Ferrata Storti Foundation

Haematologica 2021
Volume 106(2):464-473

Hemoglobin switching in mice carrying the *Klf1*^{Nan} variant

Anne Korporaal,[1] Nynke Gillemans,[1] Steven Heshusius,[1,2] Ileana Cantú,[1] Emile van den Akker,[2] Thamar B. van Dijk,[1] Marieke von Lindern[2] and Sjaak Philipsen[1]

[1]Erasmus MC Department of Cell Biology, Rotterdam and [2]Sanquin Research Department of Hematopoiesis, Amsterdam and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands

ABSTRACT

Haploinsufficiency for transcription factor KLF1 causes a variety of human erythroid phenotypes, such as the In(Lu) blood type, increased HbA2 levels, and hereditary persistence of fetal hemoglobin. Severe dominant congenital dyserythropoietic anemia IV (CDA-IV) (OMIM 613673) is associated with the KLF1 p.E325K variant. CDA-IV patients display ineffective erythropoiesis and hemolysis resulting in anemia, accompanied by persistently high levels of embryonic and fetal hemoglobin. The mouse Nan strain carries a variant in the orthologous residue, KLF1 p.E339D. *Klf1*^{Nan} causes dominant hemolytic anemia with many similarities to CDA-IV. Here we investigated the impact of *Klf1*^{Nan} on the developmental expression patterns of the endogenous α-like and β-like globins, and the human β-like globins carried on a *HBB* locus transgene. We observe that the switch from primitive, yolk sac-derived, erythropoiesis to definitive, fetal liver-derived, erythropoiesis is delayed in *Klf1*$^{wt/Nan}$ embryos. This is reflected in globin expression patterns measured between embryonic day 12.5 (E12.5) and E14.5. Cultured *Klf1*$^{wt/Nan}$ E12.5 fetal liver cells display growth- and differentiation defects. These defects likely contribute to the delayed appearance of definitive erythrocytes in the circulation of *Klf1*$^{wt/Nan}$ embryos. After E14.5, expression of the embryonic/fetal globin genes is silenced rapidly. In adult *Klf1*$^{wt/Nan}$ animals, silencing of the embryonic/fetal globin genes is impeded, but only minute amounts are expressed. Thus, in contrast to human KLF1 p.E325K, mouse KLF1 p.E339D does not lead to persistent high levels of embryonic/fetal globins. Our results support the notion that KLF1 affects gene expression in a variant-specific manner, highlighting the necessity to characterize KLF1 variant-specific phenotypes of patients in detail.

Introduction

KLF1 is an erythroid-specific transcription factor with diverse and essential roles during terminal erythroid differentiation.[1] Cloned from mouse erythroleukemia cells it was initially called **e**rythroid **K**rüppel-**l**ike **f**actor (EKLF) in honor of its erythroid-specific expression and DNA-binding domain.[2] This domain is composed of three Cys_2-His_2 zinc fingers similar to those found in the *Drosophila* Krüppel transcription factor. KLF1 is the founding member of the KLF branch of the 27-strong SP/KLF family.[3] Despite the fact that other SP/KLF factors such as SP1, SP3, KLF2, KLF3 and KLF8 are abundantly expressed in erythroid cells,[4,5] gene inactivation in mice demonstrated that KLF1 is essential for definitive erythropoiesis.[6,7] Specifically, activation of β-globin expression was strongly impaired leading to a severe β-thalassemia phenotype. Restoring globin chain imbalance with a human γ-globin transgene failed to rescue the embryonic lethality of KLF1 deficiency,[8] indicating that other KLF1 target genes contribute to the erythroid defects. This was confirmed by genome-wide expression analyses which established that KLF1 is involved in virtually every aspect of terminal erythroid differentiation.[9-12] In humans, the first *KLF1* variants reported were identified as the molecular basis of the rare blood type In(Lu).[13] In all cases one normal *KLF1* allele was present showing that, similar to *Klf1*$^{wt/ko}$ mice,[6,7] this is sufficient to sustain erythropoiesis. These

Correspondence:

SJAAK PHILIPSEN
j.philipsen@erasmusmc.nl

Received: October 4, 2019.

Accepted: January 23, 2020.

Pre-published: May 28, 2020.

https://doi.org/10.3324/haematol.2019.239830

observations were extended by the discovery that *KLF1* haploinsufficiency caused hereditary persistence of fetal hemoglobin (HPFH) in a Maltese pedigree.[14] To date, over 140 different KLF1 variants have been reported, and these have been linked to a broad range of hitherto unrelated human red cell disorders.[1] In Sardinia, Thailand and southern China the frequency of *KLF1* variants reaches endemic proportions, e.g., 1.25% in southern China.[15] In these populations, cases with compound heterozygosity for *KLF1* variants occur.[16-18] In such cases, one allele invariably carries a missense variant which retains partial activity. *KLF1* compound heterozygotes display more pronounced phenotypes, including HbF levels of >20%,[16-18] persistence of embryonic globins,[18] microcytic hypochromic anemia,[16,18] and pyruvate kinase deficiency.[18] One case of a *KLF1 null* neonate was reported.[19] This infant displayed severe non-spherocytic hemolytic anemia with elevated HbF levels (>70%). Thus, the vast majority of *KLF1* variants displays classical autosomal recessive inheritance, and *KLF1* haploinsufficiency is associated with mild erythroid phenotypes. The exception is a *KLF1* variant in which an ultra-conserved residue in the second zinc finger is affected. This KLF1 p.E325K variant causes congenital dyserythropoietic anemia type IV (CDA-IV; OMIM 613673).[20-22] CDA-IV patients suffer from severe hemolytic anemia, splenomegaly, elevated HbF, iron overload, and dyserythropoiesis. Notably, the mouse neonatal anemia (Nan) phenotype is caused by a variant of the orthologous residue in mouse KLF1 p.E339D.[23,24] *Klf1*^{Nan} displays semidominant inheritance. *Klf1*$^{wt/Nan}$ animals suffer from hemolytic anemia while *Klf1*$^{Nan/Nan}$ embryos die around embryonic day 10.5 (E10.5) due to failure of pri-mitive erythropoiesis.[23,24] This phenotype is more severe than that of *Klf1*$^{ko/ko}$ embryos which die around E14.5 due to failure of definitive erythropoiesis.[6,7] During definitive erythropoiesis KLF1^{Nan} is thought to exert a dominant-ne-gative effect on the function of wild-type KLF1.[23,24] In primitive erythropoiesis, KLF1 and KLF2 have compensatory roles[25] and the early lethality of *Klf1*$^{Nan/Nan}$ embryos could therefore be due to interference of KLF1^{Nan} with normal KLF2 function. In addition, KLF1^{Nan} leads to aberrant gene expression which exerts negative effects on erythropoiesis.[26,27]

In human *HBB* locus transgenic mice, the fetal *HBG1/2* genes are expressed highly in primitive erythrocytes and early definitive erythrocytes. Switching to expression of the adult *HBB* gene occurs in the fetal liver between E12.5 and E14.5.[28] Given the profound deregulation of embryonic and fetal globin genes in CDA-IV patients, we investigated expression of the α-like and β-like globins in *Klf1*$^{wt/Nan}$ mice carrying a single-copy human *HBB* locus transgene[29] at embryonic, fetal and adult stages of development.

Methods

Animals

All animal studies were approved by the Erasmus MC Animal Ethics Committee. Mouse strains used were *Klf1*$^{wt/Nan}$ (*C3H101H-Klf1*^{Nan}/H[30]) crossed with PAC8.1 mice carrying a single-copy human *HBB* locus transgene (*Tg(HBB)8.1Gvs*[29]). For details see the *Online Supplementary Materials and Methods*.

Culture of mouse erythroid progenitors

E12.5 fetal livers were disrupted and single-cell suspensions were cultured as described.[31] For details see the *Online Supplementary Materials and Methods*.

RNA isolation and RT-qPCR analyses

RNA was extracted using TRI reagent (Sigma-Aldrich). For details see the *Online Supplementary Materials and Methods*.

