Splenomegaly and Modified Erythropoiesis in KLF13$^{-/-}$ Mice*

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To study the function of the Krüppel-like transcription factor KLF13 in vivo, we generated mice with a disrupted Klf13 allele. Although Klf13$^{-/-}$ mice are viable, fewer mice were present at 3 weeks than predicted by Mendelian inheritance. Viable Klf13$^{-/-}$ mice had reduced numbers of circulating erythrocytes and a larger spleen. The spleen contained an increased number of Ter119medCD71hi, Ter119hiCD71hi, and Ter119hiCD71med cells but not Ter119hiCD71* cells, indicating an increase in less mature erythroblasts. A higher proportion of the Ter119medCD71hi cells were proliferating, indicating that the mice were under a degree of erythropoietic stress. These data indicate that KLF13 is involved in the normal control of erythropoiesis.

Erythrocytes differentiate from multi-potential hematopoietic stem cells through a series of intermediates. The first committed erythrocyte precursors are the burst-forming unit cells (BFU-E) which differentiate into colony-forming units (CFU-E) and then to proerythroblasts (1). As these cells differentiate further through the basophilic, polychromatophilic, and orthochromatic erythroblast stages, the nucleus shrinks and is finally shed as the cells become reticulocytes before becoming erythrocytes (2). During these later stages of differentiation, the cells lose expression of CD71 and gain the expression of ter119 so that these cell surface markers can be used to differentiate the different cell stages (3). The process of erythrocyte differentiation is controlled by a number of different transcription factors and regulated such that under normal conditions sufficient erythrocytes are produced predominantly by the bone marrow, but under conditions of erythropoietic stress (e.g. hypoxia) the number of erythrocytes can be increased; this occurs predominantly in the spleen (4, 5).

Analysis of the transcriptional program controlling erythroblast differentiation has identified a number of different transcription factors that are critical to the normal development of erythrocytes (1, 2, 6). The best studied of these factors regulating erythroid differentiation is GATA-1, a zinc finger transcription factor initially identified as a protein that bound to the globin genes and activated their expression (7). Loss of GATA-1 leads to a failure of erythropoiesis early in gestation, resulting in the death of the embryos from severe anemia by E10.5 (8). The erythroblasts in these embryos fail to develop past a pro-erythroblast-like stage.

Another transcription factor that has been studied extensively in erythrocytes is the Krüppel-like factor (KLF) EKLF/KLF1, a member of a family of at least 16 transcription factors (9, 10). Members of this family can both activate and repress transcription through interaction with a range of different transcription factors (e.g. the GATA factors and NF-κB) and co-factors (e.g. p300 and mSin3A) (11–14). KLF1 was first identified as a CACC box-binding factor expressed in erythroleukemia cells but not in monocytes/macrophages (15). Mice that lack expression of Klf1 die at E16 from a severe anemia, indicating a critical role for KLF1 in the formation of erythrocytes (16). At least eight other xKLF proteins (KLF2–5, 8, and 11–13) are expressed in erythroid cell lines. Of these KLF2, 5, 8, and 13 have been shown to bind to CACC boxes in globin promoters and can modify promoter activity in transfection assays (17). KLF2 null mice die before E14.5 from a defect in the formation of the endothelium but also have reduced expression of embryonic ε-globin, although expression of adult globin genes is normal (18).

KLF13 (KLF13/BTEB3/FKL2/FRLAT) is another xKLF protein expressed in the hematopoietic system (19, 20). Binding of KLF13 activates the promoters of a number of erythroid genes including γ-globin, GATA-1, porphobilinogen deaminase, and ferrochelatase in vitro (21). KLF13 is highly expressed in human peripheral blood lymphocytes and binds to and activates the RANTES promoter in late activated T cells (20, 22). We showed that murine KLF13 was widely expressed during embryogenesis with high levels of expression in the developing heart, thymus, and liver (19).

We have generated mice with a targeted deletion of exon 1 of the Klf13 allele and found that these mice have enlarged hearts.

