

HEMATOPOIESIS AND STEM CELLS

Conditional knockout mice demonstrate function of *Klf5* as a myeloid transcription factorNur Hezrin Shahrin,^{1,4} Sonya Diakiw,^{2,3} Lindsay A. Dent,¹ Anna L. Brown,^{2,4-6,*} and Richard J. D'Andrea^{2-5,*}

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Key Points

- *Klf5* functions in hematopoiesis to regulate HSC and progenitor proliferation and localization in the bone marrow.
- *Klf5* is required in the granulocyte lineage and positively affects neutrophil output at the expense of eosinophil production.

Krüppel-like factor 5 (*Klf5*) encodes a zinc-finger transcription factor and has been reported to be a direct target of C/EBP α , a master transcription factor critical for formation of granulocyte-macrophage progenitors (GMP) and leukemic GMP. Using an in vivo hematopoietic-specific gene ablation model, we demonstrate that loss of *Klf5* function leads to a progressive increase in peripheral white blood cells, associated with increasing splenomegaly. Long-term hematopoietic stem cells (HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs) were all significantly reduced in *Klf5*^{Δ/Δ} mice, and knockdown of KLF5 in human CD34⁺ cells suppressed colony-forming potential. ST-HSCs, MPPs, and total numbers of committed progenitors were increased in the spleen of *Klf5*^{Δ/Δ} mice, and reduced β 1- and β 2-integrin expression on hematopoietic progenitors suggests that increased splenic hematopoiesis results from increased stem and progenitor mobilization. *Klf5*^{Δ/Δ} mice show a significant reduction in the fraction of Gr1⁺Mac1⁺ cells (neutrophils) in peripheral blood and bone marrow and increased frequency of eosinophils in the peripheral blood, bone marrow, and lung. Thus, these studies demonstrate dual

functions of KLF5 in regulating hematopoietic stem and progenitor proliferation and localization in the bone marrow, as well as lineage choice after GMP, promoting increased neutrophil output at the expense of eosinophil production. (*Blood*. 2016;128(1):55-59)

Introduction

Krüppel-like-factor 5 (KLF5) is a member of the Kruppel-like family of transcription factors, several of which have been shown to regulate hematopoietic stem and progenitor cell function and lineage fate.¹⁻⁴ *Klf5* is a master regulator of growth, differentiation, migration, and apoptosis, is implicated as both a tumor suppressor and oncogene in the pathogenesis of many cancers, and is increasingly of interest as a therapeutic target.^{5,6} Previous in vitro studies have indicated that *Klf5* plays a role in regulating myeloid growth and differentiation, particularly in the neutrophil lineage.^{7,8} Hypermethylation of *KLF5* intron 1 occurs in 41% of acute myeloid leukemia (AML) patients, is an independent predictor of poor overall survival,⁹ and is associated with reduced expression of *KLF5* mRNA.^{7,8} Thus, *KLF5* may act as a tumor suppressor in AML, and further investigation of *KLF5* function in the myeloid compartment is important for the understanding of this tumor suppressor activity. The *KLF5* promoter is a direct binding target of C/EBP α ,^{10,11} which is a master regulator of myelopoiesis, being essential for the transition of common myeloid progenitors (CMP) to granulocyte-macrophage progenitors (GMP).^{10,12} *KLF5* gene expression is directly regulated by C/EBP α ,¹⁰ consistent with reduced *KLF5* expression in the *CEBPA*-mutated AML subgroup.^{13,14} Most recently, experiments in murine models have shown that C/EBP α -induced

GMP formation is an essential step in leukemic transformation by the MLL-AF9 and MOZ-TIF2 oncogenes.¹² Thus, we used the *Cre-Lox* system to generate mice with targeted ablation of *Klf5* in the hematopoietic compartment. A detailed analysis of myeloid progenitor and mature cell compartments in these mice revealed that, although *Klf5* is not absolutely required for CMP or GMP formation, it is important for neutrophil and eosinophil production, potentially functioning as a downstream target of C/EBP α after GMP formation to control lineage fate of myelomonocytic precursors.