Flow cytometry analysis

Single-cell suspensions were washed twice with PBS and resuspended in FACS buffer (PBS containing 1% [w/v] bovine serum albumin, 2 mM EDTA). Approximately 10^6 cells were incubated for 30 minutes at room temperature with the appropriate antibodies. Data were acquired on a Fortessa instrument (BD Biosciences), and analyzed with FlowJo software v10 (Tree Star). For details see the *Online Supplementary Materials and Methods*.

Cell morphology

Cell morphology was analyzed using cytospins stained with May Grünwald-Giemsa (Medion Diagnostics) and O-dianisidine (Sigma-Aldrich).[32] Pictures were taken with a BX40 microscope (40x objective, NA 0.65) equipped with a DP50 CCD camera and Viewfinder Lite 1.0 acquisition software (all Olympus).

Statistical analysis

Statistical analysis of gene expression data was performed by using Mann-Whitney tests (GraphPad Prism). Excel 2010 was used to draw the graphs. Standard deviations and *P*-values <0.05 are displayed in the relevant figures (*).

Results

Expression patterns of the globin genes in *Klf1*$^{wt/Nan}$ mice

In order to assess the impact of KLF1^{Nan} on developmental regulation of globin gene expression, the *Klf1*$^{wt/Nan}$ strain[30] was crossed with PAC8.1 mice carrying a single-copy human *HBB* locus transgene.[29] RT-qPCR analysis was performed to determine α-like and β-like globin expression in *Klf1*$^{wt/wt::HBB}$ (control) and *Klf1*$^{wt/Nan::HBB}$ (*Klf1*$^{wt/Nan}$) embryos at E11.5, E12.5, E13.5, E14.5 and E16.5. Primer pairs were designed to amplify the mouse and human embryonic, fetal and adult globin mRNA specifically, aiming to minimize inter-globin and inter-species cross-reactivity. Of note, the human *HBG1* and *HBG2* genes, encoding Aγ- and Gγ-globin respectively, arose by a recent duplication event and expression of these genes is assessed by a single primer pair. The same is true for the mouse *Hba-a1/Hba-a2* genes, encoding α1- and α2-globin respectively, and *Hbb-b1/Hbb-b2* genes, encoding major- and minor-globin respectively (see the *Online Supplementary Information*). Thus, we measured expression of mouse α-like globins (ζ and α1/2), mouse β-like globins (εy, βh1 and β major/minor) and human β-like globins (ε, Aγ/Gγ, and β). For reasons of clarity we will refer to mouse α-like globins as mζ and mα, mouse β-like globins as mεy, mβh1 and mβ, and human β-like globins as hε, hγ, and hβ in the remainder of this paper.

Globin gene expression patterns during development

The first erythroid cells are derived extra-embryonically from the blood islands in the yolk sac. They enter the cir-

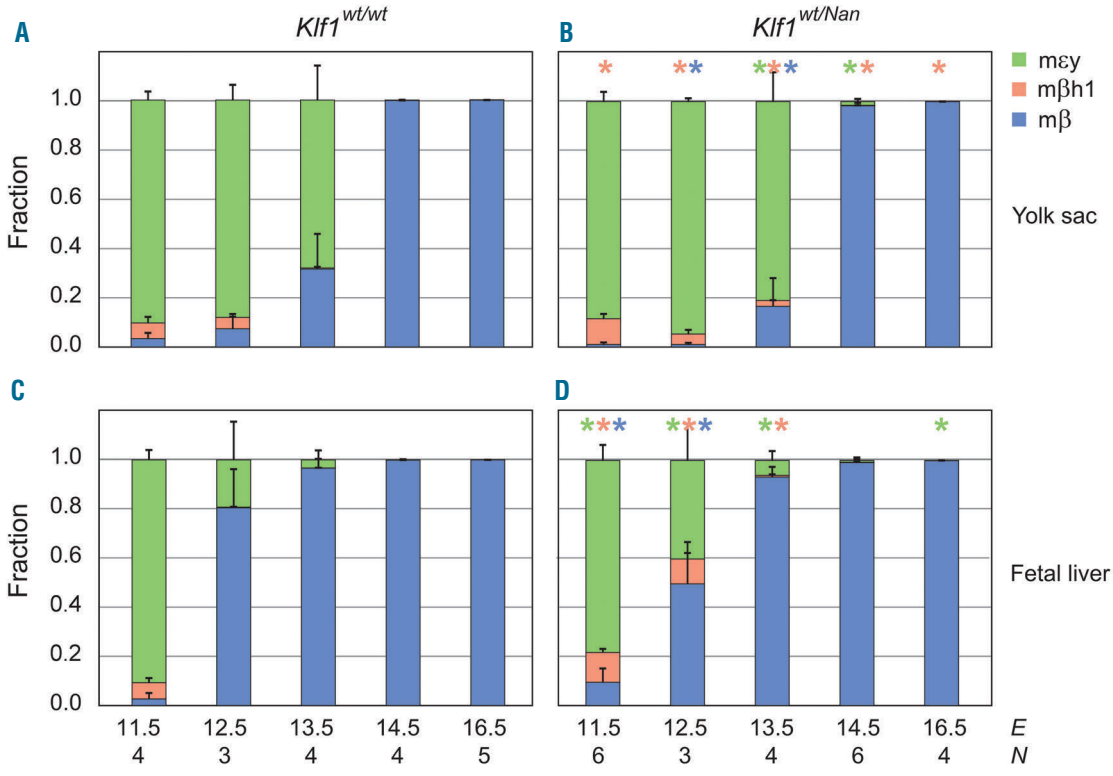

Figure 1. Developmental expression patterns of mouse β-like globins in control and *Klf1*$^{wt/Nan}$ embryos. Expression of mεy, mβh1 and mβ was determined by reverse transcriptase quantitatice PCR. Data are displayed as fraction of total mβ-like globin (mεy+mβh1+mβ) expression. Embryonic day (E) and number of embryos (N) are indicated. *P<0.05; error bars indicate standard deviations.

culation in the embryo properly after E8.5 as large nucleated cells referred to as primitive erythrocytes.[33] Expression of embryonic globins is a distinctive hallmark of primitive erythrocytes. The first intra-embryonically derived erythrocytes appear in the circulation around E12.5. These enucleated cells are generated in the fetal liver and referred to as definitive erythrocytes. They are characterized by predominant expression of adult globins; unlike the human *HBB* locus the mouse *Hbb* locus does not harbor fetal β-like globin genes. Nevertheless, mice carrying human *HBB* locus transgenes have been extensively used to study fetal-to-adult hemoglobin switching.[34]

In order to analyze the developmental dynamics of globin expression in *Klf1*$^{wt/Nan}$ embryos, we determined the globin expression profiles in RNA isolated from yolk sacs and fetal livers harvested between E11.5 and E16.5. Both the yolk sac and the fetal liver contain tissue cells plus circulating blood cells. First, we assessed expression of mβ-like globins. At E11.5, both the yolk sac and the fetal liver of control mice contained mainly mεε globin, reflecting the presence of primitive erythrocytes in the circulation and the fact that the fetal liver only just starts to produce definitive erythroid cells (Figure 1 A and C). In E12.5 fetal liver the production of large numbers of definitive erythroid cells was reflected in the dominant expression of mβ, while mεγ still constituted >90% of mβ-like globins in the yolk sac (Figure 1 A and C). At E13.5, expression of mβ-like globins detected in yolk sac was 70% mεε and 30% mβ, whereas >95% of fetal liver mβ-like globin was mβ. Finally, mainly mβ was detected in fetal liver and yolk sac from E14.5 onward (Figure 1 A and C). In comparison, *Klf1*$^{wt/Nan}$ yolk sac and fetal liver expressed a larger fraction of mβ at both E12.5 and day E13.5 (Figure 1 B and D). The increase of mβ expression over time is delayed in *Klf1*$^{wt/Nan}$ yolk sac and fetal liver (Figure 1B-D), indicating a delayed shift in the expression of primitive to definitive mβ-like globins. Next, we investigated whether this delay is specific for the *Hbb* locus, or whether it also occurs in the *Hba* locus. Yolk sacs and fetal livers from E11.5 control embryos expressed mζ as the major α-like globin, with mα contributing 25-30% to total α-like globin expression (Figure 2 A and C). At E12.5 mα became the dominant α-like globin in fetal liver, mζ was gradually replaced in the yolk sac by mα at E12.5 and E13.5, with the major shift to mα expression completed by E14.5 (Figure 2A), corresponding with fetal liver output in circulation. A different pattern was observed in yolk sacs and fetal livers from *Klf1*$^{wt/Nan}$ embryos. Compared to the controls, at E11.5 the contribution of mζ was increased at the expense of mα (Figure 2B and D). At E12.5 and E13.5 there was no increase in mα globin expression in the yolk sac (Figure 2B), and the increase in expression of mα in fetal livers was reduced compared to control fetal livers (Figure 2B and D). Thus, *Klf1*$^{wt/Nan}$ affects the developmental expression patterns of the mouse α-like and β-like globins in a very similar manner, displaying a delayed switch to expression of the adult genes.