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3 The abbreviations used are: En, embryonic day; n, Krüppel-like factor; PBS, phosphate-buffered saline; FCS, fetal calf serum; BM, bone marrow; FACs, fluorescence-activated cell sorting; ES, embryonic stem.
Erythropoiesis in Klf13\textsuperscript{−/−} Mice

and an increased susceptibility to the formation of cardiac vascular lesions.\textsuperscript{4} To determine the role of Klf13 in the differentiation of erythroid cells, we have compared erythropoiesis in Klf13\textsuperscript{−/−} and Klf13\textsuperscript{+/+} mice.

**EXPERIMENTAL PROCEDURES**

Mice—Klf13\textsuperscript{−/−} mice generated in the CCB (129S6Sv/Ev) ES cell line were maintained on this genetic background. Genotyping of tail DNA was performed by PCR using primers CAG3F (GGCTCCGCTGAGCAGCTCCTAAGA) and CAG3R (GCCGCCTCTTTCTCGGGCTG) to amplify the wild type allele and INTF (CTGGGGATGCGGTGGGCTCTA) and INTR (GGTGCGAGACTCCAGCTC) to amplify the Klf13 mutated allele. All of the mice were bred and maintained at the University of Cambridge Combined Animal Facility in accordance with the Animals (Scientific Procedures) Act 1986.

**Blood Analysis**—Blood was taken from the vena cava of Klf13\textsuperscript{+/+} and Klf13\textsuperscript{−/−} mice. Clotting was prevented by the addition of 0.5 M tri-potassium-EDTA. Each sample was read in triplicate on an ABC Vet Automated Blood Counter (Woodley Equipment Company Ltd., Bolton, UK).

**Flow Cytometry**—Spleen and femur bones were dissected from 7–8-week-old female Klf13\textsuperscript{−/−} mice and Klf13\textsuperscript{+/+}. Body and spleen weights were recorded, and splenocyte suspensions were made by mechanical dissociation through a 70-μm strainer in the presence of cold PBS and 2% fetal calf serum (PBS, 2% FCS). Bone marrow (BM) cells were extracted from femur bones using a 25-gauge needle and resuspended in PBS, 2% FCS.

The cells were stained using combinations of directly conjugated antibodies obtained from BD Pharmingen (San Diego, CA; anti-CD4\textsuperscript{FITC}, anti-CD4\textsuperscript{PE}, anti-CD4\textsuperscript{Cychrome}, anti-CD8\textsuperscript{FITC}, anti-CD8\textsuperscript{PE}, anti-CD8\textsuperscript{Cychrome}, anti-CD3\textsuperscript{FITC}, anti-CD71\textsuperscript{FITC}, anti-Ter119\textsuperscript{PE}, anti-CD45\textsuperscript{PE}, and anti-c-Kit\textsuperscript{FITC}).

Cell suspensions were stained with the antibodies for 30 min on ice in 50 μl of Dulbecco’s modified medium (Invitrogen), supplemented with 5% FCS and 0.01% sodium azide. The cells were washed in this medium between incubations and prior to analysis on the FACScan (fluorescence-activated cell scan; Becton Dickinson, Franklin Lakes, NJ). The events were collected in list mode using CellQuest software (Becton Dickinson), and the data were analyzed using CellQuest Pro software. Live cells were gated according to their forward scatter and side scatter profiles. The data are representative of at least three experiments. Intracellular staining for cyclin B1 was performed on cells stained for surface markers as above following fixation and permeabilization with the Cytofix/Cytoperm\textsuperscript{TM} solutions (BD biosciences) according to the manufacturer’s instructions.

**Fluorescence-activated Cell Sorting (FACS) and Quantitative Reverse Transcription PCR**—Splenocyte suspensions were stained with antibodies against CD19\textsuperscript{PE} (BD Pharmingen, San Diego, CA), Ter119\textsuperscript{PE}, and CD71\textsuperscript{FITC} (eBioscience, San Diego, CA) before being sorted on a DakoCytomation MoFlO sorter. Only cells falling within the forward scatter/side scatter live gate were sorted.

