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Hematopoietic Stem Cell Expansion Precedes the Generation of Committed Myeloid Leukemia-Initiating Cells in C/EBPα Mutant AML

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SUMMARY

We here use knockin mutagenesis in the mouse to model the spectrum of acquired *CEBPA* mutations in human acute myeloid leukemia. We find that C-terminal C/EBPα mutations increase the proliferation of long-term hematopoietic stem cells (LT-HSCs) in a cell-intrinsic manner and override normal HSC homeostasis, leading to expansion of premalignant HSCs. However, such mutations impair myeloid programming of HSCs and block myeloid lineage commitment when homozygous. In contrast, N-terminal C/EBPα mutations are silent with regards to HSC expansion, but allow the formation of committed myeloid progenitors, the templates for leukemia-initiating cells. The combination of N- and C-terminal C/EBPα mutations incorporates both features, accelerating disease development and explaining the clinical prevalence of this configuration of *CEBPA* mutations.

INTRODUCTION

A large body of evidence indicates that acute myeloid leukemia (AML) arises through the stepwise acquisition of genetic and epigenetic changes. In particular, tumorigenesis is promoted by the collaboration between mutations affecting signaling pathways (class I or signaling mutations; *FLT3*, *RAS* and *KIT* mutations) and mutations with a transcriptional/nuclear function (class II or initiating mutations, balanced translocations, and *CEBPA* and *NPM1* mutations) (Kelly and Gilliland, 2002; Moore, 2005). Because the hematopoietic stem cell (HSC) is the only long-lived cell within the myeloid differentiation hierarchy, it is the primary candidate cell for the accumulation of these successive leukemogenic events. Studies of AML patients relapsing from complete remission after chemotherapy are consistent with class II (or initiating) mutations, including balanced translocations and *CEBPA* mutations, being very early events in this process, because these events are generally conserved in the relapse tumor (Shih et al., 2006). In contrast, *KIT*, *FLT3,* and *RAS* mutations (class I or signaling mutations) frequently differ between initial and relapse tumors (Kottaridis et al., 2002; Shih et al., 2002, 2004), indicating that they occur subsequently to the highly conserved class II mutations. However, despite the inferred founding nature of class II mutations, limited information exists about their effect on HSCs, and in particular their ability to cause expansion of premalignant stem cell populations, thereby increasing the probability of acquisition of collaborating genetic/epigenetic events.

This issue has been highlighted by the recent controversy regarding the role of the HSC compartment in AML maintenance. The original observations by Dick and colleagues identified the leukemia-initiating cell (LIC) as $CD34^{+}CD38^{-}$ (Bonnet and Dick, 1997; Lapidot et al., 1994), consistent with an immature

SIGNIFICANCE

Acquired mutations in the *CEBPA* gene occur in ca. 9% of human AML patients. However, so far no explanation existed for the observation that ca. 90% of patients have distinct mutations on the two *CEBPA* alleles. Our findings provide a cellular basis for the observed clinical mutation pattern. Significantly, the leukemia-initiating cells that develop in mice with HSC expansion are committed progenitors, suggesting that leukemogenic mutations may have crucial functions in cell types not found in the final transformed clone. Finally, we demonstrate that impaired myeloid lineage commitment of *Cebpa* mutant HSCs is preceded by a loss of myeloid gene expression at the HSC level, providing a link between myeloid programming of HSCs and their lineage potential.

HSC-like phenotype. Here mutations that increase HSC proliferation and/or self-renewal would provide a selective advantage to premalignant HSCs, and in cooperation with second hits lead to transformation. However, recent results indicate that the lack of detectable LIC activity in the $CD34^+CD38^+$ progenitor fraction was due primarily to Fc-mediated immune clearance of anti-CD38 conjugated cells. If this is circumvented, LICs with a myeloid progenitor phenotype ($CD34^+CD38^+$) are more abundant in the majority of AML cases studied (Taussig et al., 2008). In such a scenario, premalignant HSC expansion, lineage commitment, and target cell transformation could become rate-limiting features of initiating mutations, requiring the effects of oncogenic mutations on several aspects of hematopoiesis to be examined. For proper assessment of these effects, the disease models need to model the human disease as accurately as possible. As an example, viral expression of MLL-AF9 allows leukemia initiation from a committed granulocyte-macrophage progenitor (GMP) (Cozzio et al., 2003), whereas a knockin *Mll-Af9* allele, driven by the endogenous *Mll1* promoter, could initiate AML only from HSCs (Chen et al., 2008), presumably because of the low activity of the *Mll1* promoter in myeloid progenitors.