Study design

Mice

Details for the mice used in this study are described in the supplemental Materials, available on the *Blood* Web site.

Flow cytometry analysis

Cells from peripheral blood and bone marrow were stained and analyzed according to previously described protocols.^{15,16} For specification of antibodies, see supplemental Materials.

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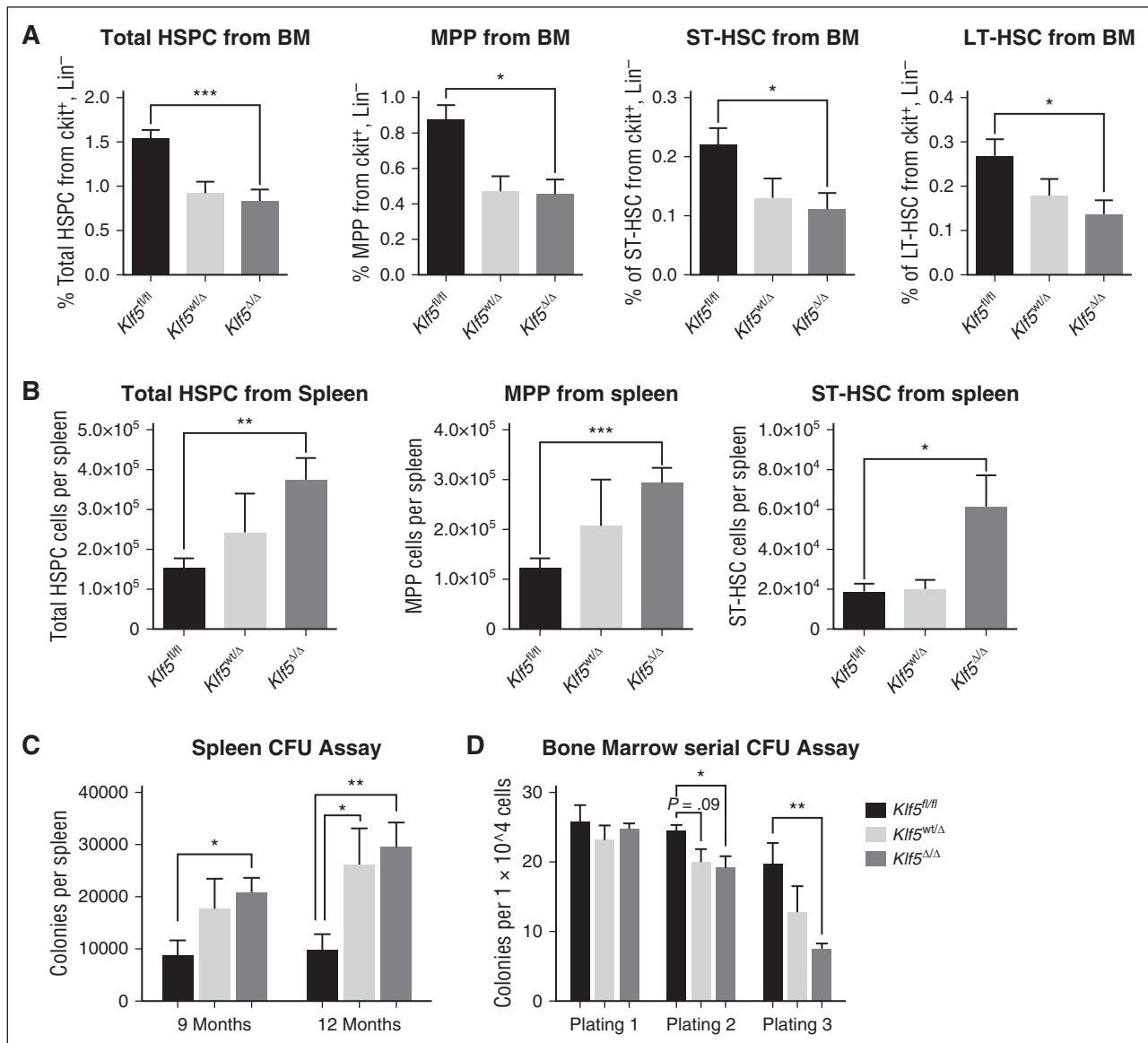


