MYELOID NEOPLASIA

Runx3 deficiency results in myeloproliferative disorder in aged mice

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Key Points

- *Runx3* conditional knockout mice develop a myeloproliferative disorder when aged.
- *Runx3*-deficient cells show hypersensitivity to G-CSF.

The *RUNX* family genes encode transcription factors that are involved in development and human diseases. *RUNX1* is one of the most frequently mutated genes in human hematological malignancies and is a critical factor for the generation and maintenance of hematopoietic stem cells. Another *Runx* family gene, *Runx3*, is known to be expressed in hematopoietic cells. However, its involvement in hematopoiesis remains unclear. Here we show the hematopoietic phenotypes in *Runx3* conditional knockout (KO) mice (*Runx3*$^{fl/fl}$; *Mx1-Cre*$^{+}$): whereas young *Runx3* KO mice did not exhibit any significant hematopoietic defects, aged *Runx3* KO mice developed a myeloproliferative disorder characterized by myeloid-dominant leukocytosis, splenomegaly, and an increase of hematopoietic stem/progenitor cells (HSPCs). Notably, *Runx3*-deficient cells showed hypersensitivity to granulocyte-colony stimulating factor, suggesting enhanced proliferative and mobilization capability of *Runx3*-deficient HSPCs when stimulated. These results suggest that, besides *Runx1, Runx3* also plays a role in hematopoiesis. (*Blood*. 2013;122(4):562-566)

Introduction

The *Runx* genes belong to the Polyomavirus enhancer binding protein 2/core binding factor family of heterodimeric transcription factors that are important for development and are implicated in various human diseases.[1-3] Of 3 family genes, *RUNX1* is a well-documented hematopoietic and leukemia factor. *RUNX1* is abrogated by multiple types of genetic alterations in human hematological malignancies[3-5] and is critical for the generation and maintenance of hematopoietic stem cells (HSCs).[6-10] *Runx1* regulates the expression of stemness- and niche-related factors, such as *Bmi1*, *Cxcr4,* and *integrin* α_2, deregulation of which in adult *Runx1* knockout (KO) mice led to an expanded hematopoietic stem/progenitor cell (HSPC) compartment and subsequent stem cell exhaustion.[6-12] In addition, adult *Runx1* KO mice show abnormal megakaryocytic differentiation and defective lymphoid development.[11-14] In contrast, the role of *Runx3* in hematopoiesis remains elusive, despite the fact that Runx3 is expressed in various hematopoietic tissues in adult mice.[15] We therefore sought to examine its effects on hematopoiesis in *Runx3*-deficient mice.

Study design

Mice

Runx3$^{fl/fl}$ chimeric mice obtained from genetically manipulated 129P2/O1aHsd-derived E14 embryonic stem cells injected into C57BL/6 blastocysts[16] were backcrossed onto C57BL/6 inbred mice for ≥3 generations before being used for subsequent crosses with *Mx1-Cre*$^{+}$ mice. For induction of the Mx-Cre transgene, 4-week-old *Runx3*$^{fl/fl}$;*Mx1-Cre*$^{+}$ mice were injected intraperitoneally with 600 μg of polyinosinic-polycytidylic acid (pIpC; Sigma-Aldrich) on 7 alternate days.

G-CSF stimulation

Mice were subcutaneously injected with 300 μg/kg/day murine granulocyte-colony stimulating factor (G-CSF; PeproTech) daily for 4 days. Twenty microliters of peripheral blood (PB) was obtained for the colony-forming unit culture (CFU-C) assay.

Additional procedures

For complete information on hematological analyses, flow cytometric analyses, CFU-C assay, bone marrow transplantation (BMT) procedures, and quantitative real-time polymerase chain reaction analysis, see supplemental Methods on the *Blood* website.

Results and discussion

Generation of *Runx3*$^{fl/fl}$;*Mx1-Cre*$^{+}$ mice

Due to the neonatal lethality of conventional *Runx3*$^{-/-}$ mice, conditional *Runx3* KO mice were generated. To induce Cre expression in hematopoietic cells, *Runx3*$^{fl/fl}$;*Mx1-Cre*$^{+}$ mice and *Runx3*$^{fl/fl}$;*Mx1-Cre*$^{-}$ littermates were injected with pIpC (hitherto referred to as *Runx3* KO

