population II from less than 10% in untreated animals (Figure 3) to more than 50% at day 5 after PHZ treatment (Figure 6B). Interestingly, we also observed an increase in erythropoiesis in the BM after PHZ treatment in the Dhh−/− mice. At day 5 after treatment, the proportion of erythroblast population II was increased to 47% in the Dhh−/− BM, from approximately 27% in untreated Dhh−/− controls (Figures 3D and 6B). Seven days after treatment, the RBC count and reticulocyte count were significantly increased in the Dhh−/− blood compared with WT, although the proportion of reticulocytes was equivalent. Nine days after treatment, the Dhh−/− spleen was smaller than WT, and the proportion of blood reticulocytes was equivalent. Nine days after treatment, the Dhh−/− spleen was smaller than WT, and the proportion of blood reticulocytes was equivalent. Nine days after treatment, the Dhh−/− spleen was smaller than WT, and the proportion of blood reticulocytes was equivalent. Nine days after treatment, the Dhh−/− spleen was smaller than WT, and the proportion of blood reticulocytes was equivalent. Nine days after treatment, the Dhh−/− spleen was smaller than WT, and the proportion of blood reticulocytes was equivalent. Nine days after treatment, the Dhh−/− spleen was smaller than WT, and the proportion of blood reticulocytes was equivalent. Nine days after treatment, the Dhh−/− spleen was smaller than WT, and the proportion of blood reticulocytes was equivalent.
cells (neutrophils) was significantly reduced in the Dhh\(^{-/-}\) BM compared with WT (Figure 6D).

**Dhh\(^{-/-}\) spleen contains more RP areas than WT, and histology returns to normal more quickly on recovery from stress-induced erythropoiesis**

Given that erythropoiesis was increased in Dhh\(^{-/-}\) spleen compared with WT, both in normal conditions and after induction of anemia, we tested whether Dhh\(^{-/-}\) spleen showed abnormal histology or a difference in the proportion of RP area, compared with WT. Hematoxinlin and eosin staining on paraffin-embedded longitudinal spleen sections revealed normal histology, and RP and WP areas in both WT and Dhh\(^{-/-}\) spleen (Figure 7A-B). We measured RP and WP surface area across the entire surface area of the central longitudinal spleen section and calculated the RP/WP ratio and the percentage of RP (Table 1). Consistent with the increased erythropoiesis in Dhh\(^{-/-}\) spleen, the ratio of RP/WP was increased from 1.4 in WT to 2.1 in Dhh\(^{-/-}\) spleen, and the percentage of RP increased from 59% in WT to 68% in Dhh\(^{-/-}\) spleen. We then compared the induction of RP during stress-induced erythropoiesis caused by anemia after PHZ treatment (Figure 7C-J). In both WT and Dhh\(^{-/-}\), the RP area was increased at 5 days after treatment to 84% and 83%, respectively (Figure 7C-D; Table 1). The RP area remained high at days 7 and 9 after treatment but resolved more quickly in the Dhh\(^{-/-}\) than in the WT (Figure 7I-J), consistent with the faster resolution of the proportion of reticulocytes in the blood in the Dhh\(^{-/-}\) (Figure 6A). Thus, on day 14 after treatment, the RP/WP ratio was reduced to 2.5 in Dhh\(^{-/-}\) spleen and 3.7 in WT spleen. The dynamics of RP induction and resolution therefore mirror the kinetics of erythrocyte differentiation in Dhh\(^{-/-}\) and WT spleen, both under normal conditions (Figure 7A-B) and during stress-induced anemia (Figure 7C-J).
Discussion

This analysis of Dhh mutant mice showed that Dhh is a negative regulator of differentiation of erythroid progenitors, as multiple stages of their development. Differentiation from CMP to GMP was decreased in Dhh−/− BM, indicating that Dhh is required for granulocyte/macrophage lineage differentiation. Interestingly, in the Dhh−/− BM, although the proportion of GMP was decreased, there was an increase in both CMP and MEP populations, so that in the absence of Dhh, erythroid differentiation was favored. Analysis of subsequent BFU-E and erythroblast populations revealed that Dhh negatively regulated multiple stages of erythroid lineage differentiation. In addition, after recovery from nonlethal radiation, and on induction of stress-induced anemia by PHZ treatment, differentiation in both spleen and BM was accelerated. We did not find evidence that absence of Dhh increased proliferation in the erythroblast populations, so the increased number of erythroblasts in the spleen and BM seemed to be the result of both the increase in early progenitors (CMP and MEP) and increased rate of differentiation.