For purification using magnetic beads, splenocytes were incubated on ice with biotin labeled anti-Ter119 at 1 μg/10\textsuperscript{6} cells for 30 min before 50 μL of streptavidin magnetic beads were added. After a further 10 min on ice, the samples were placed against a magnet until the beads were against the side of the tube. The samples were washed by resuspension in PBS, 2% FCS three times before being resuspended in 300 μL of RLT buffer (Qiagen) for RNA purification.

Total RNA from sorted cells was extracted using RNAeasy (Qiagen). Reverse transcription and quantitative real time PCR were performed as described previously (23). Primer sequences are available on request. Klf13 transcript levels for each sample were normalized to rRNA levels, which were amplified using TaqMan Universal PCR Master Mix (Applied Biosystems, Warrington, UK).

**BFU-E and CFU-E Colony Forming Assays**—BFU-E and CFU-E cultures were performed using the methocult system (StemCell Technologies) according to the manufacturer’s instructions. In brief, BM and spleen cells were plated at a density of 2×10\textsuperscript{5} cells/ml 1% methylcellulose medium containing 15% fetal bovine serum, 1% bovine serum albumin, 10 μg/ml insulin, 200 μg/ml transferrin, and 3 units/ml erthropoietin. The cultures were incubated at 37 °C in 5% CO\textsubscript{2}. CFU-E were enumerated after 2 days in culture, and BFU-E were enumerated after 3–4 days in culture. All of the cultures were done in duplicate, and each experiment was repeated eight times.

**Statistical Analysis**—Statistical analyses for differences between Klf13\textsuperscript{+/+} and Klf13\textsuperscript{−/−} mice were performed using the two-tailed Student’s t test, assuming unequal variance. The data are presented as the means ± S.D. with the n values given in the corresponding figure legends.

**RESULTS**

**Generation of the Targeting Construct**—A genomic clone containing the mouse Klf13 gene (PAC F69-B12) was obtained by screening the mouse genome filter set RCP121 with the full-length (1200 bp) Klf13 coding sequence (GenBank\textsuperscript{TM} accession number AJ245644). In silico comparison of the Klf13 coding sequence with the genomic sequence (ENS-MUSG00000052040) together with experimental verification by Southern analysis and PCR showed that the Klf13 gene consisted of two exons. Exon 1 codes for the transactivation domain and the first zinc finger of the protein, and exon 2 codes for the other two zinc fingers. To identify the start of the first exon, the 5’ end of the known coding sequence together with 200 bp of presumed upstream sequence identified from the mouse htgs data base was compared with the expressed sequence tag data base using the BLAST algorithm. The majority of the expressed sequence tag sequences containing the 5’ part of the Klf13 coding sequence stopped at one of two positions separated by 30 nucleotides, indicating the presence of two transcriptional start sites (Fig. 1A). The sequence upstream of the start sites did not contain a TATA box, indicating that Klf13 has a TATA-less promoter. To generate a vector to delete the first exon of Klf13, a 1.5-kb fragment (mouse chromosome 7, nucleotides 59688488–59689967) of the promoter region and 4.9 kb of the intron (chromosome 7, nucleotides 59682698–59687527) were amplified by PCR and cloned into...
FIGURE 1. Targeting the Klf13 locus. A, the 5′ region of the Klf13 mRNA. Start sites identified by BLAST comparison with the expressed sequence tag data base are indicated by filled rectangles. The boxes marked Neo and DT-A show the positions of the neomycin and diphtheria toxin A selection cassettes, respectively. Arrows mark the positions of primers used for genotyping. Arrow 1, B3F; arrow 2, B3R; arrow 3, MutF; arrow 4, MutR. The positions of EcoRI sites are marked by thick line. C, Southern analysis of ES cell clones. DNA extracted from ES cells was digested with EcoRI and analyzed on a 1% agarose gel. The bands sizes are 185 bp (wild type) and 226 bp (mutant).