Figure 1. Measurement and functional characterization of HSPCs in *Klf5*^{Δ/Δ} mice. (A) Measurement of total HSPC (defined by IL7R α ⁻c-Kit⁺Sca-1⁺), LT-HSCs (defined by c-kit⁺Lin⁻IL7R α ⁻Sca-1⁺CD48⁻CD150⁺), ST-HSCs (defined by c-kit⁺Lin⁻IL7R α ⁻Sca-1⁺CD48⁺CD150⁺), and MPPs (defined by c-kit⁺Lin⁻IL7R α ⁻Sca-1⁺CD48⁻CD150⁻) as a percentage of the cKit⁺Lin⁻ compartment from bone marrow of mice at 9 months of age using flow cytometry. Results were obtained from n = 8 *Klf5*^{fl/fl}, n = 8 *Klf5*^{w/wΔ}, and n = 11 *Klf5*^{Δ/Δ} mice. (B) Total HSPCs, MPPs, and ST-HSCs (as a percentage of the cKit⁺Lin⁻ compartment) in the spleen of 12-month-old mice. Results were obtained from n = 7 *Klf5*^{fl/fl}, n = 5 *Klf5*^{w/wΔ}, and n = 8 *Klf5*^{Δ/Δ} mice. (C) Enumeration of myelo-erythroid colonies from spleen cells of *Klf5*^{Δ/Δ} mice at 9 and 12 months old. Results were obtained from n = 6 *Klf5*^{fl/fl}, n = 5 *Klf5*^{w/wΔ}, and n = 7 *Klf5*^{Δ/Δ} mice at 3 months and n = 6 *Klf5*^{fl/fl}, n = 4 *Klf5*^{w/wΔ}, and n = 6 *Klf5*^{Δ/Δ} mice at 9 months. (D) Quantification of myelo-erythroid progenitor self-renewal from bone marrow using serial colony-forming assays at 9 months. Results were obtained from n = 4 *Klf5*^{fl/fl}, n = 5 *Klf5*^{w/wΔ}, and n = 5 *Klf5*^{Δ/Δ} mice. Mean and SEM are shown for all measurements. *P* values were obtained from 2-tailed *t* tests conducted between each genotype group: ****P* < .001, ***P* < .01, and **P* < .05.

Colony-forming unit assay

Methylcellulose assays for colony-forming cells (CFCs)¹⁷ were performed using MethoCult GF M3434 (mouse) or MethoCult H4034 Optimum (human) according to the manufacturer's protocol.

For detailed methods regarding immunofluorescence, short hairpin RNA (shRNA) knockdown, and *KLF5* overexpression, see supplemental Materials.

Results and discussion

To investigate the impact of *Klf5* loss in hematopoiesis, we generated a conditional *Klf5* gene ablation model (*Klf5*^{Δ/Δ} mice) using the well-characterized *Vav-cre* transgene,¹⁸ to induce loss of *Klf5* exon 2 in the

hematopoietic system, thus removing the majority of the protein coding potential (supplemental Figure 1A). Quantitative polymerase chain reaction and western blot analysis indicated that *Klf5* mRNA and protein were significantly reduced in whole bone marrow cells and CD45⁺ cells, respectively (supplemental Figure 1B-C). *Klf5*^{Δ/Δ} mice were viable and born in expected Mendelian ratios with no observed effect on mortality in the time monitored (age, to 18 months; data not shown). However, longitudinal analysis identified a blood phenotype, characterized by a progressive increase in peripheral white blood cells and associated with increasing splenomegaly over time (supplemental Figure 2A-B).