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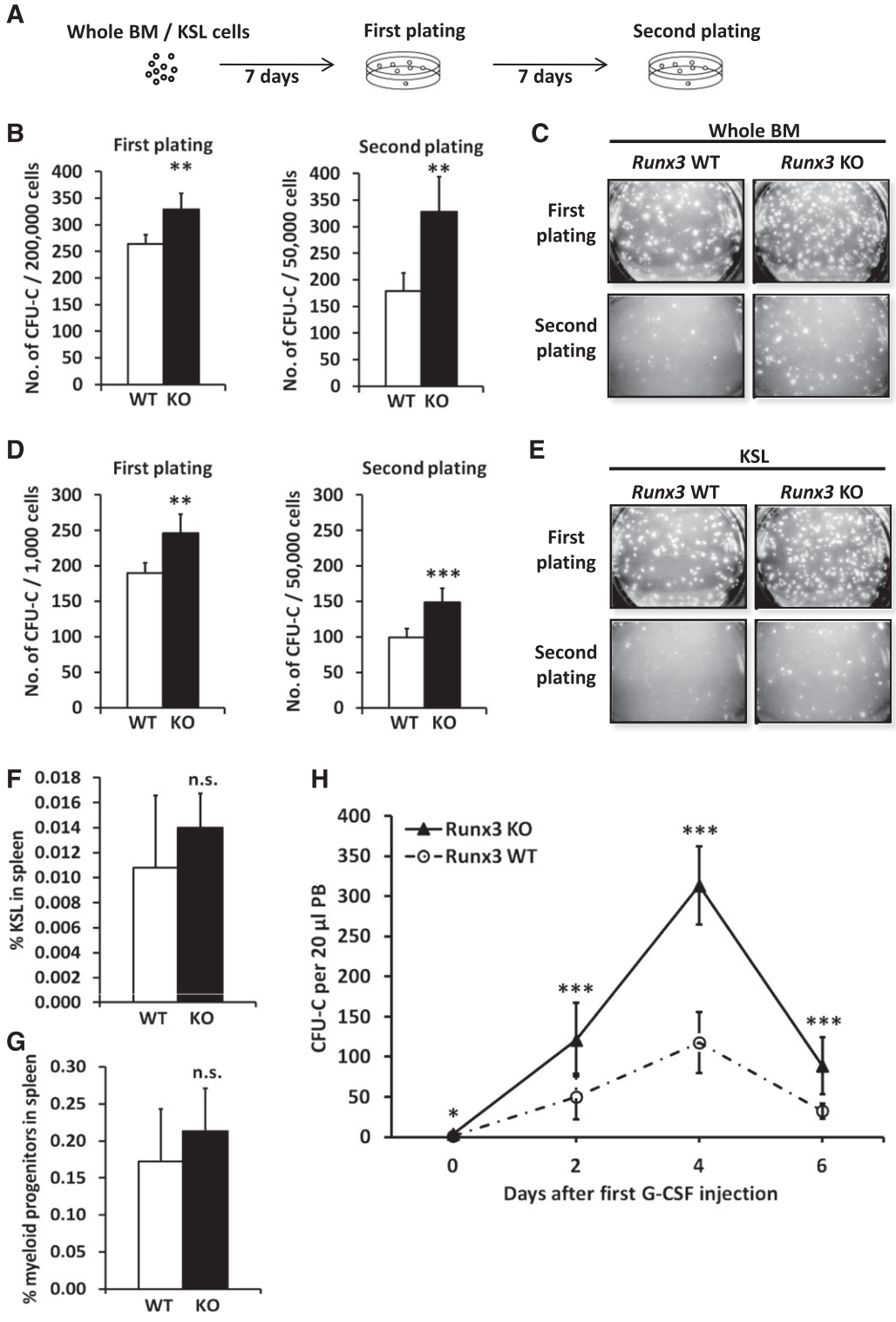

Figure 1. HSPCs from young *Runx3* KO mice show enhanced proliferative and mobilization ability when stimulated. (A) Schematic diagram showing an overview of the CFU-C assay, including serial replating. Colonies were scored 7 days after plating. (B,D) Colony-forming potential of (B) whole bone marrow (BM) cells and (D) BM KSL cells. Mean ± standard deviation (SD) is shown (n = 2/genotype). All colonies were counted from triplicate samples. Two independent experiments were performed. Asterisks represent significant differences (**$P < .01$; ***$P < .001$, Student *t* test). (C,E) Morphology of colonies formed on each plate. Representative plates in first and second platings from B and D are shown. (F-G) Flow cytometric analysis of the KSL and c-Kit$^+$Sca-1$^-$lineage$^-$ (myeloid progenitor) compartment in the spleen at 6 weeks after pIpC treatment. Mean ± SD of percentage of (F) KSL and (G) myeloid progenitors in spleen is shown (n = 5/genotype). Three independent experiments were performed. n.s., no significant difference (Student *t* test). (H) G-CSF mobilization assay of cells from *Runx3* KO mice (n = 7) and *Runx3* WT mice (n = 5). Time course of progenitor cell numbers in 20 µL PB after in vivo G-CSF stimulation (300 µg/kg/day for 4 days) is shown. Mean ± SD of CFU-C numbers is shown. Asterisks represent significant differences (*$P <$ .05; ***$P < .001$, Student *t* test).