D, reverse transcription-PCR for KLF13 mRNA. Start sites identified by BLAST comparison with the expressed sequence tag data base are indicated by filled triangles and filled rectangles. Klf13 mRNA was not detectable by this method in Klf13−/− mice.

E, reverse transcription-PCR for KLF13 mRNA produces a 185-bp product. No products were observed in the absence of reverse transcription, indicating that the samples were not contaminated with genomic DNA. F, relative expression of KLF13 determined by quantitative real time PCR of cDNA from the right ventricle (n = 5 samples for each genotype). Messenger RNAs were quantified by calculating Ct and normalized to the Ct value for rRNA as described previously (14).

TABLE 1

| Genotype ratios of mice born to KLF13+/− parents | 
|-----------------------------------------------|
| x² = 10.3 p < 0.01. | 
| Offspring number | +/+ | +/− | −/− |
| Actual | 131 | 285 | 98 |
| Predicted | 129 | 258 | 98 |

The vector pKO-DTA along with a neomycin resistance cassette. All of the fragments and the final targeting vector were sequenced to confirm the PCR fidelity and cloning. The targeted vector was expected to result in a null allele. The resulting vector pDPN is shown in Fig. 1B.

To generate recombinant ES cells with a targeted mutation in Klf13, pDPN was linearized in the vector backbone with PvuII and electroporated into ES cells. The cells were selected in G418, and resistant clones were picked and expanded.

DNA was extracted from 45 clones and amplified by PCR using primer PF and PR to identify clones with the appropriate 5′ insertion of the DNA. This process identified 14 clones, and DNA from these clones was digested with EcoRI and analyzed by Southern blot. Probing the blot with a DNA probe to the 5-kb intron gave a band of >13 kb for the wild type locus and an 11-kb band for the targeted locus (Fig. 1C). Twelve of 45 clones contained the targeted locus, giving an overall targeting frequency of 27% of the clones picked. The targeted ES cells were injected into C57Bl/6 blastocysts to generate transgenic mice.

Klf13 Null Mice—Genotyping of the offspring of the mice was performed by PCR for both the wild type and mutant alleles as shown in Fig. 1D. This analysis showed that although null mice were detected after weaning, the numbers were less than that predicted by Mendelian inheritance (Table 1). To determine whether the lethality arose from an early embryonic loss, embryos from timed matings of Klf13+/− females with Klf13−/− males were examined and genotyped at E10.5 and E13.5. At neither of these time points was there any detectable deviation from Mendelian ratios (n = 31 embryos at E10.5 with 14 Klf13−/− and 17 Klf13+/− embryos; n = 59 embryos at E13.5 with 30 Klf13+/− embryos and 29 Klf13−/− embryos). The cause and time of onset of the lethality has yet to be determined. To confirm that the Klf13−/− mice did not express KLF13 mRNA, RNA from the heart (a tissue that expresses high levels of KLF13) was reverse transcribed and amplified by PCR. KLF13 mRNA was not detectable in Klf13−/−
Erythropoiesis in Klf13\(^{-/-}\) Mice