This consideration is particularly relevant in the case of *CEBPA* mutations because deletion of the *Cebpa* gene from murine HSCs was found to make HSCs more competitive against wild-type HSCs (Zhang et al., 2004). Although this would be consistent with *CEBPA* mutations having a similar function in preleukemic HSCs, such an interpretation is complicated by the finding that leukemia-derived *CEBPA* mutations are virtually never null mutations. Rather, *CEBPA* mutations are divided into two major groups: N-terminal mutations that block the translation of the 42 kDa isoform (p42) while allowing the 30 kDa isoform (p30) to be expressed and C-terminal mutations that generate in-frame insertions/deletions within the basic region-leucine zipper DNA binding domain (Leroy et al., 2005; Nerlov, 2004). Notably, >90% of leukemias with bialleleic *CEBPA* mutations harbor one allele with a C-terminal mutation and one with an N-terminal mutation, indicating selection of this mutation pattern during tumorigenesis. In contrast, tumors with homozygous N- or C-terminal mutations are comparatively rare (Pabst and Mueller, 2007). This indicates that the two mutations provide distinct leukemogenic functions and thus are able to collaborate. The importance of specific alterations of C/EBPα function is further supported by the finding that complete deletion of *Cebpa* does not lead to leukemia in the mouse (Heath et al., 2004; Zhang et al., 2004). C/EBPα is generally considered an inducer of terminal differentiation due to its ability to couple induction of cell-type-specific gene expression to cell-cycle arrest (Nerlov, 2007), and in the mouse *Cebpa* mutations that impair E2F repression by C/EBPα increase myeloid progenitor proliferation (Kirstetter et al., 2008; Porse et al., 2005) and impair terminal granulopoiesis (Porse et al., 2001). Complete deletion of *Cebpa* blocks the formation of GMPs and thus myeloid lineage commitment (Zhang et al., 2004). However, despite the relevance of C/EBPα to HSC function, little is known about its role in HSC gene expression, and how this may impact proliferation, lineage commitment, and leukemogenesis. In particular, the complex nature of AML-derived *CEBPA* mutations indicates a need to understand the specific impact of patient-derived mutant alleles on HSC homeostasis and the myeloid differentiation pathway.

RESULTS

To resolve this question, we decided to generate an accurate mouse model for the spectrum of *CEBPA* mutations occurring in AML (Figure 1A). We generated a knockin allele mimicking one of the common C-terminal mutations (K313 duplication: K313KK or K allele; Figure 1B). By combining this mutation with an existing knockin of an N-terminal mutation (Lp30 or L allele [Kirstetter et al., 2008]) (Figure 1B), the spectrum of patient mutations could be generated. Fetal liver (FL) cells from K/K, K/L, L/L, and +/+ mice (CD45.2 allotype) were competitively transplanted into lethally irradiated CD45.1/2 recipients along with CD45.1 wild-type competitor bone marrow cells (Figure 1C). In this manner, lethality due to dysfunction of *Cebpa* mutant cells was avoided, and mutant HSC behavior could be studied while competing with wild-type HSCs, a situation more similar to that encountered in the premalignant state. Importantly, the frequency of phenotypic HSCs in the FL, measured either as Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ cells (LSK cells; data not shown) or LSK $AA4.1^+$ cells (repopulating HSC fraction (Jordan et al., 1995); Figure 1D), did not differ between the mutant genotypes.

Analysis of mice 4.5 weeks after competitive transplantation revealed that the frequency of LSK cells was increased relative to +/+ controls in K/L and K/K (but not L/L) mice, indicating loss of HSC homeostasis (Figure 2A). This increase was due to specific expansion of the mutant HSC pool (Figure 2B), demonstrating a cell-intrinsic effect of these *Cebpa* mutations. Fractionation of the experimental ($CD45.2^+CD45.1^-$) HSC compartment using CD150 and Flt3 expression (Figure 2C) showed expansion of all phenotypic HSC subsets, most significantly the short-term (ST)-HSC ($CD150^-Flt3^-$) and lymphoid-primed multipotent progenitor (LMPP) ($CD150^-Flt3^+$) populations, although mild expansion was observed also of long-term (LT)-HSCs ($CD150^+$ $Flt3^-$) (Figure 2D). Interestingly, K/+ HSCs did not expand in this setting (see Figure S1 available online), suggesting that mutation of both *Cebpa* alleles is required for the effect of C-terminal C/EBPα mutation on HSC expansion to take effect. Importantly, *Cebpa* mRNA was expressed in all three HSC fractions (Figure 2E), consistent with the effects of *Cebpa* mutation being cell intrinsic.

To probe the molecular basis for the observed HSC expansion, we performed Affymetrix-based global gene expression analysis on sorted $CD45.2^+CD45.1^-$ LSK cells from competitively transplanted animals. LIMMA analysis of differentially expressed genes (Table S1) followed by unsupervised clustering showed that the nonhomeostatic K/L and K/K LSK populations coclustered, as did the L/L and +/+ LSK cells (Figure 3A). Gene expression signatures of expanding HSC populations have been generated by microarray analysis of the quiescence-proliferation-quiescence sequence induced by 5-fluorouracil treatment (Venezia et al., 2004). Using gene set enrichment analysis (GSEA), we observed that the expression of quiescence-associated genes (Q-Sig) was significantly decreased in expanding (K/K and K/L) relative to homeostatic (L/L and +/+) LSK cells, whereas no significant enrichment of proliferation-associated gene expression was observed (Figure 3B). Comparison of individual mutant LSK populations to +/+ LSK cells showed that K/K and K/L LSK cells, but not L/L LSK cells, were depleted of Q-Sig genes (Figure 3C). Because the LT-HSC population gives rise to