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Oncogenic Kras Initiates Leukemia in Hematopoietic Stem Cells

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How oncogenes modulate the self-renewal properties of cancer-initiating cells is incompletely understood. Activating KRAS and NRAS mutations are among the most common oncogenic lesions detected in human cancer, and occur in myeloproliferative disorders (MPDs) and leukemias. We investigated the effects of expressing oncogenic KrasG12D from its endogenous locus on the proliferation and tumor-initiating properties of murine hematopoietic stem and progenitor cells. MPD could be initiated by KrasG12D expression in a highly restricted population enriched for hematopoietic stem cells (HSCs), but not in common myeloid progenitors. KrasG12D HSCs demonstrated a marked in vivo competitive advantage over wild-type cells. KrasG12D expression also increased the fraction of proliferating HSCs and reduced the overall size of this compartment. Transplanted KrasG12D HSCs efficiently initiated acute T-lineage leukemia/lymphoma, which was associated with secondary Notch1 mutations in thymocytes. We conclude that MPD-initiating activity is restricted to the HSC compartment in KrasG12D mice, and that distinct self-renewing populations with cooperating mutations emerge during cancer progression.

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Introduction

Self-renewal is integral to the malignant phenotype [1]. In principle, the ability of cancer cells to self-renew may be intrinsic to the compartment in which the tumor-initiating mutation occurs, or may be acquired as a consequence of mutations in more differentiated cells. The hematopoietic system has proven highly informative for addressing how cancer-associated mutations and cell of origin interact to establish malignant self-renewing populations. Accumulating evidence supports the idea that many hematopoietic malignancies exist in a hierarchy of differentiation with only a minor population capable of propagating and maintaining the disease in vivo [2]. These cells are termed leukemia-initiating cells or leukemia stem cells (LSCs), and manifest some biologic properties of normal hematopoietic stem cells (HSCs). However, the precise relationship between these populations is uncertain and appears to depend, in part, on both the leukemia subtype and on the effects of specific mutations. For example, overexpressing MLL fusion proteins found in human acute myeloid leukemia transforms both murine HSCs and more differentiated progenitors [3,4]. By contrast, inactivation of the JunB transcription factor must occur in the HSC compartment for initiation of myeloid malignancies [5]. These proof-of-concept experiments underscore the importance of understanding how oncogenes and tumor suppressors that are commonly mutated in human cancers perturb self-renewal and growth control. Importantly, the functional characteristics of LSCs that distinguish them from HSCs and how these properties are modulated by oncogenes are poorly understood.

RAS gene mutations are highly prevalent in pancreatic (>80%), colorectal (40%–50%), endometrial (40%), lung (30%), and cervical cancers (20%–30%), as well as in myeloid malignancies (20%–40%) [6]. Of the genes in the canonical RAS family, KRAS accounts for ~90% of cancer-associated mutations, whereas HRAS mutations are rare. In hematologic cancers, NRAS is mutated 2–3 times more often than KRAS [6]. Cancer-associated RAS mutations, which introduce amino acid substitutions at codons 12, 13, or 61, result in oncogenic RAS proteins that accumulate in the active, GTP-bound conformation because of defective guanine nucleotide hydrolysis [7]. Elevated levels of GTP-bound Ras, in turn, deregulate signaling in cancer cells by altering the activation of effector cascades that include the Raf/MEK/ERK, phosphatidylinositol 3-kinase (PI3K)/Akt, and Ras-GDS pathways [8].

Chronic and juvenile myelomonocytic leukemias (CML and JMML) are aggressive myeloid malignancies that are classified as myeloproliferative disorders (MPDs) [9]. Both diseases are characterized by leukocytosis with excess monocytes in blood and bone marrow, and by significant infiltration of malignant myeloid cells into the liver, spleen, and other organs. Hyperactive Ras is strongly implicated in the pathogenesis of JMML and CML. Somatic NRAS and KRAS

Abbreviations: 2-AAD, 2-aminoacetonic acid; CFU-GM, granulocyte-macrophage colony forming unit; CFU-S, day 8 spleen colony-forming unit; CML, chronic myelomonocytic leukemia; CMP, common myeloid progenitor; DN, double negative; HSC, hematopoietic stem cell; JMML, juvenile myelomonocytic leukemia; LSK, leukemia stem cell; LSK, Lin−/loSca1−/c-kithi; NSCL, NSCL, lung Sca1−/C0; pIpC, polyinosinic-polycytidylic acid; SEM, standard error of the mean; T-ALL, T lineage acute lymphoblastic leukemia/lymphoma; WT, wild type; YFP, yellow fluorescent protein

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Author Summary

Ras proteins act as molecular switches that relay growth signals from outside the cell. This mechanism is often subverted in cancer, and Ras proteins are activated directly by RAS gene mutations in approximately one-third of human malignancies. We have modeled this in mice engineered to have a Ras mutation. These mice develop a disease similar to chronic leukemias in humans called myeloproliferative disorders. It is marked by a fatal accumulation of mature and immature cells in the blood and bone marrow. We investigated whether some or all of these neoplastic cells were immortal. In agreement with the "cancer stem cell" hypothesis, we found that immortal cells were extremely rare in the bone marrow of diseased mice. They were found only in the same cell populations that contain normal bone marrow stem cells. However, these cells had high rates of replication and produced large numbers of daughter cells. Furthermore, many mice went on to develop acute lymphoid leukemia after acquiring additional mutations in maturing lymphoid cells. These studies exemplify the evolution of malignant stem cells during cancer progression. They also highlight the importance of rare, long-lived cells in the genesis and, potentially, therapy of high-risk chronic leukemias caused by abnormal Ras proteins.