**TABLE 2**

Blood cell counts

|                      | Klf13\(^{+/+}\) | Klf13\(^{-/-}\) | t test | p value |
|----------------------|----------------|----------------|--------|---------|
| Mean (n = 5)         | Reference range | Mean (n = 4) | STDEV  | STDEV   |
| WBC                  | 9.36 ± 0.78     | 10.9 ± 7.8    | 6.75   | 0.89    | 0.003   |
| RBC                  | 10.54 ± 0.52    | 11.5 ± 9.5    | 9.54   | 0.55    | 0.03    |
| HGB                  | 16.7 ± 0.5      | 17.6 ± 15.7   | 16.0   | 0.84    | NS      |
| HCT                  | 51 ± 1.50       | 53.9 ± 47.9   | 49.0   | 2.44    | NS      |
| MCV                  | 48.3 ± 1.55     | 51.4 ± 45.2   | 51.5   | 0.43    | 0.008   |
| MCH                  | 15.9 ± 0.46     | 16.7 ± 14.9   | 16.8   | 0.19    | 0.006   |
| MCHC                 | 32.7 ± 0.24     | 33.1 ± 32.5   | 32.7   | 0.08    | NS      |
| PLTS                 | 458 ± 50        | 557–358       | 437    | 113.42  | NS      |
| MPV                  | 5.03 ± 0.09     | 5.21–4.85     | 4.98   | 0.13    | NS      |
| RDW                  | 13.71 ± 0.42    | 14.5–12.9     | 14.36  | 0.11    | 0.024   |
| LYMMP                | 6.50 ± 0.69     | 7.88–12.12    | 4.56   | 0.51    | 0.002   |
| MONO                 | 1.33 ± 0.06     | 1.45–121      | 1.01   | 0.21    | 0.054   |
| GRAN                 | 1.53 ± 0.22     | 1.97–1.09     | 1.18   | 0.28    | 0.08    |

**FIGURE 2.** Deletion of Klf13 results in increased spleen size. Spleens were removed from 7-week-old female Klf13\(^{-/-}\) mice and their wild type counterparts. The spleens were weighed and compared with the body weight of the mouse (n = 26 of each genotype). (A) Splenocyte suspensions were prepared as described under “Experimental Procedures,” and the cells were counted on a hemocytometer (n = 7 of each genotype). (B) Cell suspensions were stained with anti-CD19 and anti-CD3 and analyzed by flow cytometry. (C) The number of cells in each area was quantified and multiplied by the total number of cells in each sample. This total cell number was then normalized to the average for wild type mice (E). All of the data are the means ± S.D., \(*, p < 0.02; **, p < 0.005; ***, p < 0.0001 wild type versus Klf13\(^{-/-}\).
Erythropoiesis in Klf13<sup>-/-</sup> Mice

Increased erythropoiesis in Klf13<sup>-/-</sup> mice. A, Ter119 and CD71 staining of splenocytes from Klf13<sup>-/-</sup> and wild type mice identified four erythroblast populations (I–IV; the values given are the mean percentages corrected for spleen size). The overlay plots show the CD71 fluorescence profile of populations II and III from a wild type and klf13<sup>-/-</sup> mouse. The four populations identified in populations (A) were quantified by multiplying the number of cells in each region by the total number of cells in the spleen (B) and normalized to the average number in wild type mice (the data are the means ± S.D., n = 6 mice for each line). The open bars represent wild type, and the black bars represent Klf13<sup>-/-</sup> mice. C, splenocytes from wild type mice were stained with antibodies to Ter119 and CD71 and sorted by FACS. A minimum of 10<sup>6</sup> cells were collected for each population, and the RNA was extracted. RNA was reverse transcribed and amplified in real time PCRs using primers for Klf13. Klf13 mRNA was normalized to rRNA. D, femoral bone marrow cells were stained with Ter119 and CD71 and separated by FACS. The four erythroblast populations were quantified and normalized to the average number in wild type mice (n = 6 mice of each genotype). Splenocytes and bone marrow cells from wild type and Klf13<sup>-/-</sup> mice were cultured in methylcellulose as described under “Experimental Procedures.” The number of CFU-E colonies (E) and BFU-E colonies (F) on each plate were normalized to the average number of colonies on the plates from wild type mice (n = 8 for each line). All of the data are presented as the means ± S.D.; *, p < 0.05; **, p < 0.01 wild type versus Klf13<sup>-/-</sup>.