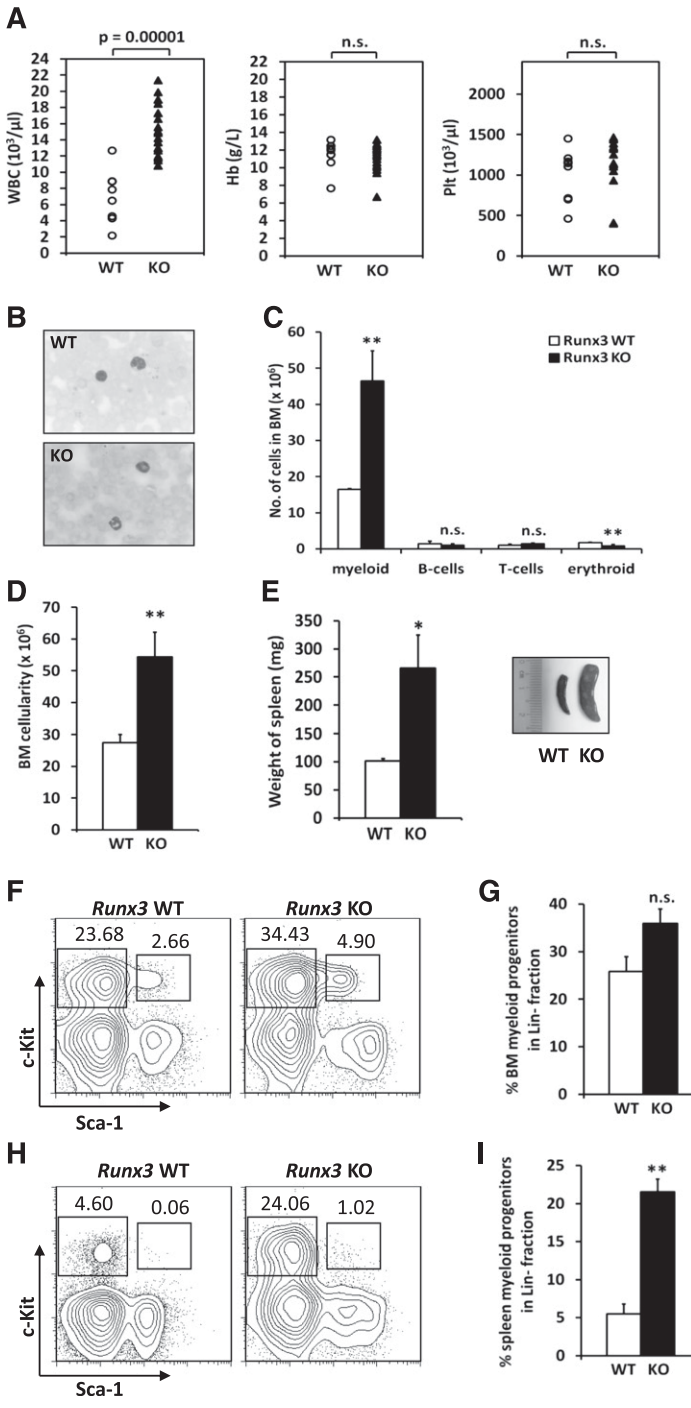

Figure 2. Aged *Runx3* KO mice shows enhanced myeloproliferation with an expanded HSPC compartment. (A) Complete blood counts performed on 18-month-old *Runx3* WT (n = 9) and *Runx3* KO mice (n = 21). WBC, Hb, and Plt counts are shown. The statistical significance (*P* value, Student *t* test) is shown at the top. (B) May-Grünwald-Giemsa staining of PB cells in aged mice. Representative pictures of cells are shown. (C) Number of cells of each lineage in BM of 18-month-old mice. Lineage markers: myeloid, Mac-1$^+$ and Gr-1$^+$; B-cells, B220$^+$CD19$^+$; T cells, CD3$^+$; erythroid, Ter119$^+$. Mean ± standard deviation (SD) is shown (WT, n = 2; KO, n = 4). Three independent experiments were performed. Asterisks represent significant difference (**$P < .01$, Student *t* test). (D) BM cellularity of aged mice. Mean ± SD of the numbers of cells in BM are shown (WT, n = 2; KO, n = 4). Asterisks represent significant difference (**$P < .01$, Student *t* test). (E) Spleen of aged *Runx3* KO mice. (Left) Graphical representation of spleen weight in the aged mice. Mean ± SD is shown (WT, n = 2; KO, n = 4). Asterisk represents significant difference (*$P < .05$, Student *t* test). Three independent experiments were performed. (Right) Representative pictures of spleens in the aged mice. (F,H) Flow cytometric analysis of the HSPC compartment in (F) BM and (H) spleen of 18-month-old mice. Representative FACS plots of 200 000 cells gated on viable Lineage$^-$ cells are shown. (G,I) Graphical representations of results presented in F and H are shown in G and I, respectively. Mean ± SD of percentage of c-Kit$^+$Sca-1$^-$Lineage$^-$ (myeloid progenitors) and KSL within the BM Lineage$^-$ population is shown (WT: n = 2; KO: n = 4). Three independent experiments were performed. (J) Time course PB chimerism analysis of recipient mice after BMT. CD45.1/CD45.2 competitive cells were cotransplanted with *Runx3* WT or KO BM cells (CD45.2/CD45.2) into CD45.1/CD45.1 sublethally irradiated (8 Gy) mice in a 1:1 ratio. Percentage contribution of CD45.2/CD45.2 donor cells to total PB, myeloid, B-cell, and T-cell populations at indicated time points after BMT are shown. Mean ± SD is shown (WT, n = 3; KO, n = 6). Five of 8 mice receiving *Runx3* WT donor cells and 3 of 9 mice receiving *Runx3* KO donor cells that failed to show contribution of CD45.2/CD45.2 cells to PB of recipient mice are excluded. Asterisk represents significant differences (*$P < .05$, Student *t* test).