The reduction in differentiation from CMP to GMP, mature granulocyte numbers, proportion of macrophages after irradiation, and of neutrophils after PHZ treatment, in the Dhh−/− BM is consistent with a recent study showing that Gli1 plays a role in myeloid differentiation. In that study, the influence of Gli1 deficiency on erythroblast differentiation was not investigated. It is, however, in contrast to recent reports that conditional deletion of Smo from hematopoietic lineage cells has no impact on any stage of hematopoiesis. The reason for this difference is not clear, although it has been suggested that noncanonical Gli1-activation might be involved in the regulation of hematopoiesis.

The impact of conditional deletion of Smo from hematopoietic cells has also been examined in stress-induced hematopoiesis in the spleen. Smo-deficient cells were found to be unable to respond to BMP4 and to recover normally after stress induction. Although this study would seem to contradict reports that Smo deficiency has no impact on hematopoiesis, it is possible that specific Smo-dependent mechanisms are important during the stress response in the spleen. In contrast, our data indicate that recovery and erythropoiesis in spleen and BM are accelerated in Dhh−/− mice. The reason for this discrepancy may lie in the fact that Smo is thought to be the essential nonredundant signal transduction component of the Hh signaling pathway, so Smo-deficient cells should be unable to transduce a Hh signal. Dhh, however, is one of 3 Hh family members, and both Ihh and Shh are also expressed in the spleen. Thus, although erythroblasts undergoing differentiation in the Dhh−/− spleen would have reduced Hh pathway activation, some Hh signal transduction was present (Figure 2), whereas Smo-deficient erythroblasts would be assumed to have none. Hh proteins can function as morphogens, signaling for distinct outcomes dependent on strength and duration of signal received. Thus, it is possible that reduction in Hh signal (by Dhh deficiency) could produce the distinct outcome of accelerating stress-induced erythropoiesis in the spleen, compared with Smo deficiency, which resulted in reduced stress-induced erythropoiesis. Indeed, in the thymus, several experimental systems have shown that reduction of Hh signal accelerated pre-TCR induced differentiation, although some Hh signal transduction was still required for differentiation.

Table 1. The ratio of red pulp/white pulp and percentage of red pulp in control and PHZ-treated WT and Dhh−/− spleen

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Red pulp and white pulp and entire surface area of longitudinal sections of paraffin-embedded spleen from WT and Dhh−/− mice, untreated (day 0), and at time points after PHZ treatment. Ratio of red pulp and white pulp and percentage of red pulp were quantified using ImageJ software.
In contrast to our finding that Dhh negatively regulates erythrocyte differentiation, Ihh has been shown to promote the earliest stages of hematopoiesis and vasculogenesis\(^13\) and to support definitive erythropoiesis\(^10\) in the mouse embryo. These opposing functions of Ihh and Dhh can be accounted for the different spatial and temporal expression patterns of the 2 genes, strength of signal transduced by each protein, and stage of differentiation on which they act, or by differences between embryonic and adult erythropoiesis. In our study, Dhh acted at multiple stages of erythrocyte differentiation from the LSK stage onwards, but we did not find an influence of Dhh deficiency on the LSK stem cells.

In conclusion, we have shown that Dhh signaling is a negative regulator of erythropoiesis, thus adding erythropoiesis to the very few functions currently ascribed to Dhh. This finding is of general importance to an understanding of erythropoiesis and may in the future have relevance to the treatment of human hematologic disease, including blood cancers and anemia.

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Authorship

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