As *C/EBP α* acts as a master transcription factor early in adult hematopoiesis to regulate myelomonocytic cell output via GMP production, we next used flow cytometry to analyze the hematopoietic

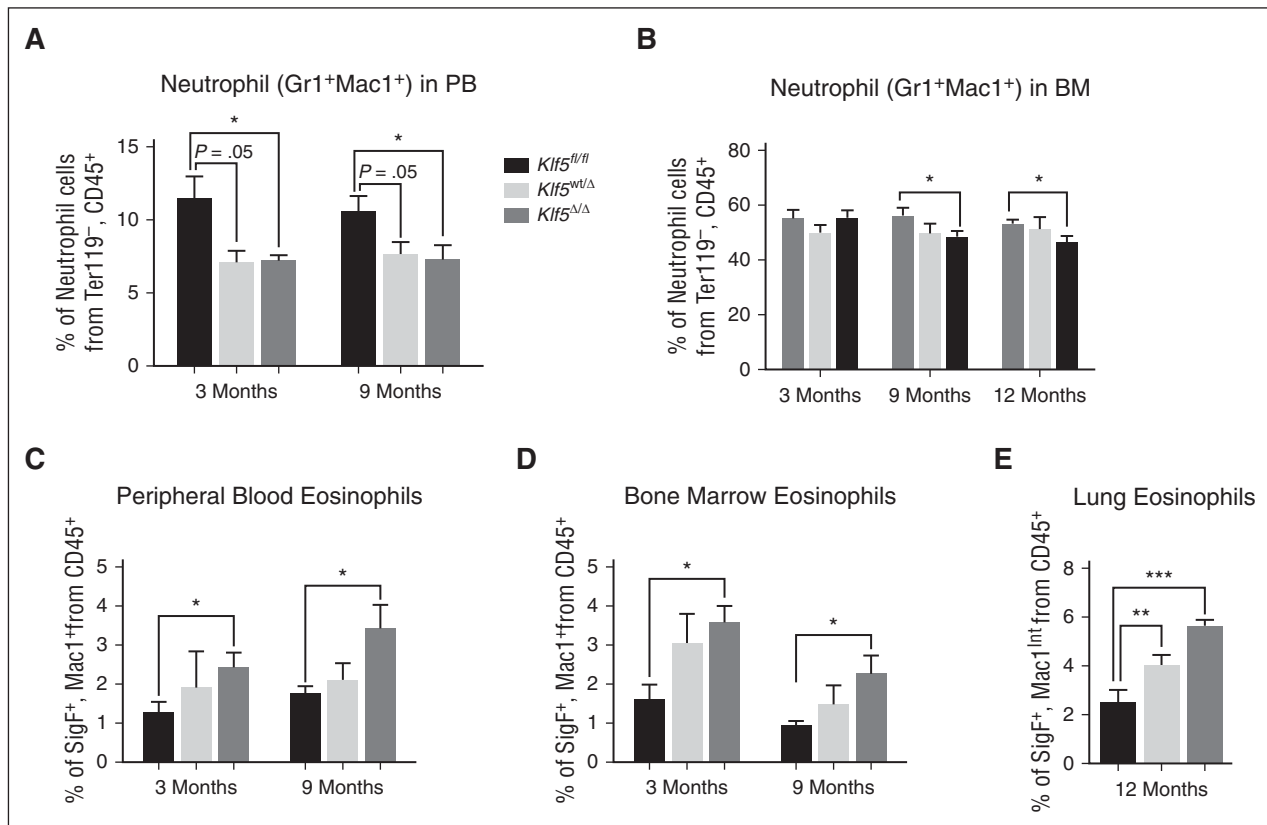


Figure 2. Characterization of mature myeloid cells in *Klf5* $\Delta\Delta$ mice using flow cytometry. Measurement of neutrophils ($\text{Gr1}^+\text{Mac1}^+$) from (A) peripheral blood and (B) bone marrow at 3 and 9 months. Eosinophils, measured as $\text{Mac1}^+\text{SigF}^+$ cells, from (C) peripheral blood, (D) bone marrow, and (E) lung ($\text{Mac1}^{\text{int}}\text{SigF}^+$). Results from peripheral blood and bone marrow were obtained from $n = 10$ *Klf5* $^{\text{fl/fl}}$, $n = 6$ *Klf5* $^{\text{wt}/\Delta}$, and $n = 10$ *Klf5* $\Delta\Delta$ mice at 3 months and $n = 8$ *Klf5* $^{\text{fl/fl}}$, $n = 8$ *Klf5* $^{\text{wt}/\Delta}$, and $n = 10$ *Klf5* $\Delta\Delta$ mice at 9 months. For lung eosinophil analysis, results were obtained from $n = 6$ *Klf5* $^{\text{fl/fl}}$, $n = 4$ *Klf5* $^{\text{wt}/\Delta}$, and $n = 6$ *Klf5* $\Delta\Delta$ at 12 months. Mean and SEM are shown for all measurements. *P* values were obtained from 2-tailed *t* tests conducted between each genotype group: ****P* < .001, ***P* < .01, or **P* < .05.

stem and progenitor cell (HSPC) fractions from bone marrow of *Klf5* $\Delta\Delta$ mice, using *Klf5* $^{\text{fl/fl}}$ littermates as controls. The fraction of long-term hematopoietic stem cells (LT-HSCs), short-term HSCs (ST-HSCs), and multipotent progenitors (MPPs) were all significantly reduced in *Klf5* $\Delta\Delta$ mice (Figure 1A), consistent with high relative expression of *KLF5* in both mouse and human HSC compartments (supplemental Figure 3) and the previously reported function of Klf5 in hematopoietic stem cells.¹⁹ As Ishikawa et al¹⁹ showed that Klf5 regulates homing and adhesion of HSCs in the bone marrow, we hypothesized that the reduction of HSCs in the bone marrow may be associated with splenic myeloopoiesis due to increased mobility of *Klf5* $\Delta\Delta$ HSCs. As shown in Figure 1B, flow cytometric analysis revealed that HSPCs (predominantly the ST-HSCs and MPPs) were increased in the spleen of *Klf5* $\Delta\Delta$ mice as a fraction of total $\text{Lin}^- \text{c-Kit}^+$ cells. The total number of committed progenitors, as determined from clonal assays, was also increased in the spleen of *Klf5* $\Delta\Delta$ mice (Figure 1C). Reduced levels of $\beta 1$ - and $\beta 2$ -integrin proteins in c-Kit^+ cells from bone marrow were observed (supplemental Figure 