Numbers did not cause the splenomegaly. Staining the cells for CD45 identified three cell populations CD45<sup>-</sup> (Fig. 2D), CD45<sup>med</sup> (Fig. 2D, lower rectangle) and CD45<sup>hi</sup> (Fig. 2D, upper rectangle). The number of CD45<sup>hi</sup> cells did not change (Klf13<sup>+/+</sup>; 1.05 ± 0.06 × 10<sup>8</sup> cells/spleen and Klf13<sup>-/-</sup>; 0.98 ± 0.2 × 10<sup>8</sup> cells/spleen), but there was a marked 7-fold increase (Klf13<sup>+/+</sup>, 5.8 ± 2.3 × 10<sup>6</sup> cells/spleen and Klf13<sup>-/-</sup>, 43.7 ± 11.0 × 10<sup>6</sup> cells/spleen) in the number of CD45<sup>med</sup> cells and a 3-fold increase in the number of CD45<sup>-</sup> cells in the spleens of Klf13<sup>-/-</sup> mice (Klf13<sup>+/+</sup>, 1.26 ± 0.17 × 10<sup>9</sup> cells/spleen and Klf13<sup>-/-</sup>, 41.8 ± 0.74 × 10<sup>9</sup> cells/spleen; Fig. 2E). Because CD45 is highly expressed on the majority of lymphoid and myeloid cells, it was unlikely that the increased spleen mass in Klf13<sup>-/-</sup> mice was attributable to cells of these lineages. However, during erythroid cell development, expression of CD45 decreases as erythroblasts mature into erythrocytes, suggesting that the increase in spleen size in Klf13<sup>-/-</sup> mice could be due to alterations in the erythroid lineage.

Abnormal Erythroblast Maturation in Klf13<sup>-/-</sup> Mice—To determine whether the increase in cell number was due to an expansion of cells of the erythroblast lineage, splenocytes from Klf13<sup>-/-</sup> and Klf13<sup>+/+</sup> mice were stained for Ter119 and CD71. The expression of these markers distinguishes at least four populations of erythroid cells that are, from least to most differentiated, Ter119<sup>med</sup>CD71<sup>hi</sup>, Ter119<sup>hi</sup>CD71<sup>hi</sup>, Ter119<sup>hi</sup>CD71<sup>med</sup>, and Ter119<sup>hi</sup>CD71<sup>low</sup> (Fig. 3A) (3). These populations are represented in Fig. 3B as I, II, III, and IV, respectively, and can be further characterized by their size with an overall decrease in cell size from type I to Type IV (3). Flow cytometric analysis (Fig. 3A) showed a 7-fold expansion of Ter119<sup>med</sup>CD71<sup>hi</sup> from 2.2 ± 1.0 × 10<sup>5</sup> cells/spleen in the Klf13<sup>+/+</sup> mice to 15.6 ± 8.9 × 10<sup>5</sup> cells/spleen in the Klf13<sup>-/-</sup> mice, and Ter119<sup>hi</sup>CD71<sup>hi</sup> from 3.4 ± 2.3 × 10<sup>6</sup> cells/spleen in the Klf13<sup>-/-</sup> mice to 26 ± 11 × 10<sup>6</sup> cells/spleen in the Klf13<sup>-/-</sup> mice and a 2-fold expansion of Ter119<sup>hi</sup>CD71<sup>med</sup> from 9.3 × 10<sup>5</sup> cells/spleen in the Klf13<sup>-/-</sup> mice to 21.9 × 10<sup>6</sup> ± 0.4 cells/spleen in the Klf13<sup>-/-</sup> mice (Fig. 3B). The increase in CD71<sup>hi</sup> cells in the Ter119<sup>hi</sup> population is illustrated in Fig. 3A. However, despite this increase in erythroid precursors, numbers of late erythroblasts (Ter119<sup>hi</sup>CD71<sup>low</sup>, chromatophilic erythroblasts to reticulocytes) remained unaltered.