As CDA-IV patients display very high HbF levels,[20-22] we extended the observations on the mouse globins to the human β-like globin genes thus adding analysis of the developmental expression patterns of fetal-stage globin

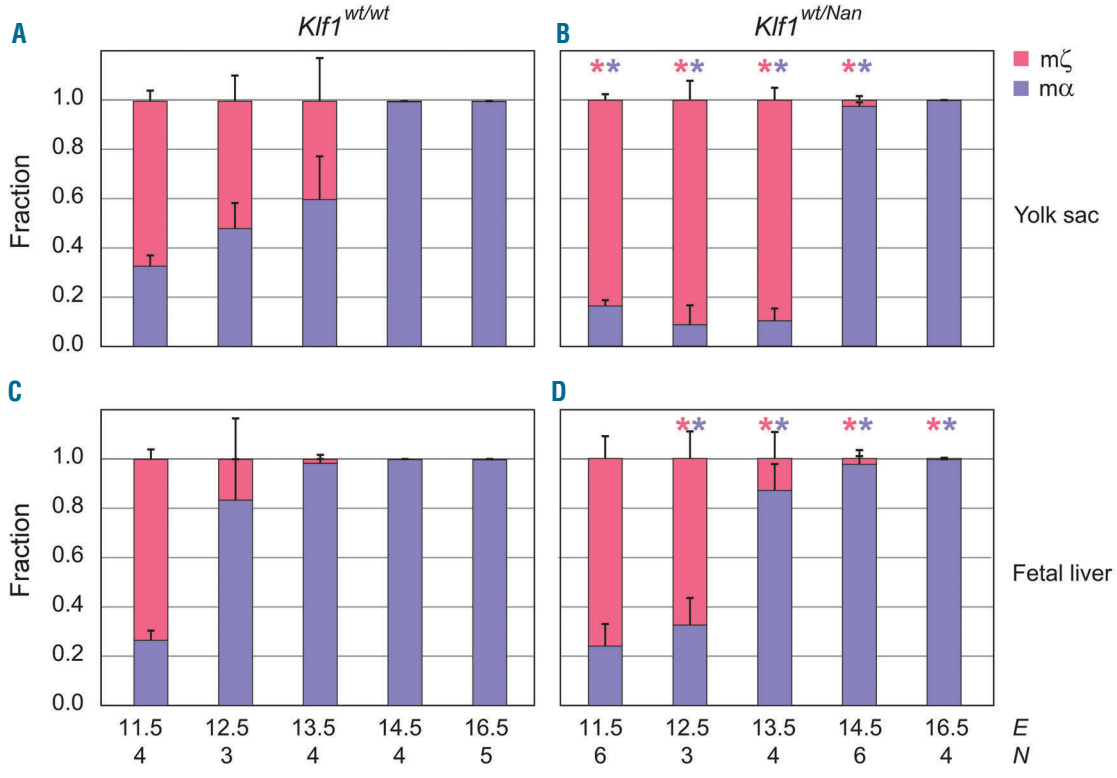

Figure 2. Developmental expression patterns of mouse α-like globins in control and *Klf1*$^{wt/Nan}$ embryos. Expression of mζ and mα was determined by reverse transcriptase quantitative PCR. Data are displayed as fraction of total mα-like globin (mζ+mα) expression. Embryonic day (E) and number of embryos (N) are indicated. *P<0.05; error bars indicate standard deviations.

genes. The E11.5 yolk sac and fetal liver of control embryos expressed very similar ratios of hε and hγ, while expression of hβ was very low (Figure 3A). Compared to the controls, E11.5 *Klf1*$^{wt/Nan}$ yolk sac and fetal liver displayed a small but significant shift to hε expression. Relatively increased hε expression was also observed in *Klf1wt/Nan* E12.5 yolk sac and fetal liver (Figure 3B and D).

Next to the difference in hε expression at E12.5, hβ made up ~75% of total hβ-like globins in control compared to ~43% in *Klf1*$^{wt/Nan}$ fetal liver. This apparent lag in switching to hβ expression was also observed in *Klf1*$^{wt/Nan}$ E13.5 fetal liver, and in E13.5 and E14.5 *Klf1*$^{wt/Nan}$ yolk sacs (Figure 3 B and D). In control yolk sacs, expression of hβ increased rapidly to ~35% at E13.5, with hβ remaining the most abundantly expressed hβ-like globin accounting for ~50% of the total output of the *HBB* locus (Figure 3A). Compared to control yolk sacs, expression of hγ in *Klf1*$^{wt/Nan}$ E13.5 yolk sacs was even higher at ~60%, with hβ expression also rapidly increasing but reaching a lower level of ~25% of hβ-like globins (Figure 3B). At E14.5, the hγ:hβ ratio shifted to 4:96 in control yolk sacs, while in *Klf1*$^{wt/Nan}$ yolk sacs this ratio remained higher at 28:72 (Figure 3A and B). At E16.5, hβ expression accounted for >97% of total hβ-like globin in all yolk sacs and fetal livers, showing that hemoglobin switching had quantitatively proceeded to the adult profile in both genotypes (Figure 3). We conclude that expression of hε is maintained at higher levels in E11.5-E12.5 *Klf1*$^{wt/Nan}$ embryos. This is followed by a lag in switching to hβ expression, which favors expression of hγ, in E13.5-E14.5 *Klf1*$^{wt/Nan}$ embryos. Nevertheless, at E16.5 expression of hε and hγ has receded to <3% of total hβ-like globins.

Delayed appearance of definitive erythrocytes in the circulation of *Klf1*$^{wt/Nan}$ embryos

Having established that the shift from embryonic to fetal and adult globin expression is delayed in *Klf1*$^{wt/Nan}$ embryos, we investigated whether this could be due to a delay in embryonic development. The erythroid compartment changes dynamically during mouse development,[33] and any alterations in this dynamic change would be reflected in globin expression patterns. Primitive erythrocytes originate in the yolk sac and are the sole erythrocytes in the circulation until E12.5, when the first definitive erythrocytes are released from the fetal liver. As fetal liver erythropoiesis gathers momentum, the majority of cells in the circulation are definitive erythrocytes by E14.5. In contrast to primitive erythrocytes, definitive erythrocytes enucleate before they are released in the circulation. We used this characteristic to determine the contribution of primitive cells to the circulation by making cytospins of peripheral blood collected from E14.5 and E16.5 control and *Klf1*$^{wt/Nan}$ embryos. Compared to the controls, nucleated erythrocytes were more abundant in cytospins of *Klf1*$^{wt/Nan}$ blood. They were still easily detected in E16.5 cytospins of *Klf1*$^{wt/Nan}$ blood, while such cells were virtually absent in control samples (Figure 4A). In order to assess the switch from primitive to definitive erythropoiesis, E14.5 cytospins were split into early, mid and late of litter harvest (Figure 4B). Consistent with the previous results, we observed that the fraction of nucleated cells declined very rapidly at this stage of development, in the controls from ~0.34 at early E14.5 to ~0.01 at late E14.5, and in the *Klf1*$^{wt/Nan}$ samples from ~0.52 at early E14.5 to ~0.13 at late E14.5. Importantly, compared to the controls the fraction