and *Runx3* wild-type [WT] mice, respectively). Deletion of the *Runx3* locus was nearly complete in the BM of *Runx3* KO mice (supplemental Figure 1A).

Runx3 inactivation in young adult mice does not result in gross hematological abnormalities but leads to enhanced proliferative and mobilization ability of HSPCs when stimulated

Young *Runx3* KO mice (6-8 weeks old) did not display gross hematopoietic abnormalities (supplemental Figure 2), evidenced by normal white blood cell (WBC), hemoglobin (Hb), and platelet (Plt) counts. Consistent with these findings, flow cytometric analysis showed no significant changes in various lineage populations, namely the myeloid, lymphoid, and erythroid lineages. There were no significant differences in BM cellularity, spleen weight, and thymus weight between *Runx3* KO and *Runx3* WT mice.

The HSPC compartment, immunophenotypically defined as a c-Kit$^+$Sca-1$^+$Lin$^-$ (KSL) population, in the BM of *Runx3* KO mice exhibited a slight increase, although this was statistically not significant ($P = .11$, Student *t* test; supplemental Figure 2D). In addition, a trend toward an increase in short-term HSC frequency with a marginal decrease in long-term HSC frequency in *Runx3* KO mice was observed (supplemental Figure 2E). Interestingly, a significant increase in

CFU-C activity was observed in cells from *Runx3* KO mice (Figure 1A-E). The increased CFU-C activity was further pronounced after the replating assay (Figure 1B-D). Notably, *Runx3* KO cells do not replate for a longer period of time compared with the WT cells. These results suggest that that *Runx3* KO mice have enhanced CFU-C forming capacity of HSPCs compared with their WT littermates.

HSPC analysis in the spleen of *Runx3* KO mice revealed a marginal increase in the KSL and myeloid progenitor (c-Kit$^+$Sca-1$^-$Lin$^-$) populations (Figure 1F-G). As increased HSPC numbers in the spleen is indicative of an enhanced mobilization ability, a mobilization assay was conducted using G-CSF, which is widely known to enhance the mobilization of HSPCs.[17] The result showed that the number of CFU-Cs in the PB of *Runx3* KO mice was significantly greater than that in *Runx3* WT mice at all time points (Figure 1H). Therefore, *Runx3* KO HSPCs appear to be easily mobilized into the periphery by G-CSF.