4), which also correlated with decreased mRNA in total bone marrow from *Klf5* $\Delta\Delta$ mice. Consistent with previous studies of hematopoietic stem and progenitor cells from Klf5-deficient mice,¹⁹ *Rab5b*, which is an upstream regulator of the β -integrins and a direct Klf5 target, also had decreased mRNA in total bone marrow from *Klf5* $\Delta\Delta$ mice (supplemental Figure 4). Therefore, it is likely that that reduced adhesion to bone marrow stroma contributes to increased HSC mobilization, resulting in the increased stem and progenitor numbers observed in the spleen. We also observed a change in replating capacity for *Klf5* $\Delta\Delta$ committed progenitors. Clonal serial

replating assays using bone marrow mononuclear cells demonstrated a reduction in colony numbers for *Klf5* $\Delta\Delta$ cells following secondary and tertiary plating (Figure 1D). This is consistent with reduced self-renewal capacity associated with loss of Klf5 function, and this defect may be inherited from the HSCs as a previous report found that the repopulation ability of the long-term HSC compartment was impaired in *Klf5*-deficient mice.¹⁹ To investigate the effect of reduced KLF5 in human stem and progenitor cells, we targeted KLF5 for knockdown in CD34^+ bone marrow cells. As shown in supplemental Figure 5, lentiviral-mediated targeting of KLF5 with 2 independent validated shRNA triggers reduced total CFCs, consistent with KLF5 having a conserved role in proliferation and survival of both mouse and human primitive hematopoietic cells. Examination of more committed progenitors in mouse bone marrow did not identify significant differences in the relative fraction of GMP or pre-GMP as a percentage of $\text{Lin}^- \text{c-Kit}^+$ cells (supplemental Figure 6). Thus, although *KLF5* is a known target of C/EBP α , it is not essential for C/EBP α -induced GMP production in the mouse. We next investigated changes in the neutrophil, eosinophil, T-cell, and B-cell fractions in total leukocytes from peripheral blood and bone marrow of adult *Klf5* $\Delta\Delta$ mice. A significant reduction in the fraction of $\text{Gr1}^+\text{Mac1}^+$ cells (neutrophils) as early as 3 months of age in peripheral blood was observed, and this was still evident in bone marrow until ≥ 12 months of age (Figure 2A-B). Analysis of eosinophil levels using flow cytometry revealed an increase in the eosinophil fraction of total leukocytes in the peripheral blood, bone marrow, and lungs of *Klf5* $\Delta\Delta$ mice (Figure 2C,D,E, respectively), suggesting that Klf5 may function as a negative regulator