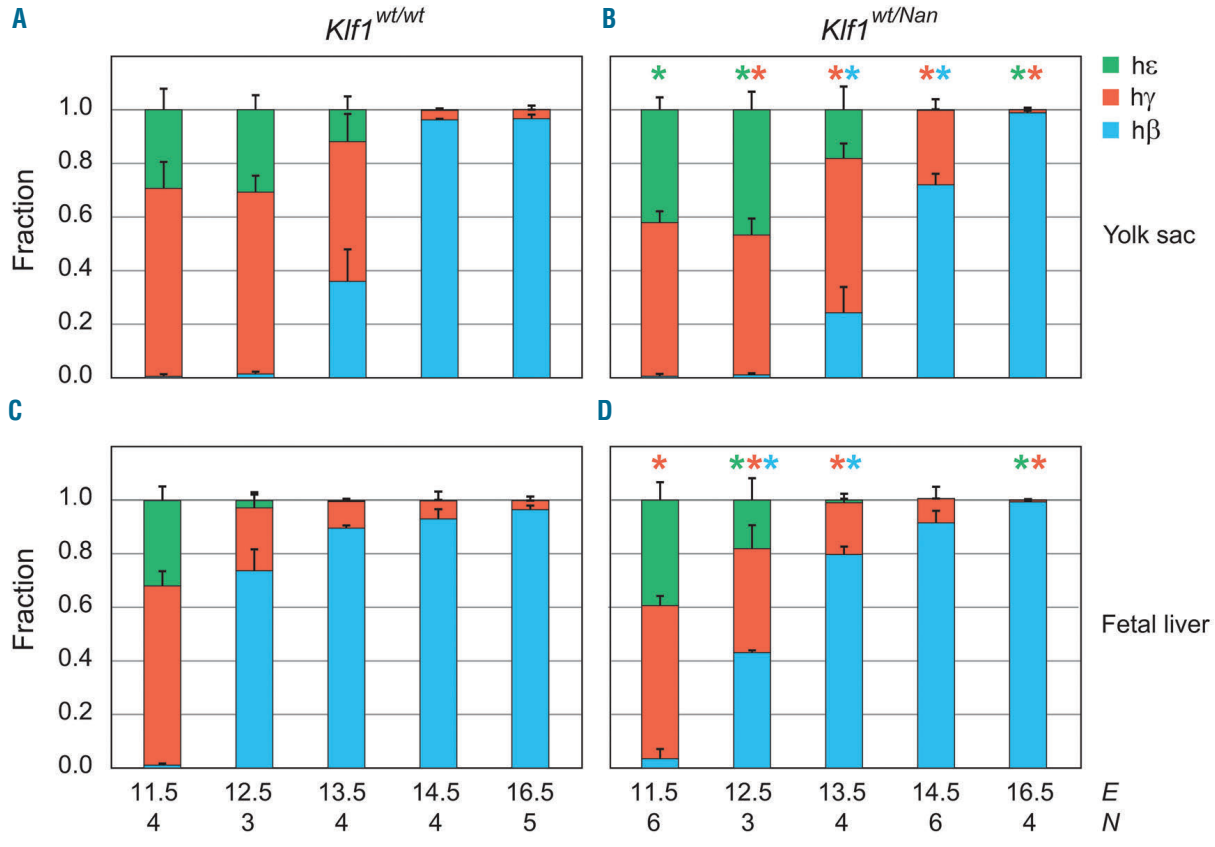

Figure 3. Developmental expression patterns of human β-like globins in control and *Klf1*$^{wt/Nan}$ embryos. Expression of hε, hγ and hβ was determined by reverse transcriptase quantitative PCR. Data are displayed as fraction of total hβ-like globin (hε+hγ+hβ) expression. Embryonic day (E) and number of embryos (N) are indicated. *P<0.05; error bars indicate standard deviations.

of nucleated cells remained significantly higher in the *Klf1*$^{wt/Nan}$ samples in all E14.5 litters.

Using a CASY cell counter, we determined the cell size distributions in E14.5 blood samples. In E14.5 control samples, the two peaks representing primitive (large) and definitive (small) cells are clearly separated (Figure 4C). In E14.5 *Klf1*$^{wt/Nan}$ samples, these two peaks are not clearly separated. The apparent continuum of cell sizes is in agreement with the rampant anisocytosis observed in the cytospins of E14.5 *Klf1*$^{wt/Nan}$ blood (Figure 4A). Finally, we sought to use flow cytometry as an orthogonal approach to determine the contribution of primitive erythrocytes to the circulation. In an attempt to better distinguish primitive from definitive erythrocytes, we performed flow cytometry using CD71 (transferrin receptor), Ter119, and CD9 (Tetraspanin) which is a marker for primitive erythrocytes.[35] Compared to the controls, expression of CD71 was slightly increased on *Klf1*$^{wt/Nan}$ E10.5 primitive cells (Figure 4D). This might indicate a delay in maturation, similar to what has been proposed for *Klf1*$^{wt/ko}$ reticulocytes.[36] Expression of Ter119 was virtually absent in E10.5 *Klf1*$^{wt/Nan}$ erythrocytes, while CD9 expression was strongly reduced (Figure 4D). At E14.5, CD9 was unable to distinguish primitive from definitive erythrocytes in *Klf1*$^{wt/Nan}$ blood, in contrast to E14.5 *Klf1*$^{wt/Nan}$ blood in which a distinct fraction of CD9^{+} primitive cells was observed (Figure 4D, arrow). Collectively, we conclude that the contribution of primitive erythrocytes to the circulation of *Klf1*$^{wt/Nan}$ embryos cannot be determined by flow cytometry using CD71, Ter119 and CD9 as markers. Despite these technical limitations, the analysis of blood samples is consistent with the notion that, compared to control embryos, the contribution of primitive erythrocytes to the pool of circulating erythrocytes in *Klf1*$^{wt/Nan}$ embryos is extended during development.

Klf1$^{wt/Nan}$ erythroblasts display impaired proliferation and differentiation

The delays in hemoglobin switching and appearance of definitive erythrocytes in the circulation of *Klf1*$^{wt/Nan}$ embryos suggest that the production of definitive cells in the fetal liver might be affected by impaired proliferation or differentiation. Since KLF1 is critically involved in regulation of the erythroid cell cycle,[11,37,38] central to both these processes, we cultured primary cells derived from E12.5 fetal livers to assess the proliferative capacity of *Klf1*$^{wt/Nan}$ erythroblasts. RT-qPCR analysis at day 6 of culture showed deregulated expression of the embryonic/fetal globin genes in the *Klf1*$^{wt/Nan}$ cells compared to the controls, demonstrating that this aspect of the phenotype is maintained in the culture system (*Online Supplementary Table S1*). We have previously shown that *Klf1*$^{ko/ko}$ E12.5 fetal liver cells expand well when grown under selfrenewal conditions.[9] In contrast, *Klf1*$^{wt/Nan}$ erythroblasts from E12.5 fetal liver expanded very poorly under these growth conditions (Figure 5A). Up to day 3-4 of culture, *Klf1*$^{wt/Nan}$ fetal liver cells expanded similar to those derived from control embryos. After day 4, expansion of the *Klf1*$^{wt/Nan}$ cultures