Results

A Small Kras\textsuperscript{G12D} Population Dominates Both Primitive and Differentiated Hematopoietic Compartments in Mx1-Cre, Kras\textsuperscript{LSL-G12D} Mice

Although basal Mx1-Cre activity is low \cite{18,19}, many Mx1-Cre, Kras\textsuperscript{LSL-G12D} mice that are not injected with pIpC ultimately succumb with MPD \cite{14} and unpublished data). This observation suggests that HSCs and/or progenitor cells that activate Kras\textsuperscript{G12D} expression have a substantial proliferative advantage in vivo. To assess the kinetics of this process, we analyzed recombination in the myeloid progenitors of young Mx1-Cre, Kras\textsuperscript{LSL-G12D} mice that were not treated with pIpC. Bone marrow cells from 3–5-wk-old animals were plated in methylcellulose medium to enumerate granulocyte-macrophage colony forming unit progenitors (CFU-GM), and individual myeloid colonies were genotyped by PCR. Surprisingly, nearly all CFU-GM in untreated animals were recombined as early as 3 wk of age (Figure 1A). We then examined more primitive populations in Mx1-Cre, Kras\textsuperscript{LSL-G12D} mice crossed to a ROSA26 yellow fluorescent protein (YFP) reporter strain (Figure 1B) \cite{20}. In these mice, cells expressing Cre are identified by YFP expression. The frequency of YFP+ cells in wild-type (WT) mice was within the expected range of background Cre expression \cite{18,19}. However, we found a much higher incidence of YFP expression in bone marrow cells of Mx1-Cre, Kras\textsuperscript{LSL-G12D}, ROSA26-YFP mice. This result was consistent among all populations analyzed, including the primitive Flk2\textsuperscript{−}Lin\textsuperscript{−}Sca1\textsuperscript{+}c-kit\textsuperscript{+} (Flk2\textsuperscript{−}LSK) compartment, which is highly enriched for HSCs \cite{21}. To confirm that YFP expression correlated with Kras\textsuperscript{G12D} expression, we also directly genotyped colonies formed by single Flk2\textsuperscript{−}LSK cells. This method again revealed a predominance of Kras\textsuperscript{G12D} expressing cells in naive Mx1-Cre, Kras\textsuperscript{LSL-G12D} mice (Figure 1C). Together, these data are consistent with an advantage for Cre-expressing cells in untreated Mx1-Cre, Kras\textsuperscript{LSL-G12D} mice.

Increased Cell Cycle Entry of Kras\textsuperscript{G12D} HSCs

The apparent outgrowth of Kras\textsuperscript{G12D} Flk2\textsuperscript{−} LSK cells in mice that were not injected with pIpC suggested that Kras\textsuperscript{G12D} expression might increase proliferation in this compartment. To test this hypothesis, we stained bone marrow cells collected from Kras\textsuperscript{G12D} mice and WT littermates 2 wk after pIpC treatment with antibodies to cell surface proteins and with dyes that stain DNA and RNA (7-aminoactinomycin D [7-AAD] and pyronin Y, respectively). These studies revealed a significant reduction in the number of quiescent Flk2\textsuperscript{−}LSK cells in Kras\textsuperscript{G12D} animals, which are identified by having a 2n DNA content and low pyronin Y staining (Figure 2A and 2B). Whereas the Flk2\textsuperscript{−} subset of WT LSK contains roughly 80% cells in the G0 phase of the cell cycle, Kras\textsuperscript{G12D} Flk2\textsuperscript{−} LSK are only 50% quiescent. As an initial exploration of mechanisms
regulating cell cycle progression, we analyzed D- and E-type cyclin expression by quantitative PCR in sorted Flk2^+ LSK cells from Kras^G12D^ mice and WT mice. Kras^G12D^ Flk2^+ LSK cells had significantly higher expression of cyclin D1 (Figure 2C).

Self-Renewal Is Restricted to Primitive Hematopoietic Cells in Kras^G12D^ Mice

Bone marrow mononuclear cells from Kras^G12D^ mice display a hypersensitive pattern of CFU-GM progenitor growth, which is a cellular hallmark of JMML [12–14]. We found that this abnormal CFU-GM activity resides primarily in the common myeloid progenitor (CMP) compartment, and that these cells demonstrate enhanced proliferation in vivo ([22] and unpublished data). To investigate if this population could initiate and maintain MPD, we collected Lin^−^Scal^−^c-kit^+^CD34^+^FcγRII/III^+^ CMPs from 5-wk-old Kras^G12D^ mice and WT littermates by FACs, and transferred 10,000 of these cells into lethally irradiated recipients with 10^7^ WT marrow cells for radioprotective support. Transplanted tester cells, recipient cells, and support cells were marked by expression of different isoforms of CD45, allowing them to be distinguished by flow cytometry. Transplanted Kras^G12D^ or WT CMPs demonstrated robust day 8 spleen colony-forming unit (CFU-S8) activity, with colony size somewhat larger for Kras^G12D^ input CMPs (Figure 3A). However, we detected less than 0.1% of circulating myeloid cells derived from transplanted Kras^G12D^ or WT CMPs 1 mo after transplantation (Figure 3B and 3C). As expected from previous studies [23,24], Kras^G12D^ and WT CMPs made minor contributions to the circulating B cell compartment with a statistically insignificant trend towards greater B cell production from Kras^G12D^ cells. Taken together, these data indicate that Kras^G12D^ CMPs do not initiate a hematologic disease. By contrast, transferring 500 Flk2^−^ LSK cells from Kras^G12D^ animals into lethally irradiated recipients rapidly resulted in durable multilineage reconstitution. Kras^G12D^ cells dominated the T cell and B cell compartments sooner and to a higher degree than the progeny of WT Flk2^−^ LSK cells in control animals. The myeloid series demonstrated a more variable time course, but Kras^G12D^ derived cells also eventually out-competed WT cells (Figure 4).