of adult eosinophil development. Changes to other compartments in *Klf5*^{ΔΔ} mice, included an increase in the fraction of T cells (CD3⁺ cells) in the peripheral blood, but not in the bone marrow or spleen, and transient differences in the fraction of B cells (B220⁺ cells) in peripheral blood, bone marrow, and spleen (supplemental Figure 7). Flow cytometric analysis of erythroid and megakaryocyte progenitors also did not identify significant differences in the *Klf5*^{ΔΔ} mice (supplemental Figure 8).^{15,20}

Thus, these studies, and those of Ishikawa et al¹⁹ in independent *Klf5* murine gene ablation models, demonstrate that *Klf5* functions in hematopoiesis to regulate HSC and progenitor mobilization and granulocyte-macrophage progenitor lineage choice after GMP, positively affecting neutrophil output at the expense of eosinophil production. Increased expression of *KLF5* mRNA is observed in the granulocyte lineage during both mouse and human hematopoiesis⁷ (supplemental Figure 3), and *KLF5* overexpression promotes differentiation in human U937 cells following retroviral transduction with increased granulocytes (evident morphologically and by CD11b expression), which is particularly observed following induction with ATRA (supplemental Figure 9). As we previously showed that *KLF5* is silenced by methylation in U937 cells,⁷ this also has therapeutic relevance to hematopoietic disease states.

In addition to C/EBPα, other members of the CEBP family may also be important as *Klf5* interacts directly with C/EBPβ,²¹ which has been reported as a regulator of eosinophil gene expression.²² *Klf5* has also been reported as a C/EBPβ target gene,²¹ and therefore *Klf5* may interact with, and function downstream of, a range of other hematopoietic transcription factors involved in specification of the granulocyte-macrophage lineage. For example, GFI1 and KLF4 have been shown to bind the *KLF5* promoter.¹¹ Interestingly, KLF4 and KLF5 are often coexpressed and are known to coordinately or antagonistically regulate cellular differentiation in a range of contexts.^{5,23} KLF4 controls monocyte lineage fate and is also a target of gene silencing in AML.³ Other KLFs also have roles in hematopoiesis and leukemia; for example, KLF1 is a master regulator of erythropoiesis and variation at the *KLF1* locus underlies a large spectrum of red blood cell disorders.² KLF7 regulates function of primitive hematopoietic cells,⁴ and KLF2 has been reported to function in lymphoid development and disease and in regulating adhesion of myeloid cells.²⁴ Therefore, further work to dissect the

functional relationships of different members of the KLF family in normal and malignant hematopoiesis will be important. Future experiments to define both the direct upstream regulators and downstream targets of KLF5 in the hematopoietic compartment will add to our knowledge of stem cell regulation and myeloid differentiation. Finally, reduced KLF5 activity associated with hypermethylation of *KLF5* intron 1 may contribute to the mobilization of preleukemic or leukemic stem and progenitor cells, thus separating these cells from critical regulators of growth and differentiation.

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Authorship

Contribution: N.H.S. contributed to research design, performed the research, analyzed the data, and drafted the manuscript; S.D. contributed to research design, performed research, and analyzed the data; L.A.D. contributed to research design and critically reviewed the manuscript; and A.L.B. and R.J.D. contributed to research design, supervised the research, and critically reviewed and wrote the manuscript.

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