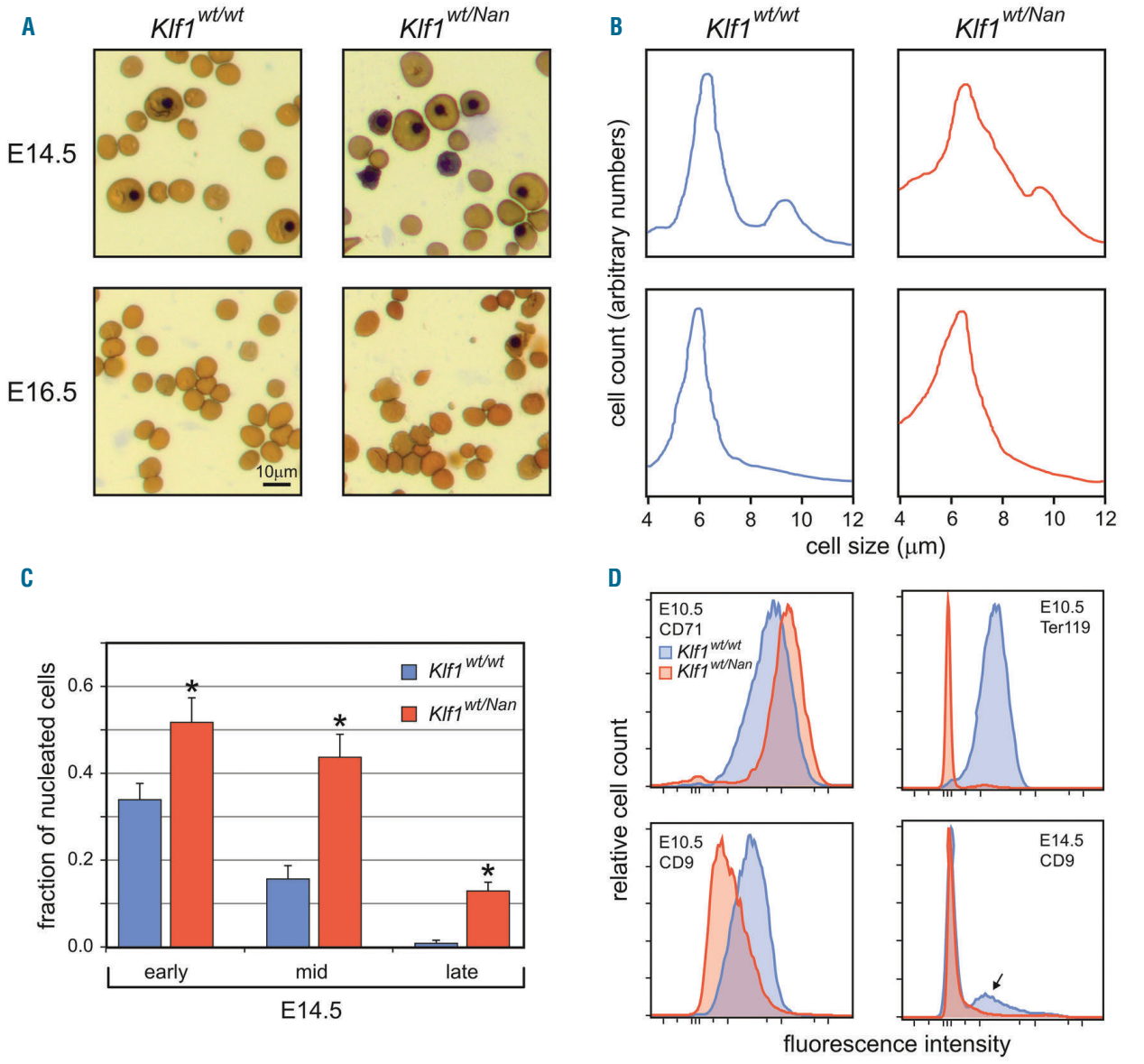

Figure 4. Analysis of blood from control and *Klf1*$^{wt/Nan}$ embryos. (A) Cytospins of E14.5 and E16.5 blood isolated from control (*Klf1*$^{wt/wt}$) and *Klf1*$^{wt/Nan}$ embryos, stained with dianisidine and histological dyes.[32] (B) Fraction of nucleated cells in blood isolated from control (*Klf1*$^{wt/wt}$) and *Klf1*$^{wt/Nan}$ littermates at early, mid and late E14.5. *P<0.05; error bars indicate standard deviations. N=3 for each genotype at each developmental time point. (C) Cell size analysis of E14.5 and E16.5 blood isolated from control (*Klf1*$^{wt/wt}$) and *Klf1*$^{wt/Nan}$ embryos, obtained with a CASY cell counter. (D) Histograms of flow cytometry analysis of blood isolated at E10.5 and E14.5 from control (*Klf1*$^{wt/wt}$) and *Klf1*$^{wt/Nan}$ embryos. Arrow indicates CD9^{+} primitive erythrocytes in *Klf1*$^{wt/wt}$ E14.5 blood.

slowed down and the percentage of smaller cells increased, suggesting spontaneous differentiation. Consistent with previously reported RT-qPCR data of *Klf1*$^{wt/Nan}$ fetal liver RNA,[24] expression of cell cycle regulators *E2F2, E2F4,* and *P18*, all known *KLF1* target genes,[11,37,38] was downregulated in *Klf1*$^{wt/Nan}$ cells compared to the controls, while expression of *P21* was unchanged (Figure 5B).

Next, the cells were switched to differentiation medium at day 6. During differentiation the control cells, but not the *Klf1*$^{wt/Nan}$ cells, displayed the characteristic differenti-ation divisions, i.e., the cell number increased while cell size decreased (Figure 5 C and D). Cytospins taken at day 2 of differentiation revealed many mature, enucleated and hemoglobinised cells in the control cultures (Figure 5Ea). Rare macrophages still present in the cultures were surrounded by healthy maturing erythroblasts (Figure 5Eb). Macrophage inclusions resembled nuclei, presumably resulting from phagocytosis of pyrenocytes (expelled erythroid nuclei[33]). In contrast, the *Klf1*$^{wt/Nan}$ cultures showed few enucleated cells and the cells displayed much larger nuclei (Figure 5Ec). Macrophages were not surrounded by enucleating erythroblasts, but appeared to engulf the entire erythroblast (Figure 5Ed). Combined these observations suggest that *Klf1*$^{wt/Nan}$ erythroblasts are impaired in both proliferation and differentiation. It is known that KLF1 blocks progression to myeloid lineages.[39] In the *Klf1*$^{wt/Nan}$ cultures we observed cells with morphological features of megakaryocytes (Figure 5Ee). Flow cytometry analysis of the cultured cells at day 9 revealed that control cultures were essentially free of non-erythroid cells, while *Klf1*$^{wt/Nan}$ cultures displayed pan-