Recipients of Kras^G12D^ Flk2^−^ LSK cells that were euthanized 3 mo after transplantation had mild to moderate MPD, manifested as leucocytosis and splenomegaly with myeloid and erythroid infiltration (Figure 5). As discussed below, we also found that all recipients of Kras^G12D^ Flk2^−^ LSK cells developed T-ALL 2–4 mo after adoptive transfer. Although early mortality from T-ALL precluded analyzing recipient mice beyond 3 mo, Kras^G12D^ Flk2^−^ LSK cells recapitulate the essential features of MPD seen in the original Mx1-Cre, Kras^LSL-G12D^ model [13,14].

HSCs Are Less Frequent in Kras^G12D^ Mice but Have Enhanced Repopulating Ability

We next asked how Kras^G12D^ expression affects HSC function. Immunophenotypic analysis revealed a 2-fold reduction in the number of marrow Flk2^−^ LSK cells in Kras^G12D^ mice 2 wk after plpC injection (Figure 6A). This was mostly offset by an increased number of splenic Flk2^−^ LSK cells. The reduction in marrow Flk2^−^ LSK cells persisted in older animals (Figure S2). We also performed limited dilution studies to assess functional HSC activity. In these experiments, lethally irradiated recipients received decreasing numbers of whole bone marrow cells from either Kras^G12D^ mice or WT littermates that had been injected with plpC 2 wk earlier. Recipients were bled monthly, and flow cytometry was performed to assess whether CD45.1^+^ donor cells were able to provide durable (>2 mo) multilineage (myeloid, B cell, and T cell) engraftment. These studies demonstrated a striking 10-fold decrease in the number of long-term repopulating stem cells in Kras^G12D^ animals compared to the WT littermate controls (Figure 6B; Table S1).

Progression of MPD despite a reduction in the size of the HSC compartment suggests an increased production of mature cells by each Kras^G12D^ HSC. To address this possibility, we examined the patterns of reconstitution from either

Figure 1. Spontaneous Kras^G12D^ Activation in CFU-GM and HSCs from Mx1-Cre, Kras^LSL-G12D^ Mice

(A) Bone marrow was harvested at 24 d or 5 wk of life from mice that either received a single dose of plpC at 21 d of life or were left untreated, and plated in methylcellulose medium with GM-CSF. Individual colonies were isolated and genotyped by PCR to assess for Cre-mediated excision of the LSL cassette (n = 18 colonies from two uninjected animals, and 34 colonies from two injected animals, compiled from two independent experiments; error bars show SEM).

(B) Mx1-Cre (filled bars) and Mx1-Cre, Kras^LSL-G12D^ (open bars) mice that inherited an LSL-YFP reporter were sacrificed at 5 wk of life, without ever being injected with plpC. Expression of YFP requires excision of the LSL cassette from the reporter gene by Cre recombinase. The percentage of YFP^+^ cells in all bone marrow cells (Bulk), Lin^−^c-kit^+^Sca1^+^ myeloid progenitors (MP), LSK cells, and Flk2^+^ LSK cells was assessed by flow cytometry. (n = 4 for WT and for Mx1-Cre, Kras^LSL-G12D^ mice compiled from three independent experiments; error bars show SEM).

(C) Flk2^+^ LSK cells were sorted from bone marrow of 4-wk-old Mx1-Cre, Kras^LSL-G12D^ mice that had not received plpC, and plated in methylcellulose medium for a culture period of 14 d. Individual colonies were genotyped by PCR (n = 44 colonies from 6 mice in 3 independent experiments; error bar shows SEM).

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or WT HSCs in mice that received the limit dilution dose (1×10^5 Kras^{G12D} and 1×10^4 WT bone marrow cells). As ~50% of these recipients were engrafted with donor cells, the Poisson distribution predicts that approximately 70% of the engrafting mice received a single HSC and ~24% received two HSC. By 2 mo after transplantation, donor Kras^{G12D} marrow cells made a markedly greater contribution to recipient hematopoiesis than WT cells (Figure 6C).

We were able to compare repopulation of the stem cell compartment by WT and Kras^{G12D} HSCs in a few lethally irradiated recipient mice before the onset of T-ALL. In these experiments, lethally irradiated recipients (CD45.2) were reconstituted with equal numbers of Kras^{G12D} (CD45.1) and WT (CD45.1) Flk2^-LSK cells, as well as 10^6 CD45.2 whole bone marrow cells (CD45.2) for radioprotection. Whereas only one of six recipients euthanized 2 mo after transplantation showed a clear bias towards Kras^{G12D}-derived Flk2^-LSK cells, mice that survived for 3 mo without evidence of diffuse T-ALL demonstrated an overwhelming bias towards Kras^{G12D}-derived Flk2^-LSK cells, myeloid progenitors, and mature myeloid cells (Figure S3).

**T-ALL Arises from Transplanted Kras^{G12D} HSCs in Thymocytes That Acquire Somatic Notch1 Mutations**

Recipient mice that were injected with Kras^{G12D} Flk2^-LSK cells, either alone or in a 1:1 ratio with WT Flk2^-LSK cells, uniformly became moribund 8–14 wk after transplantation.