The alterations in erythropoiesis may have resulted from indirect effects rather than loss of KLF13 in the erythroblasts. We therefore determined whether Klf13 was expressed in erythroblasts. Quantifying Klf13 mRNA in erythroblasts sorted by FACS from the spleens of Klf13<sup>+/+</sup> mice showed that Klf13 expression increased between Ter119<sup>+</sup>CD71<sup>+</sup> erythroblasts and Ter119<sup>+</sup>CD71<sup>-</sup> erythroblasts (2.5-fold; Fig. 3C). These levels of Klf13 mRNA in Ter119<sup>+</sup>CD71<sup>-</sup> erythroblasts are similar to those in splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells CD19<sup>+</sup> and 10-fold higher than splenic B cells. Therefore, Klf13 expression increases as erythroid precursors mature, suggesting a direct involvement of KLF13 in erythroblast differentiation.

Erythropoiesis in the BM—Although some erythropoiesis occurs in the spleen under normal conditions, most splenic erythropoiesis occurs as a response to hematologic stress (5). The process of normal erythropoiesis occurs mostly in the BM. Therefore to determine whether the same defects occurred in erythropoiesis in the BM, we determined the proportion of type I to type IV cells in the BM. This analysis showed an increase in type II erythroblasts (p < 0.03) in the BM and a trend to an increase in the number of type I cells, but this did not reach significance (p = 0.06). There was no effect on the other erythroblast populations (Fig. 3D).

To analyze the ability of progenitors from Klf13<sup>-/-</sup> mice to differentiate along the erythropoietic lineage, we determined the number of BFU-Es and CFU-Es in the spleen. Consistent

5 S. V. Outram, A. R. Gordon, A. L. Hager-Theodorides, J. Metcalfe, T. Crompton, and P. Kemp, submitted for publication.
with the increase in Ter119medCD71hi cells both BFU-Es and CFU-Es were increased in the spleen (Fig. 3, E and F). In the BM there was no change in the number of CFU-E or BFU-E. Together with the CD71/Ter119 staining, these data suggest that loss of KLF13 does not inhibit the commitment of cells to the erythrocyte lineage.

Expression of Transcription Factors Involved in Erythropoiesis—To determine whether the expression of other transcription factors known to be involved in the differentiation of erythrocytes was altered by the lack of Klf13, we determined the expression of GATA-1, Klf1, and Klf3 by quantitative reverse transcription-PCR in Ter119+ cells isolated by magnetic bead separation from the spleens of Klf13−/− and Klf13+/+ mice. The mRNA for all of these transcription factors increased at least 3-fold in the erythroblasts of Klf13−/− mice compared with their wild type counterparts (Fig. 5B). To determine whether this increase in Bcl-xL was due to the increase in the proportion of CD71-positive cells in the Ter119+ population Bcl-xL, Bad, and Bcl2 were measured in FACs sorted populations of CD71+;Ter119− and CD71+:Ter119+ splenocytes from wild type mice. This analysis showed that Bcl-xL expression was higher in the CD71+:Ter119− cells than in the CD71+;Ter119+ cells; consequently much of the increased amount of Bcl-xL observed in the KLF13−/− mice is likely to arise from the increase in proportion of immature cells in the spleens of these mice (Fig. 5C). Consistent with these observations, we did not observe a significant reduction in apoptosis in Ter119+ cells by annexin staining. During the preparation of this paper Zhou et al. (24) published their analysis of a similar line of KLF13−/− mice. This analysis showed a marked reduction in apoptosis and increased expression of Bcl-xL in thymocytes. Thus it appears that Bcl-xL is a major target of KLF13 in thymocytes but not in erythrocytes.

DISCUSSION

Previous studies have implicated Klf13 in the differentiation of erythrocytes in vitro (17, 22). Our data demonstrate a role for Klf13 in these cells in vivo by showing that there is an increase in the number of immature erythrocytes in the spleens of mice that lack expression of Klf13. Indeed the reduced levels of other cell types in the circulation of the Klf13−/− mice suggest that Klf13 is required for the differentiation and/or proliferation of other hematopoietic cell lineages.