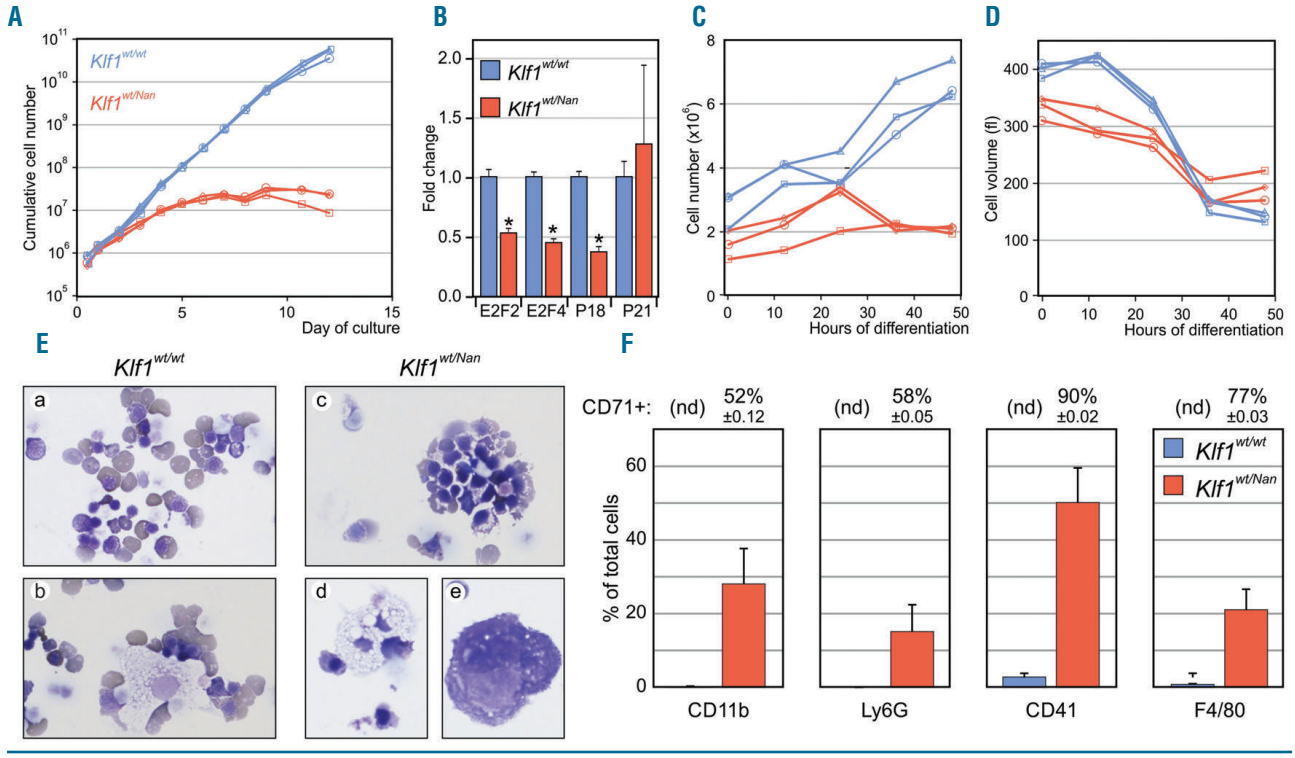

Figure 5. Erythroid progenitor cultures of control and *Klf1*$^{wt/Nan}$ embryonic day 12.5 fetal liver cells. (A) Growth curves of primary erythroid progenitors cultured from control (*Klf1*$^{wt/wt}$) and *Klf1*$^{wt/Nan}$ E12.5 fetal livers in StemPro medium containing SCF, dex and EPO. (B) Reverse transcriptase quantitative PCR analysis of cell cycle regulators in cultured erythroid progenitors (n=3 for each group). *P*-values<0.05; error bars indicate standard deviations. (C-D) Differentiation was induced on day 6, transferring the cells to StemPro medium supplemented with EPO and transferrin. Cell number (C) and cell volume (D) was recorded. (E) At day 2 of differentiation cells were centrifuged on glass slides, fixed and stained with dianisidine and histological dyes.[32] (F) Flow cytometry analysis of cells cultured from control (*Klf1*$^{wt/wt}$) and *Klf1*$^{wt/Nan}$ fetal livers at day 9 of culture. Cells were stained with antibodies indicated. The percentage of cells double-positive for the erythroid marker CD71 is shown on top, ± standard deviation. (nd): not detectable, due to the virtual absence of expression of myeloid markers on the control cells.

myeloid markers on 20-50% of the cells (Figure 5F). Of note, the majority of these cells were positive for the erythroid marker CD71. Collectively, these data suggest that compromised lineage fidelity, reduced proliferative capacity and impaired terminal differentiation all contribute to the delay in abundance of definitive erythrocytes in the circulation of *Klf1*$^{wt/Nan}$ embryos, thus bearing weight on the observed changes in globin expression during embryonic development.

Expression of globins in adult *Klf1*$^{wt/Nan}$ mice

The analysis of developmental expression patterns of the globins demonstrated that by E16.5 *Klf1*$^{wt/Nan}$ embryos had quantitatively switched to expression of the adult genes (Figures 1-3). However, it remains possible that the maintenance of embryonic/fetal globin silencing is perturbed in adult *Klf1*$^{wt/Nan}$ mice. Indeed, derepression of embryonic globin genes in the spleen of *Klf1*$^{wt/Nan}$ mice has been reported.[24] In order to investigate this further, we isolated RNA from spleen and bone marrow derived from control and *Klf1*$^{wt/Nan}$ mice. By RT-qPCR analysis we found that mζ and mβh1, but not mε, expression was increased between 35-800-fold in *Klf1*$^{wt/Nan}$ samples check comparison to control samples (Figure 6). For the human β-like globins, we observed 4-9-fold increased expression of hε and hγ. In quantitative terms, even in the case of the most highly expressed embryonic globin mζ, this amounted to less than 0.3% of total α-like globin. We conclude that maintenance of embryonic/fetal globin silencing is perturbed in the bone marrow and spleen of adult *Klf1*$^{wt/Nan}$ mice. Since the amount of embryonic/fetal globins produced remains below 0.3% of the total amount of globins, this is a qua-litative rather than a quantitative trait.

Discussion

In humans, reduced KLF1 activity has been associated with persistent expression of fetal hemoglobin in adults.[1,14] A severe phenotype of hemolytic anemia characterizes patients suffering from CDA-IV, caused by the p.E325K variant in the DNA binding domain of KLF1. In the *Klf1*$^{wt/Nan}$ mouse, the orthologous glutamic acid residue (p.E339) is changed. Biochemically, these amino acid substitutions are very different. In CDA-IV, the glutamic acid (E) is replaced by a basic amino acid (lysine, K) while in KLF1^{Nan} it is replaced by aspartic acid (D), an acidic amino acid. Despite these biochemically opposing properties, the erythroid phenotypes of CDA-IV patients and *Klf1*$^{wt/Nan}$ mice share many similarities. A hallmark of CDA-IV patients is that they maintain high expression levels of embryonic and fetal globins. In order to investigate whether this is also the case in *Klf1*$^{wt/Nan}$ mice, we surveyed expression of the α-like and β-like globins during development. Our main observations are summarized in the *Online Supplementary Figure S1*. We found that in *Klf1*$^{wt/Nan}$ embryos, switching from embryonic/fetal to adult globin genes is delayed in the endogenous *Hbb* and *Hba* loci, and in the single-copy human *HBB* locus trans-

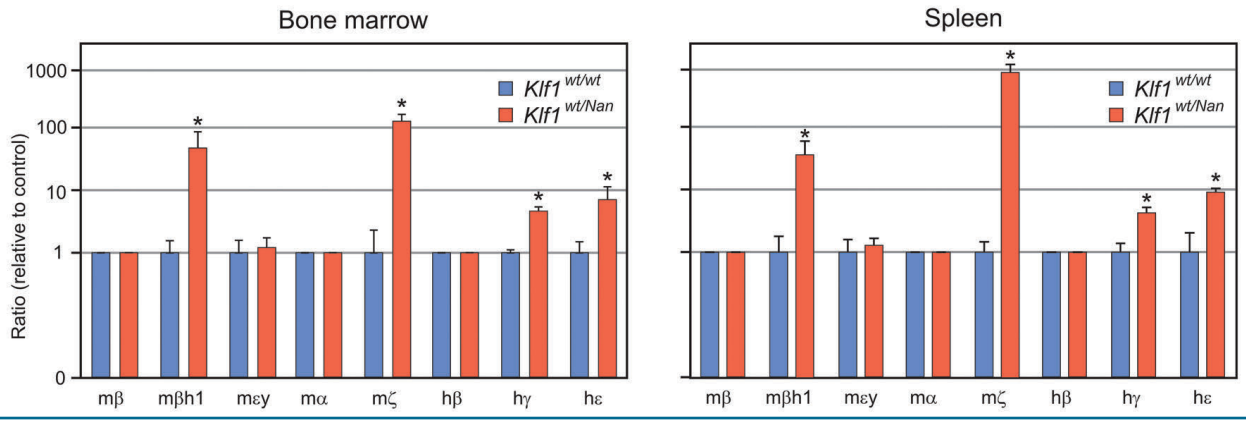

Figure 6. Expression of human and mouse globins in bone marrow and spleen of adult control and *Klf1*$^{wt/Nan}$ animals. Expression of mouse and human globins was determined by reverse transcriptase quantitative PCR. Expression ratios of individual globins in *Klf1*$^{wt/Nan}$ samples were calculated relative to those observed for the control samples (*Klf1*$^{wt/wt}$). Note logarithmic scale of the y-axis. N=3 for each group. *P<0.05; error bars indicate standard deviations.