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**Figure 2. Increased Proliferation of Kras^{G12D} HSC**

Bone marrow from 5-wk-old WT and Kras^{G12D} mice was stained with 7-AAD and pyronin Y (PY) for DNA and RNA quantitation, along with surface markers for Flk2^-LSK cells.

(A) Gating is shown for cell cycle analysis of WT and Kras^{G12D} Flk2^-LSK cells.

(B) Summary of replicate samples from WT (closed circles) and Kras^{G12D} (open circles) mice (n = 3 or 4 as shown; compiled from two independent experiments). Means and SEM are WT: G0 81.8 ± 4.72, G1 5.66 ± 1.76, S-G2-M 11.7 ± 2.56; and Kras^{G12D}: G0 43.6 ± 3.19, G1 34.9 ± 4.85, S-G2-M 21.4 ± 3.14. p-Values by unpaired t-test are indicated: ***, p < 0.001; **, p < 0.01; *, p < 0.05.

(C) RNA from doubly sorted Flk2^-LSK cells was isolated, and quantitative PCR performed on cDNA to test expression levels of selected cyclins (n = 3 or 4 as shown; bar shows geometric mean). Results are expressed as fold change in expression compared to WT Flk2^-LSK cells, after normalization to β-actin expression. These results represent three or four independent experiments as shown, each performed with pooled bone marrow from three to five animals. The geometric mean of cyclin D1 expression is 2.4-fold over WT (95% confidence interval 1.2–4.9). Purity of sorted cells is shown in Figure S1.
Examination of euthanized mice revealed massive thymic enlargement with an arrest in T cell development at the CD4/CD8 double positive stage, and variable infiltration of blast cells within the liver, spleen, and bone marrow (Figure 7A). Importantly, two of three animals from the bone marrow limit dilution transplantation assay that were engrafted with a single Kras\textsubscript{G12D} repopulating unit developed T-ALL that was identical to that seen in animals repopulated with 500 Kras\textsubscript{G12D} Flk2\textsuperscript{–} LSK cells (unpublished data).

Next, we asked if T-ALL arose within the bone marrow or the thymus of mice transplanted with Kras\textsubscript{G12D} HSCs. To do this, we took advantage of our prior observation that recipients conditioned with sublethal irradiation fail to engraft with Kras\textsubscript{G12D} bone marrow [13]. Therefore, sublethally irradiated recipients can exclusively select for hematologic malignancies with more aggressive biologic behavior. We isolated bone marrow cells and thymocytes from primary recipients of Kras\textsubscript{G12D} Flk2\textsuperscript{–} LSK cells that developed T-ALL, and injected each population into sublethally irradiated secondary recipients (Figure 7B). As expected, no animals demonstrated multilineage engraftment or MPD. Animals transplanted with thymocytes quickly succumbed with an identical T-ALL as primary recipients; however, none of the mice transplanted with bone marrow developed leukemia. Thus, whereas bone marrow-derived Kras\textsubscript{G12D} HSCs efficiently give rise to T-ALL, the T-ALL LSC population is initially restricted to the thymus in primary recipient mice.

These results suggested that one or more secondary mutations might have developed in a novel T-lymphoid clone. Somatic NOTCH1 mutations are common in human and murine T-ALL [25–28]. To determine if a similar mechanism might contribute to the evolution of Kras\textsubscript{G12D} HSCs to T-ALL LSCs, we performed Western blot analysis to detect cleaved (activated) Notch1 protein. Cleaved Notch1 was observed in thymocytes from diseased primary recipients, but not in bone marrow cells (Figure 7C), a finding that is consistent with the secondary transplant data. Direct sequencing around the PEST domain uncovered frameshift mutations in exon 34 of Notch1 in thymocytes from five of six animals transplanted with Kras\textsubscript{G12D} Flk2\textsuperscript{–} LSK cells that developed T-ALL, but no mutations in thymocytes from control mice that received WT HSCs alone and remained well. The presence of a somatically acquired Notch1 mutation in a large fraction of the tumor provides a molecular indication of clonality.

Thymic Abnormalities in Kras\textsubscript{G12D} Mice

The propensity of Kras\textsubscript{G12D} HSCs to generate T-ALL led us to investigate the effects of Kras\textsubscript{G12D} expression in early T-
Figure 5. Transplanted Kras$^{G12D}$ Flk2$^{-}$ LSK Cells Induce MPD
Mice were analyzed 3 mo after transplantation with either Kras$^{G12D}$ or WT Flk2$^{-}$ LSK cells for evidence of MPD (n = 3 WT, n = 6 Kras$^{G12D}$ in a single experiment).
(A) Numbers of circulating leukocytes (WBC).
(B) Spleen weights.
(C) Percentages of myeloid cells (Mac1$^+$ and/or Gr1$^+$) in the spleen.
(D) Percentages of erythroid precursors (nucleated and TER119$^+$) in the spleen.
(E) A representative splenic section demonstrating infiltration by mature myeloid cells (ring-shaped nuclei) and erythroblasts (small dense nuclei).