Aged *Runx3* KO mice show enhanced myeloproliferation with an expanded HSPC compartment

To explore whether the hematopoietic abnormalities will be enhanced over time, *Runx3* KO mice were aged to 18 months old. Deletion of the *Runx3* locus in BM cells from aged *Runx3* KO mice remained almost complete (supplemental Figure 1B). There was a significant increase in WBC counts in aged *Runx3* KO mice compared with WT controls, whereas Hb and Plt counts remained unchanged (Figure 2A). Notably, the leukocytosis was dominated by a population of myeloid cells, characterized by abundant granulocytic cells in the PB and an increase in Mac-1$^+$ and Gr-1$^+$ populations in the BM and spleen of aged *Runx3* KO mice (Figure 2B-C and data not shown). Analysis of other hematopoietic lineages in the BM revealed a significant decrease in the erythroid lineage (Figure 2C). These changes were accompanied by significant increases in BM cellularity and spleen weight (Figure 2D-E). HSPC analysis in aged *Runx3* KO mice showed an increase of KSL and myeloid progenitor populations in BM and spleen compared with aged *Runx3* WT mice (Figure 2F-I). Although the frequency of KSL in BM remained unchanged, which is thought to be attributable to the decreased Lin$^-$ fraction in *Runx3* KO BM, the absolute number of KSL cells was significantly increased in the aged *Runx3* KO mice (supplemental Figure 3A-C). Analysis of the proliferative ability of KSL cells by Ki-67 staining revealed a tendency toward more Ki-67$^+$ cells in *Runx3* KO mice, suggesting that the increased HSPCs in aged mice is probably due to enhanced proliferation in a subtle but sustained manner (supplemental Figure 3D). Together, these results suggest that the increased WBC count and increased BM cellularity were due to an expansion of KSL and myeloid progenitor compartments and myeloid cells. Of note, development of spontaneous leukemia was not observed in aged *Runx3* KO mice.

The *Runx* family genes show a high degree of conservation in their amino acid sequences within the family and share the same capability to bind to their consensus DNA sequence. Therefore, the observation that Runx3 function was not fully unveiled in young *Runx3* KO mice may be attributable to functional compensation among *Runx* family genes. However, *Runx1* expression levels were not significantly altered in both young and aged *Runx3* KO mice (supplemental Figure 3E). However, aged *Runx3* KO mice partially but clearly phenocopied *Runx1* KO mice, implying that *Runx3* per se is also a critical factor for hematopoiesis. Although a clear increase of KSL fraction was observed in young *Runx1* KO mice,[6] a mild myeloid proliferation phenotype was only occasionally observed in aged *Runx1* KO mice.[11,13] In contrast, all aged *Runx3* KO mice examined showed a myeloproliferative status accompanied by a modest HSPC expansion. Consistent with the higher penetrance of MPD in *Runx3* KO mice than in *Runx1* KO mice, HSC exhaustion was not observed in the *Runx3* KO mice, unlike *Runx1* KO mice, as *Runx3* KO BM cells transplanted into WT recipient mice were not outcompeted by WT competitor cells at different doses (test: competitor ratios of 1:1, 2:1, and 4:1) in BMT experiments. In fact, transplantation with *Runx3* KO donor cells resulted in greater chimerisms and higher WBC counts in the recipient mice, as opposed to mice transplanted with *Runx3* WT BM cells (Figure 2J; supplemental Figure 3F; data not shown), implicating that *Runx1* and *Runx3* seem to play distinct roles in regulating the HSC behavior. The BMT result also suggests that the increase of phenotypic HSCs in aged *Runx3* KO mice corresponds to an increase of functional HSCs and that the phenotypes observed were due to cell autonomous mechanisms. Differentiation blocks in megakaryocytic and lymphoid lineages, which were observed in *Runx1* KO mice, were not evident in *Runx3* KO mice. Intriguingly, *Runx3*-deficient HSPCs showed enhanced HSPC mobilization suggested by hypersensitivity to G-CSF, like *RUNX1* haploinsufficient status, which is known to cause familial Plt disorder with predisposition to acute myeloid leukemia (AML).[18]

Human data further implicate *RUNX3* in leukemogenesis. Deletion in chromosome 1p36, containing the *RUNX3* locus, is frequently observed in human leukemia cells.[19-21] Methylation of the *RUNX3* promoter in AML has also been reported,[22] and RUNX3 expression is down-regulated in AML with t(8;21) or inv(16).[23,24] Collectively, our work represents the first direct evidence for the pivotal role of *Runx3* in hematopoiesis. Further analysis of *Runx3* would provide us with deeper insight into leukemogenesis and may lead to future drug development.

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Authorship

Contribution: C.Q.W. performed experiments, analyzed data, and wrote the manuscript; L.M. performed experiments; M.S., Y.I., and I.T. provided research tools; M.O. and V.T. designed research and wrote the manuscript; and C.Q.W., L.M., M.S., Y.I., I.T., M.O., and V.T. reviewed and approved the manuscript.

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