gene. Two mechanisms may contribute to this phenomenon. Firstly, decreased proliferation and differentiation of fetal erythroblasts delays the replacement of primitive by definitive erythroid cells. Since we isolated RNA from populations of cells, the ratio of primitive/definitive cells will have an impact on the globin levels measured. Secondly, although by E16.5 adult globins are quantitatively the dominant globins in *Klf1*$^{wt/Nan}$ embryos, the embryonic and fetal globin genes retained expression in adult spleen and bone marrow. Qualitatively, this persistent expression is another phenotypic similarity with CDA-IV patients. However, quantitatively there is a major difference. While in CDA-IV patients HbF levels of up to 37% of total hemoglobin have been reported,[20] even the most highly expressed embryonic globin in adult *Klf1*$^{wt/Nan}$ mice, mζ, contributes only 0.3% to the total amount of α-like globins. KLF1 activates expression of BCL11A[14,40] and LRF,[41] two transcriptional repressors directly involved in hemoglobin switching.[42,43] RT-qPCR analysis of BCL11A and LRF expression indicates that reduced expression of these two factors contributes to the sustained expression of the embryonic/fetal genes in *Klf1*$^{wt/nan}$ erythroid cells (*Online Supplementary Figure S2*). We note that expression of BCL11A is also significantly reduced in *Klf1*$^{wt/ko}$ cells with little effect on expression of the embryonic/fetal genes.[36] Mechanistically, this suggests that the repressor proteins are expressed well above the critical threshold level in mice, and a reduction to 40-60% of normal expression would still be sufficient for quantitative silencing of the embryonic/fetal genes.

The impaired proliferation of E12.5 fetal liver-derived *Klf1*$^{wt/Nan}$ erythroblasts was initially surprising because a lack of KLF1 increased proliferation, likely due to impaired spontaneous differentiation.[9] Whereas erythropoiesis in *Klf1*$^{ko/ko}$ mice is severely affected during terminal differentiation, erythropoiesis in *Klf1*$^{wt/nan}$ mice is much less affected with respect to terminal differentiation. The presence of KLF1^{Nan} not only results in the reduced expression of KLF1 target genes, but also induces expression of genes not normally regulated by KLF1.[26,27,44] The combined effects of deregulation of canonical KLF1 target genes and ectopic gene expression likely underlie the observed lineage commitment infidelity and impaired proliferation and differentiation of *Klf1*$^{wt/nan}$ erythroblast cultures. Defective growth of erythroid progenitor cultures derived from a CDA-IV patient has been reported,[45,46] indicating that impaired proliferation of erythroid progenitors is another hallmark that CDA-IV patients and *Klf1*$^{wt/Nan}$ mice have in common. In contrast, adult *Klf1*$^{wt/Nan}$ mice expressed mainly adult-type globin genes, as opposed to adult CDA-IV patients who maintain expression of embryonic and fetal globins at substantial levels.[20-22,45]

Importantly, the dominant effect of KLF1^{Nan} is illustrated by comparison with *Klf1*$^{wt/ko}$ mice which do not display deregulated expression of mouse embryonic globins in E14.5 yolk sac and fetal liver and adult bone morrow[36] (*Online Supplementary Figure S3*). We found that the effects of KLF1^{Nan} on developmental regulation of globin expression are very consistent, but surprisingly subtle. KLF1^{Nan} affects the dynamics of progression from primitive to definitive erythropoiesis during mouse development. Compared to control embryos, definitive erythrocytes emerge at a later stage as the dominant cell type in the circulation of *Klf1*$^{wt/Nan}$ embryos. We propose that this is at least in part due to the reduced expansion and differentiation capacity of the fetal liver progenitors, since cultured *Klf1*$^{wt/Nan}$ E12.5 fetal liver cells display growth and differentiation defects. Consistent with impaired erythroid diffe-rentiation, we observed aberrant expression of erythroid flow cytometry markers (CD71, Ter119, CD9). Furthermore, we found misexpression of myeloid markers, in particular the megakaryocyte marker CD41, indicating that *Klf1*$^{wt/Nan}$ erythroid progenitors display lineage infidelity. This is akin to the previously reported aberrantly activated megakaryocyte program in *Klf1*$^{ko/ko}$ erythroid cells.[39,47]

Collectively, our data support the notion that KLF1^{CDA} and KLF1^{Nan} present with similar but also variant-specific phenotypes. Thus, our study further highlights the need to investigate the effects of individual KLF1 variants in detail.[1] The recently developed human adult erythroid progenitor cell lines HUDEP-2[48] and BEL-A[49] could be combined with CRISPR-mediated homology-directed recombination[50] to investigate the impact of individual human KLF1 variants on the molecular control of erythropoiesis, with a view to increase understanding of the broad spectrum of human red blood cell disorders caused by KLF1 variants.[1]

Disclosures

No conflicts of interests to disclose.

Contributions

AK, NG, IC, SH and EvdA performed experiments. AK, TBvD, SH, SP, EvdA and MvL analyzed data. MvL and SP conceived the study. AK, MvL and SP made the figures and wrote the paper. All authors reviewed the paper and agree with its contents.

Funding

Research in our laboratories was funded by the Landsteiner Foundation for Blood Transfusion Research (LSBR 1040 and 1627), the Netherlands Organization for Scientific Research (ZonMw TOP 40-00812-98-12128), the Netherlands Genomics Initiative (NGI Zenith 93511036), and EU fp7 Specific Cooperation Research Project THALAMOSS (306201).