Discussion

We find that oncogenic Kras$^{G12D}$ expression in HSCs confers a strong in vivo growth advantage, increases proliferation, and results in MPD and T-ALL. In MPD, as in normal marrow, stem cell activity is restricted to the Flk2$^{-}$ LSK population, which represents less than 0.1% of nucleated marrow cells. While pathologic behaviors of more mature cells may contribute to tissue infiltration, anemia, and organomegaly, self-renewal is confined to this very primitive population. Therefore, hyperactive Ras signaling promotes excess proliferation in multiple hematopoietic compartments without immortalizing non-self-renewing cells. Similar data have been described in murine models of MPD based on BCR-ABL overexpression or loss of JunB, both of which also deregulate cytoplasmic signaling networks [5,30]. By contrast, recent experiments have provided direct evidence that some oncogenic transcription factors allow committed myeloid progenitors to acquire self-renewal ability [3,4]. Taken together, studies of myeloid oncogenes performed to date support the general idea that mutations that predominantly alter cytoplasmic signaling networks and those that affect transcription factors controlling cell fate decisions comprise discrete complementation groups for the fully transformed phenotype [31,32].

Our limit dilution transplantation data demonstrate that oncogenic Kras confers a dramatic growth advantage in the HSC compartment. Under stringent conditions in which the contribution of a single WT HSC can barely be detected, the progeny of one (or at most three) Kras$^{G12D}$ HSC comprise a substantial fraction of the hematopoietic compartment. These studies provide direct experimental evidence that the outgrowth of malignant cells in MPD can be attributed to hyperactive Ras signaling in HSC. Our data suggest a pathogenic model in which JMML or CMML is initiated by a somatic mutation that deregulates Ras signaling in a single HSC. This idea is consistent with limited data from human patients and xenograft studies that implicate the HSC as the cell of origin for JMML [33–38].

To begin to address the mechanism by which mutant HSCs outgrow their WT counterparts, we analyzed the cell cycle in Kras$^{G12D}$ Flk2$^{-}$ LSK cells and found they are preferentially in cycle. The overexpression of cyclin D1 in Kras$^{G12D}$ cells we observed is consistent with many prior studies in cultured cell lines engineered to overexpress oncogenic Ras [39,40]. Intriguingly, HSCs in mice lacking D-type cyclins demonstrate severe proliferative defects and accumulate in the G0 and/or G1 phases of the cell cycle [41]. If increased cyclin D1 levels conversely result in excessive proliferation of HSC, then MPD in Kras$^{G12D}$ mice may be mediated in part by cyclin D1, similar to the requirement for cyclin D1 in a model of Ras-mediated breast cancer [42].

We demonstrate a substantial early growth advantage of HSCs that express Kras$^{G12D}$; however, it is also possible that oncogenic Ras expression has a negative long-term impact on HSC function. Increased proliferation or oncogenic stress may ultimately detract from self-renewal capacity. Reduced HSC fitness was observed in Pten$^{-}$ mice, in which phosphatidylinositol 3-kinase signaling is hyperactive [43,44]. Similar effects of Kras$^{G12D}$ are suggested by the reduced numbers of HSCs in Kras$^{G12D}$ mice, although this finding could also reflect changes in the composition of the Flk2$^{-}$ LSK population or cell-extrinsic effects related to alteration of the marrow microenvironment. The rapid demise of primary Kras$^{G12D}$ mice from MPD, and of transplant recipients from T-ALL, precluded serial transplantation experiments to test the long term fitness of Kras$^{G12D}$ HSC. In addition, the 5-fold discrepancy between HSC numbers that were measured by flow cytometry versus limit dilution transplantation suggests a defect in engraftment of Kras$^{G12D}$ HSC. This idea is consistent with a prior report in which retroviral transduction of mutant NRAS appeared to reduce engraftment
Data are pooled from two independent experiments. Frequencies of Flk2
also shown (p = 0.044, B-lineage (B220−) at 2 mo to determine the percentage of circulating myeloid (Mac1/Gr1−) cells from the transplanted marrow. Filled bars represent mice receiving WT KrasG12D mice (open bars) or WT littermates that were treated with pIpC at 21 d of age and then sacrificed at 35 d of age. (A) Total numbers of Flk2− LSK cells in KrasG12D mice (open bars) or WT mice (filled bars) were quantified by flow cytometry in bone marrow (p < 0.001, t-test) and spleen (p < 0.01); total numbers (spleen + marrow) are also shown (p = 0.12); n = 6 mice per genotype, and error bars show SEM; data are pooled from two independent experiments. Frequencies of Flk2− LSK cells among viable nucleated cells were multiplied by nucleated bone marrow numbers using published distributions [75]. (B) Whole bone marrow from KrasG12D (open circles) or WT littermates (filled circles) was tested for repopulating activity in a limit dilution transplantation assay. The calculated values for frequencies of repopulating units were 1 in 16,610 nucleated bone marrow cells (NMBC) for WT marrow and 1 in 180,404 for KrasG12D marrow. Narrow lines designate 95% confidence intervals (1:7,338 to 1:37,598 for WT and 1:68,953 to 1:471,998 for KrasG12D; frequencies in WT versus KrasG12D marrow are significantly different with p = 0.0003 by two-tailed t-test). Outcomes of individual experiments are described in Table S1. (C) Mice that engrafted after transplantation with limiting numbers of whole bone marrow cells (1 × 10⁴ for WT, 1 × 10⁵ for KrasG12D) were bled at 2 mo to determine the percentage of circulating myeloid (Mac1/Gr1+), B-lineage (B220−), and T-lineage (CD3/CD5+/-) cells that were derived from the transplanted marrow. Filled bars represent mice receiving WT marrow (n = 2), open bars represent mice receiving KrasG12D marrow (n = 3; error bars show SEM; note logarithmic scale).

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Potential [45], and with extensive data showing that proliferating HSCs fail to engraft efficiently [46–50].

