Mice expressing Kras<sup>G12D</sup> in hematopoietic multipotent progenitor cells develop neonatal myeloid leukemia

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Introduction

Juvenile myelomonocytic leukemia (JMML) is a pediatric myeloproliferative neoplasm (MPN) caused by somatic mutations in the RAS/MEK/ERK pathway signaling genes, including KRAS, NRAS, PTPN11, NFI, and c-CBL (1). These mutations result in a hypersensitivity of hematopoietic progenitors to granulocyte macrophage–CSF (GM-CSF) and lead to monocytosis, anemia, thrombocytopenia, hepatosplenomegaly, and infiltration of peripheral tissues with histiocytes (2–4). Compared with other pediatric hematologic malignancies, the prognosis of patients with JMML is very poor. Allogeneic hematopoietic stem cell (HSC) transplantation is the only curative therapy, which nonetheless has a 5-year overall survival rate of only 52% (5).

The majority of JMML cases result from a mutation in a single gene (6–8). As such, disease models using the most common JMML-initiating mutations have been readily generated (9–11). The MxiCre Kras<sup>G12D</sup> mouse was the first conditional animal model of JMML and continues to be studied extensively (12, 13). However, these mice succumb with MPN that can be exacerbated by T cell leukemia/lymphoma (T-ALL) and that is confounded by nonhematopoietic expression, and the pervasive emergence of T cell acute lymphoblastic leukemia. Here, we have developed a model of JMML using mice that express Kras<sup>G12D</sup> in multipotent progenitor cells (Flt3Cre<sup>+</sup> Kras<sup>G12D</sup> mice). These mice express Kras<sup>G12D</sup> in utero, are born at normal Mendelian ratios, develop hepatosplenomegaly, anemia, and thrombocytopenia, and succumb to a rapidly progressing and fully penetrant neonatal myeloid disease. Mutant mice have altered hematopoietic stem and progenitor cell populations in the BM and spleen that are hypersensitive to granulocyte macrophage–CSF due to hyperactive RAS/ERK signaling. Biased differentiation in these progenitors results in an expansion of neutrophils and DCs and a concomitant decrease in T lymphocytes. Flt3Cre<sup>+</sup> Kras<sup>G12D</sup> fetal liver hematopoietic progenitors give rise to a myeloid disease upon transplantation. In summary, we describe a Kras<sup>G12D</sup> mouse model that reproducibly develops JMML-like disease. This model will prove useful for preclinical drug studies and for elucidating the developmental origins of pediatric neoplasms.
progenitors from Flt3Cre+ KrasG12D animals demonstrated hyper-
sensitivity to GM-CSF in colony-forming assays, which was cor-
rected by MEK inhibition (Figure 1E and Supplemental Figure 6).

To confirm that the disease in Flt3Cre+ KrasG12D animals was
initiated in utero and could be propagated autonomously in vivo,
we transplanted E14.5 fetal liver (FL) cells into adult BoyJ ani-
mals (Figure 2A). Progenitors from mutant donors showed robust
engraftment and rapidly contributed to monocytosis, anemia, and
thrombocytopenia (Figure 2, B and C, and Supplemental Figure
7). Mutant progenitors gave rise to expanded myeloid cell popu-
lations in the BM and spleen, leading to hepatosplenomegaly and
a median survival of 9 weeks (Figure 2, D–H). In stark contrast to
other KrasG12D models, primary recipients of Flt3Cre+ Kras G12D
mutant cells showed no signs of T-ALL (Figure 2I). Upon secondary
transplantation with 4 × 106 primary BM cells, Flt3Cre + Kras G12D
mutant cells engrafted, and 5 of 6 recipients rapidly succumbed
with monocytosis, splenomegaly, and thymic atrophy (Supple-
mental Figure 8). These findings indicate that temporal expres-
sion of KrasG12D in utero transforms fetal hematopoietic progeni-
tors into transplantable JMML-initiating cells.