Our data indicate that the cells were able to commit to the erythrocyte lineage because there was no change in the number of BFU-Es or CFU-Es in the bone marrow. However, the cells accumulate in the proerythroblast stage of development and have increased expression of KLF3. Mice that lack KLF3 have also been shown to have splenomegaly, although the mechanism behind this phenotype is unexplored, raising the possibility that both KLF3 and KLF3 act at the same point but that the increase in KLF3 is insufficient to overcome the loss of KLF13. An alternative explanation of these data is that KLF13 is an inhibitor of KLF3 expression in erythroblasts.
Erythropoiesis is disrupted in several other lines of mice with targeted mutations to transcription factors. For example, both GATA-1 and KLF1 are required for erythropoiesis to occur (8, 16). Mice with low levels of expression of GATA-1 survive but become increasingly anemic with age (25) and show stress erythropoiesis as a compensation for defective primary erythropoiesis. This phenotype is similar to that seen in lyn−/− mice, which have reduced expression of GATA-1 and KLF1 (26). The splenomegaly observed in our mice is also consistent with a stress erythropoietic response. However, in our mice we found little change in the expression of both Klf1 and GATA-1 in Ter119+ cells in contrast to the data from lyn−/− mice. This difference suggests that KLF13 regulates a stage of erythropoiesis different from that regulated by KLF1 and GATA-1. Furthermore, it suggests that although KLF13 is known to bind to and activate the GATA-1 promotor, it is not a major regulator of GATA-1 expression. However, it is also possible that KLF13 interacts with and/or cooperates with GATA-1 to drive a subset of GATA-1 functions in erythropoiesis. Such an interaction has recently been shown between GATA-4 and KLF13 in Xenopus and results in a failure of cardiac development and a reduction in the expression of B-type natural peptide in this model (27). The extent of any requirement for KLF13 in the function of GATA-1 is also dependent on the ability of other KLF proteins to compensate for the loss of KLF13 in some but not all activities of GATA-1.

Apoptosis of erythrocytes occurs in the spleen as a feedback mechanism to regulate the number of erythrocytes, and splenomegaly has been shown in mice with aberrant apoptosis (28, 29). It was therefore possible that loss of KLF13 resulted in a reduction in apoptosis, and consistent with this suggestion, during the preparation of this paper Zhou et al. (24) published their analysis of Klf13−/− mice and showed that KLF13 was an inhibitor of the expression of the anti-apoptotic factor Bcl-xL. However, in our mice there was no significant change in annexin staining in Ter119+ cells, suggesting that there was no increase in apoptosis in these mice. Furthermore we found no detectable increase in Bcl-xL expression once the change in proportion of immature cells was accounted for. However, we did detect an increase in the expression of cyclin B in Ter119+ cells, suggesting an increase in proliferation. Together these data suggest that increased proliferation rather than a reduction in apoptosis leads to the increase in spleen size.

**Erythropoiesis in Klf13−/− Mice**

**Figure 5. Cell proliferation and apoptosis in Klf13−/− mice.** Splenocytes were stained for Ter119 and cyclin B (A). Spleens from Klf13−/− mice had increased numbers of Ter119− cells expressing cyclin B1 compared with those of wild type mice (n = 3 mice of each genotype). RNA was extracted from Ter119− positive splenocytes isolated on magnetic beads (B) or Ter119 and CD71− stained, FACS-sorted splenocytes (C). Bcl-xL, Bad, Bax, and Bcl2 mRNAs were quantified by quantitative real time PCR normalized to Gapdh (n = 5 mice for each line; mRNA samples were prepared and analyzed from each mouse independently). The data are presented as the means ± S.D. *, p < 0.05; **, p < 0.01, wild type versus Klf13−/−. WT or wt, wild type; KO, knock-out.
In summary the data presented here identify a role for Klf13 in the maturation of erythrocytes. Loss of Klf13 results in an increase in the expression of Klf3 and cyclin B, but other Klf13 target genes in erythroblasts remain to be identified.

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