Despite the reduced number of HSC in the bone marrow of KrasG12D mice, our data are not entirely consistent with the idea that KrasG12D is a cell-intrinsic negative regulator of HSC self-renewal. If it were, we would expect specific loss of KrasG12D cells and outgrowth of WT or KrasLSL-G12D cells, because conditional models using Mx1-Cre typically retain a small pool of cells with the unarranged locus [51,52]. However, we observed preferential retention of KrasG12D cells, even within the diminishing Flk2− LSK compartment. This observation suggests that residual WT HSCs are unable to compensate for the reduced HSC number. Therefore, we favor the hypothesis that the reduction in HSC number in KrasG12D mice reflects a disordered bone marrow microenvironment with reduced supportive capacity rather than a purely cell-intrinsic effect of KrasG12D in HSC.

The natural history of hematologic disease was different in primary KrasG12D mice than in recipients of transplanted KrasG12D HSC. A subtle but important finding is that MPD is established earlier in primary KrasG12D mice than in transplanted recipients. (Figure 5 and [13]; also see [16,53]). There are several possible explanations for this observation. The hematopoietic microenvironment in young mice may be more permissive for MPD than the irradiated bone marrow of an adult. Additionally, pIpC administration in Mx1-Cre mice may quickly create a field of KrasG12D myeloid progenitors that contributes to the rapid evolution of MPD in primary mice through cytokine-mediated autocrine and/or paracrine mechanisms [54]. It is also possible that nonhematopoietic stromal cells in Mx1-Cre, KrasLSL-G12D mice express K-RasG12D and contribute to the rapid onset of MPD in primary KrasG12D mice.

The kinetics of MPD development relate directly to the high frequency of T-ALL we observed in transplanted recipients as compared to primary KrasG12D mice. The apparent incidence of T-ALL is highly subject to selection bias, because animals that die from MPD cannot be evaluated for subsequent emergence of T-ALL. For example, we have observed ~10%–15% of KrasG12D mice develop T-ALL on an inbred C57BL/6 strain background (unpublished data), but median time to death from MPD is shorter than the typical latency of T-ALL, and lymphoid tumors exclusively appear in mice with a relatively late onset of MPD. Similarly, we have not observed spontaneous T-ALL in F1 (C57BL/6 × 129Sv/Jae) mice, which die from MPD at a younger age than the C57BL/6 strain described here ([13] and unpublished data). In transplant recipients, aggressive T-ALL arose in mice that also had evidence of underlying MPD that was not yet severe enough to kill the animal. Together, these observations suggest that the attenuation of MPD in transplant recipients was central to the apparent increase in the incidence of T-ALL in the transplant setting.

Cooperation of hyperactive Ras and deregulated Notch signaling in T-ALL has recently been shown [55–57]. Our data extend these studies by demonstrating the remarkable efficiency with which KrasG12D HSCs can initiate T-ALL, and delineating how multiple cell types may participate in the stepwise acquisition of oncogenic mutations in hematologic cancers. In the Mx1-Cre, KrasLSL-G12D model, T-ALL is initiated by oncogenic Kras expression in HSC, but full transformation occurs when cooperating Notch1 mutations arise in a T-
lineage cell. In this sense, both Kras\textsuperscript{G12D} HSCs and the fully transformed thymocytes can be considered different types of malignant stem cells with distinct leukemogenic potentials.

One potential implication of these results is that the initiating Kras mutation creates conditions favorable for acquisition of cooperating mutations by increasing the size of susceptible lymphoid progenitor pools and/or conferring resistance to apoptotic signals during thymic selection. Kras\textsuperscript{G12D} appears to most greatly affect the DN population that is characteristically undergoing TCR rearrangement, selection, and proliferation [58,59]. Interestingly, K-Ras\textsuperscript{G12D} protein expression may substitute for the pre-T cell receptor rearrangement at this critical checkpoint, thereby allowing propagation of thymocytes that would normally be edited [60]. Consistent with this idea, a patient with impaired lymphoid homeostasis and multiple lymphoid malignancies was recently reported to have a germline NRAS\textsuperscript{G13D} mutation, and oncogenic NRAS suppressed apoptosis of lymphocytes after cytokine withdrawal [61]. By contrast, Kindler et al. recently reported reduced thymic cellularity in Mx1-Cre,

**Figure 7.** Transplanted Kras\textsuperscript{G12D} Flk2\textsuperscript{-} LSK Cells Initiate T-ALLs That Contain Notch1 Mutations

(A) Flow cytometry of thymocytes harvested from moribund recipients of Kras\textsuperscript{G12D} Flk2\textsuperscript{-} LSK cells shows an abnormal accumulation of CD4/CD8 double positive and immature CD8 single positive cells. Spleen histology demonstrates infiltration by monomorphic cells with open chromatin (hematoxylin/eosin).

(B) Primary recipients (n = 5) received 500 Kras\textsuperscript{G12D} Flk2\textsuperscript{-} LSK cells with or without an equal number of WT Flk2\textsuperscript{-} LSK cells after lethal irradiation (950 rad). These primary recipients were euthanized 2–3 mo later, and 10\textsuperscript{6} bone marrow cells or thymocytes were transferred into sublethally irradiated (450 rad) secondary recipients (two per primary mouse). Sublethal irradiation selectively permits transfer of acute leukemia but not Kras\textsuperscript{G12D} HSCs or MPD [13]. Thymocytes, but not bone marrow cells, transferred T-ALL.

(C) Cell lysates from thymocytes and bone marrow of animals euthanized 3 mo after transplantation with Kras\textsuperscript{G12D} or WT HSCs were blotted with an antibody specific for cleaved Notch1. Three independent primary recipients are shown. Sequence analysis demonstrates frameshift mutations near the PEST domain of Notch1 in thymocytes from five of six mice that received Kras\textsuperscript{G12D} HSC, but not in recipients receiving WT HSCs alone (reference sequence from GenBank [http://www.ncbi.nlm.nih.gov/Genbank] accession number NM_008714).