References

1. Perkins A, Xu X, Higgs DR, et al. Kruppeling erythropoiesis: an unexpected broad spectrum of human red blood cell disorders due to KLF1 variants. Blood. 2016;127(15):1856-1862.
2. Miller IJ, Bieker JJ. A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. Mol Cell Biol. 1993;13(5):2776-2786.
3. Suske G, Bruford E, Philipsen S. Mammalian SP/KLF transcription factors: bring in the family. Genomics. 2005;85(5):551-556.
4. Eaton SA, Funnell AP, Sue N, Nicholas H, Pearson RC, Crossley M. A network of Kruppel-like Factors (Klfs). Klf8 is repressed by Klf3 and activated by Klf1 in vivo. J Biol Chem. 2008;283(40):26937-26947.
5. Zhang P, Basu P, Redmond LC, et al. A functional screen for Kruppel-like factors that regulate the human gamma-globin gene through the CACCC promoter element. Blood Cells Mol Dis. 2005;35(2):227-235.
6. Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosveld F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature. 1995;375(6529):316-318.
7. Perkins AC, Sharpe AH, Orkin SH. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. Nature. 1995;375(6529):318-322.
8. Perkins AC, Peterson KR, Stamatoyannopoulos G, Witkowska HE, Orkin SH. Fetal expression of a human Agamma globin transgene rescues globin chain imbalance but not hemolysis in EKLF null mouse embryos. Blood. 2000; 95(5):1827-1833.
9. Drissen R, von Lindern M, Kolbus A, et al. The erythroid phenotype of EKLF-null mice: defects in hemoglobin metabolism and membrane stability. Mol Cell Biol. 2005;25(12):5205-5214.
10. Hodge D, Coghill E, Keys J, et al. A global role for EKLF in definitive and primitive erythropoiesis. Blood. 2006;107(8):3359-3370.
11. Pilon AM, Arcasoy MO, Dressman HK, et al. Failure of terminal erythroid differentiation in EKLF-deficient mice is associated with cell cycle perturbation and reduced expression of E2F2. Mol Cell Biol. 2008; 28(24):7394-7401.
12. Tallack MR, Magor GW, Dartigues B, et al. Novel roles for KLF1 in erythropoiesis revealed by mRNA-seq. Genome Res. 2012;22(12):2385-2398.
13. Singleton BK, Burton NM, Green C, Brady RL, Anstee DJ. Mutations in EKLF/KLF1 form the molecular basis of the rare blood group In(Lu) phenotype. Blood. 2008; 112(5):2081-2088.
14. Borg J, Papadopoulos P, Georgitsi M, et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. Nat Genet. 2010;42(9):801-805.
15. Liu D, Zhang X, Yu L, et al. KLF1 mutations are relatively more common in a thalassemia endemic region and ameliorate the severity of beta-thalassemia. Blood. 2014;124(5):803-811.
16. Huang J, Zhang X, Liu D, et al. Compound heterozygosity for KLF1 mutations is associated with microcytic hypochromic anemia and increased fetal hemoglobin. Eur J Hum Genet. 2015;23(10):1341-1348.
17. Satta S, Perseu L, Moi P, et al. Compound heterozygosity for KLF1 mutations associated with remarkable increase of fetal hemoglobin and red cell protoporphyrin. Haematologica. 2011;96(5):767-770.
18. Viprakasit V, Ekwattanakit S, Riolueang S, et al. Mutations in Kruppel-like factor 1 cause transfusion-dependent hemolytic anemia and persistence of embryonic globin gene expression. Blood. 2014; 123(10):1586-1595.
19. Magor GW, Tallack MR, Gillinder KR, et al. KLF1-null neonates display hydrops fetalis and a deranged erythroid transcriptome. Blood. 2015;125(15):2405-2417.
20. Arnaud L, Saison C, Helias V, et al. A dominant mutation in the gene encoding the erythroid transcription factor KLF1 causes a congenital dyserythropoietic anemia. Am J Hum Genet. 2010;87(5):721-727.
21. de la Iglesia-Inigo S, Moreno-Carralero MI, Lemes-Castellano A, Molero-Labarta T, Mendez M, Moran-Jimenez MJ. A case of congenital dyserythropoietic anemia type IV. Clin Case Rep. 2017;5(3):248-252.
22. Jaffray JA, Mitchell WB, Gnanapragasam MN, et al. Erythroid transcription factor EKLF/KLF1 mutation causing congenital dyserythropoietic anemia type IV in a patient of Taiwanese origin: review of all reported cases and development of a clinical diagnostic paradigm. Blood Cells Mol Dis. 2013;51(2):71-75.
23. Heruth DP, Hawkins T, Logsdon DP, et al. Mutation in erythroid specific transcription factor KLF1 causes hereditary spherocytosis in the nan hemolytic anemia mouse model. Genomics. 2010;96(5):303-307.
24. Siatecka M, Sahr KE, Andersen SG, Mezei M, Bieker JJ, Peters LL. Severe anemia in the Nan mutant mouse caused by sequence-selective disruption of erythroid Kruppel-like factor. Proc Natl Acad Sci U S A. 2010; 107(34):15151-15156.
25. Basu P, Lung TK, Lemsaddek W, et al. EKLF and KLF2 have compensatory roles in embryonic beta-globin gene expression and primitive erythropoiesis. Blood. 2007; 110(9):3417-3425.
26. Nebor D, Graber JH, Ciciotte SL, et al. Mutant KLF1 in adult anemic Nan mice leads to profound transcriptome changes and disordered erythropoiesis. Sci Rep. 2018;8(1):12793.
27. Planutis A, Xue L, Trainor CD, et al. Neomorphic effects of the neonatal anemia (Nan-Eklf) mutation contribute to deficits throughout development. Development. 2017;144(3):430-440.
28. Strouboulis J, Dillon N, Grosveld F. Developmental regulation of a complete 70-kb human beta-globin locus in transgenic mice. Genes Dev. 1992;6(10):1857-1864.
29. de Krom M, van de Corput M, von Lindern M, Grosveld F, Strouboulis J. Stochastic patterns in globin gene expression are established prior to transcriptional activation and are clonally inherited. Mol Cell. 2002;9(6):1319-1326.
30. Lyon MF, Glenister PH, Loutit JF, Peters J. Dominant haemolytic anemia. Mouse News Letters. 1983;68:68.
31. Dolznig H, Kolbus A, Leberbauer C, et al. Expansion and differentiation of immature mouse and human hematopoietic progenitors. Methods Mol Med. 2005;105:323-344.
32. Beug H, Leutz A, Kahn P, Graf T. Ts mutants of E26 leukemia virus allow transformed myeloblasts, but not erythroblasts or fibroblasts, to differentiate at the non-permissive temperature. Cell. 1984;39(3 Pt 2):579-588.
33. Palis J. Primitive and definitive erythropoiesis in mammals. Front Physiol. 2014; 5:3.
34. Peterson KR. Hemoglobin switching: new insights. Curr Opin Hematol. 2003; 10(2):123-129.
35. Isern J, Fraser ST, He Z, Zhang H, Baron MH. Dose-dependent regulation of primitive erythroid maturation and identity by the transcription factor Eklf. Blood. 2010; 116(19):3972-3980.
36. Esteghamat F, Gillemans N, Bilic I, et al. Erythropoiesis and globin switching in compound Klf1::Bcl11a mutant mice. Blood. 2013;121(13):2553-2562.
37. Gnanapragasam MN, McGrath KE, Catherman S, Xue L, Palis J, Bieker JJ. EKLF/KLF1-regulated cell cycle exit is essential for erythroblast enucleation. Blood. 2016;128(12):1631-1641.
38. Tallack MR, Keys JR, Humbert PO, Perkins AC. EKLF/KLF1 controls cell cycle entry via direct regulation of E2f2. J Biol Chem. 2009;284(31):20966-20974.
39. Tallack MR, Perkins AC. Megakaryocyte-erythroid lineage promiscuity in EKLF null mouse blood. Haematologica. 2010; 95(1):144-147.
40. Zhou D, Liu K, Sun CW, Pawlik KM, Townes TM. KLF1 regulates BCL11A expression and gamma- to beta-globin gene switching. Nat Genet. 2010;42(9):742-744.
41. Norton LJ, Funnell APW, Burdach J, et al. KLF1 directly activates expression of the novel fetal globin repressor ZBTB7A/LRF in erythroid cells. Blood Adv. 2017;1(11):685-692.

42. Masuda T, Wang X, Maeda M, et al. Transcription factors LRF and BCL11A independently repress expression of fetal hemoglobin. Science. 2016;351(6270):285-289.
43. Sankaran VG, Xu J, Ragoczy T, et al. Developmental and species-divergent globin switching are driven by BCL11A. Nature. 2009;460(7259):1093-1097.
44. Gillinder KR, Ilsley MD, Nebor D, et al. Promiscuous DNA-binding of a mutant zinc finger protein corrupts the transcriptome and diminishes cell viability. Nucleic Acids Res. 2017;45(3):1130-1143.
45. Ravindranath Y, Johnson RM, Goyette G, Buck S, Gadgeel M, Gallagher PG. KLF1 E325K-associated congenital dyserythropoietic anemia type IV: insights into the variable clinical severity. J Pediatr Hematol Oncol. 2018;40(6):e405-e409.
46. Varricchio L, Planutis A, Manwani D, et al. Genetic disarray follows mutant KLF1-E325K expression in a congenital dyserythropoietic anemia patient. Haematologica. 2019;104(12):2372-2380.
47. Frontelo P, Manwani D, Galdass M, et al. Novel role for EKLF in megakaryocyte lineage commitment. Blood. 2007; 110(12):3871-3880.
48. Kurita R, Suda N, Sudo K, et al. Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells. PLoS One. 2013; 8(3):e59890.
49. Trakarnsanga K, Griffiths RE, Wilson MC, et al. An immortalized adult human erythroid line facilitates sustainable and scalable generation of functional red cells. Nat Commun. 2017;8:14750.
50. Mali P, Esvelt KM, Church GM. Cas9 as a versatile tool for engineering biology. Nat Methods. 2013;10(10):957-963.