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Kras\textsuperscript{G12D} mice\cite{56}. We speculate that the proliferative effects of K-Ras\textsuperscript{G12D} in the T cell compartment were obscured in those studies by the short interval between pIpC injection, which induces systemic interferon production, and histologic analysis.

The idea that patients may harbor a variety of genetically distinct LSC is consistent with studies of patients with chronic myeloid leukemia in blast transformation\cite{62}, and has important therapeutic implications. The need to eliminate partially transformed but self-renewing cells, like Kras\textsuperscript{G12D} HSCs, will depend on their propensity to initiate a life-threatening disease. Targeted therapies that are directed against onco-proteins such as K-Ras\textsuperscript{G12D} will effectively eliminate premalignant clones only if the targeted lesions are initiating rather than secondary mutations. For example, inhibition of Notch signaling is an attractive therapeutic strategy for T-ALL that is being investigated in the clinic. However, if these cancers arise from aberrant HSCs that do not contain a NOTCH1 mutation and are not eradicated by treatment, relapse could occur through the acquisition of distinct cooperating mutations in a self-renewing preleukemic population. Consistent with this idea, studies of human T-ALL suggest that NOTCH1 mutation occurs as a secondary mutation in at least some cases, with some patients

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**Figure 8. Abnormal T-lymphoid Development in Kras\textsuperscript{G12D} Mice**

(A) Enumeration of Lin\textsuperscript{−} Flk\textsuperscript{−} IL-7R\textsuperscript{+} c-kit\textsuperscript{+} Sca1\textsuperscript{+} common lymphoid progenitors (CLPs) in bone marrow of WT and Kras\textsuperscript{G12D} mice. A typical comparison and gating strategy is shown, with the far right panel showing expression of c-kit and Sca1 in CLPs (black dots) compared to the larger population of Lin\textsuperscript{−} cells (gray dots). CLP frequencies are graphed (n=5 WT and 6 Kras\textsuperscript{G12D}; error bars show SEM and difference is not statistically significant by unpaired t-test).

(B) Enlarged thymi in 7-wk-old Kras\textsuperscript{G12D} mice compared with WT littermates; photograph shows a typical example, and graph shows data from a representative cohort (n=3 mice and error bars show standard deviation; p<0.05).

(C) Flow cytometry of primary thymocytes for expression of CD4 and CD8, and also CD25 and CD44 expression within DN (CD4\textsuperscript{−} CD8\textsuperscript{−}) cells; figure shows a representative example. Myeloid cells were excluded using Mac1 and Gr1 staining. Graph represents the frequency of thymocytes within in the live gate in DN, double positive (CD4\textsuperscript{+}D8\textsuperscript{+}, DP), and CD4 or CD8 single positive cells (n=3 mice per genotype and error bars show SEM; p>0.05 for all populations. Data are representative of three independent experiments).

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developing recurrent disease having distinct NOTCH1 alleles [63].

Finally, our data have implications for understanding the nature of cancer stem cell populations in nonhematopoietic malignancies. KRAS is the most frequent target of dominant oncogenic mutations in human cancer, and it is particularly important in carcinomas of the lung, pancreas, and colon. Analogous cancers arise in strains of mice expressing conditional oncogenic Kras alleles in these tissues [64–68]. Importantly, whereas oncogenic Kras expression efficiently initiates tumorigenesis in murine lung and pancreas, colon cancer is observed only when the tumor suppressor Apc is inactivated as well [68]. These data are consistent with studies of human patients, which imply that KRAS mutation occurs early in pancreatic cancer but typically after APC mutation in colon carcinoma [69–71]. Lung cancer in KrasG12D mice appears to be initiated in a distinct bronchio-alveolar stem cell population [72]. On the basis of these observations and our data, we speculate that, like HSCs, cells initiating pancreatic and lung cancer will possess inherent self-renewal potential, and that KRAS mutations only contribute to colon tumorigenesis in cells that have already acquired a mutation that enhances self-renewal. Uncovering specific proteins and pathways that are essential for the self-renewal and survival of Kras mutant cancer stem cell populations may reveal novel targets for therapeutic intervention in a variety of human cancers.

Materials and Methods

Ethics statement. All animals were handled in strict accordance with good animal practice as defined by the relevant national and local animal welfare bodies, and all animal work was approved by the institutional animal research committee (University of California, San Francisco IACUC).

Mouse strains. Animals were housed in a barrier facility at the University of California, San Francisco. All mice were of the C57BL/6 strain. As described [13], 21-d-old Mcl-1Cre, KrasLSL-G12D were injected IP with 250 μg plpc (Sigma). ROSA26 LSL-YFP reporter mice were a gift from C. Lowell. Ptf1r (CD45) congenic mice were from Jackson Labs.

Colony assays. To assay CFU-GM, nucleated bone marrow cells (10^5) or splenocytes (2 × 10^5) were suspended in 1 ml methylcellulose medium (M3231, StemCell Technologies) with 10 ng/ml murine GM-CSF (Milteny). Cell sorting was performed on a FACSaria and flow cytometry on an LSRII, both using FCSdiva software (BD). Data were analyzed using Flowjo software (TreeStar).

Transplantation. Recipients received a single fraction of 950 rads for lethal irradiation, or 450 rads for sublethal irradiation, from a cesium source. Donor cells were prepared in IL-3, IL-6, stem cell factor, and erythropoietin, and incubated at 37°C for 14 d. For recipients were transplanted with 1 × 10^5, 3 × 10^5, or 1 × 10^6 nucleated bone marrow cells along with 10^5 unfractionated WT bone marrow cells for radioprotection. Peripheral blood was analyzed monthly by flow cytometry; mice with detectable engraftment in myeloid, B and T lineages 2 mo after transplantation were scored as positive and L-Calc software (StemCell Technologies) was used for statistical analysis.

For the CFU-S assay, lethally irradiated mice received 10^5 CMPs without support cells. Spleen colonies were observed 8 d later by gross examination at harvest and after 18 h fixation in 10% formalin. Bacterial colonies were stained with crystal violet and enumerated.

Notch1 analysis. Cells were lysed in 1% NP-40 with 30 mM NaF, 30 mM β-glycerophosphate, 20 mM NaPO4, 1 mM NaVO4, and Complete (Roche) and analyzed after SDS-PAGE using cleaved Notch1 and cleaved-β-actin antibodies (Cell Signaling). Genomic DNA was PCR-amplified bidirectionally using 5’-ATACTACAT-GATGGGGCCACATA-3’ and 5’-GCTTCTGGAATGTGGGTGAT-3’ PCR primers. The PCR product was cloned into pGEMT (Promega) and sequenced bidirectionally using M13 forward and reverse primers. Sequences were analyzed using Chromas software (Technelysium).

Quantitative real-time PCR. The assay was performed as described [50]. KFlk2 KLS cells from animals pooled by genotype were double-sorted directly into RNA binding lysis buffer from the RNeasy kit (Qiagen), and total RNA was extracted per instructions. First strand cDNA synthesis was performed using SuperScript III kit (Invitrogen) per manufacturer's instructions. Reactions were performed in an ABI-7900 sequence detection system using SYBR green according to manufacturer’s instructions (Applied Biosystems). Each amplification was performed in triplicate on a template of 10 ng total RNA for 14 sorted HSCs. Each sample was tested in triplicate with each primer pair, and normalized to β-actin expression. Due to limiting numbers of doubly sorted cells, final cell purity was not analyzed; however the staining characteristics of c-kit-enriched and singly sorted cells are presented in Figure S1.

Poisson statistics. To analyze repopulation by a single HSC, we identified a cohort of mice receiving a cell dose yielding engraftment in only 50% of recipients. The Poisson distribution indicates that the probability of a mouse receiving k HSCs is given by

\[
P(k) = \frac{e^{-n} n^k}{k!}
\]

where \( n \) is the average HSC number per mouse. The average that yields \( k = 0 \) HSC at a rate of 0.5 is given by 0.5 = e\(^{-n}\), which is solved to give \( n = 0.693 \). Using this value for the average number of HSC per mouse, the Poisson distribution can be used to estimate the

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Flow cytometry was performed to enumerate Flk2

**Purity of Cells Sorted for RNA Purification**

The number of repopulating units was calculated using the number of mice transplanted with 10,000, 30,000, or 100,000 nucleated bone marrow cells, and the proportions with detectable multilineage engraftment were determined 2 mo later. The total number of repopulating units was calculated using the totals from these four experiments with L-Calc software (StemCell Technologies).

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**Supporting Information**

**Figure S1.** Purity of Cells Sorted for RNA Purification

Cells expressing c-kit were enriched by magnetic bead isolation, then doubly sorted by FACS. FACS data for the first and second sorts for typical examples of WT and KrasG12D mice are shown. Found at doi:10.1371/journal.pbio.1000059.sg001 (740 KB TIF).

**Figure S2.** Flk2−/− LSK Cell Number in 8-wk-Old KrasG12D Mice

Flow cytometry was performed to enumerate Flk2−/− LSK cells in bone marrow (n = 4; p < 0.05) and spleens (n = 6; p < 0.05) harvested from 8-wk-old KrasG12D or WT mice. Data were analyzed as shown in Figure 2. Combined numbers in marrow and spleen were not significantly different between genotypes (p = 0.27). Data are pooled from two independent experiments; error bars show standard error of the mean (SEM). Statistical significance was evaluated with the unpaired t-test. Found at doi:10.1371/journal.pbio.1000059.sg002 (80 KB TIF).

**Figure S3.** KrasG12D HSCs Dominate the Stem Cell Compartment by 3 mo After Transplantation

Mice transplanted with 500 CD45.1 KrasG12D and 500 CD45.1CD45.2 WT HSCs were sacrificed (A) 2 mo (n = 6) or (B) 3 mo (n = 2) after transplantation, and their chimerism was assessed by flow cytometry. The percentage of graft cells derived from the KrasG12D HSCs is shown for Flk2−/− LSK cells (black bars), CMPs (dark grey bars), granulocyte-macrophage progenitors (GMP, light grey bars), and mature (Mac1/Gr1+) circulating myeloid cells (open bars). GMPs were defined as Lin−/−Sca1− c-kit− Flk2−RII/III+ c-kit+ animals lacking any graft-derived contribution to the HSC compartment have been omitted. Found at doi:10.1371/journal.pbio.1000059.sg003 (168 KB TIF).

**Table S1. Outcomes of Individual Limit Dilution Transplantation Experiments**

The indicated numbers of mice were transplanted with 10,000, 30,000, or 100,000 nucleated bone marrow cells, and the proportions with detectable multilineage engraftment were determined 2 mo later. The total number of repopulating units was calculated using the totals from these four experiments with L-Calc software (StemCell Technologies).

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Competing interests

The authors have declared that no competing interests exist.

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