We proceeded to analyze the effect of fetal KrasG12D expression
on the frequency and distribution of HSCs and progenitors. In con-
trast to Mx1Cre+ KrasG12D animals (16), we observed a reduction of
HSCs (Tomato+ LSK CD150+CD48–) and MPPs (GFP+ LSK CD150–
CD48+) in both the BM and spleen of moribund Flt3Cre + KrasG12D
mice (Figure 3A). This reduction corresponded with increased
quiescence among HSCs in Flt3Cre+ KrasG12D mice (Figure 3B and
Supplemental Figure 9). These effects were mediated non–cell
autonomously, since LSL-KrasG12D was not recombined in HSCs
(Supplemental Figure 10). We proceeded to analyze the progeny of
Flt3Cre+ KrasG12D progenitors and found that BM cells cultured in
cytokine-free medium gave rise to histiocytes that expressed CD11c

Results and Discussion

We mated Flt3Cre+ ROSA<sup>tm6V/mG</sup>/tmG studs with dams bearing a con-
ditional Lox-STOP-Lox Kras<sup>G12D</sup>/+ allele (LSL-Kras<sup>G12D</sup>/+) to gen-
erate Flt3Cre+ ROSA<sup>tm6V/mG</sup> LSL-Kras<sup>G12D</sup>/+ mice (hereafter referred to
as Flt3Cre+ KrasG12D mice), in which oncogene expression could
be monitored by a switch from Tomato to GFP expression. Flt3Cre+ KrasG12D
mutants were born at expected Mendelian ratios and had
weight gain comparable to that of their littermates until 2 weeks of
age (Figure 1A and Supplemental Figure 1A; supplemental mate-
rial available online with this article; https://doi.org/10.1172/
JCI94031DS1). Mutants and littermates had equivalent activi-
ty of Flt3Cre, as measured by the percentage of GFP<sup>+</sup> cells, and
LSL-KrasG12D recombination in mutant mice was confirmed by
PCR (Supplemental Figure 1, B and C). After 2 weeks, Flt3Cre+ KrasG12D
mice showed progressive weight loss, leukocytosis, ane-
mia, thrombocytopenia, and hepatosplenomegaly and died at a
median age of 26 days (Figure 1, A–C, Supplemental Figure 2, and
Supplemental Figure 3). Histological organ examination revealed
a histiocytic infiltrate in the spleen, liver, lung, and intestines
(Supplemental Figure 4), and a markedly increased frequency of
CD11b+Gr1<sup>+</sup> cells in the BM, blood, liver, and spleen was con-
firmed by flow cytometry (Figure 1D and Supplemental Figure 5).
Notably, the frequency of CD3<sup>+</sup> T lymphocytes and B220<sup>+</sup> B
lymphocytes was decreased, and Flt3Cre+ KrasG12D mice had an atro-
phied thymus compared with that seen in the littermates (Figure
1D, Supplemental Figure 3, and Supplemental Figure 4C). Consis-
tent with a faithful model of hyperactive RAS-induced JMML, BM

Figure 1. Flt3Cre<sup>+</sup> Kras<sup>G12D</sup> mice develop a JMML-like disease. (A) Weight gain from birth (n = 12 mutants and 19 controls). (B) Overall survival (statistical
analysis by Mantel-Cox test). (C) Peripheral blood smear (n = 5). Scale bar: 100 μm. (D) Flow cytometric quantification of tissue leukocytes. (E) Seven-day BM colony formation with 100 nM PD0325901 or 0.1% DMSO (n = 3 biological replicates/group). All analyses were performed on 3- to 4-week-old moribund
Flt3Cre<sup>+</sup> Kras<sup>G12D</sup> mice and age-matched littersmates. *P < 0.05, #P < 0.01, and §P < 0.001, by unpaired, 2-tailed Student’s t test (A, D, and E).
Enhanced myeloid cell production compared with adult progenitors targeted by Mx1Cre (24, 27). This context emulates studies of JMML patients that highlighted the fetal origins of this disease: the causative somatic mutation commonly occurs before birth, and BM cells have a gene expression signature that is characteristic of fetal progenitors (22, 23). Therefore, in contrast to Mx1Cre, Flt3Cre targets KrasG12D expression to hematopoietic progenitors at the appropriate developmental stage to recapitulate the origin of JMML.

The identity of the JMML-initiating cell has been controversial. On the one hand, case reports have shown that CD34+CD38− phenotypic HSCs express disease-initiating mutations (6, 35) and that xenotransplantation of patients’ progenitors gives rise to mutated myeloid, B, and T cells with a common clonal origin (36). On the other hand, circulating T lymphocytes from most patients do not express the disease-initiating mutation (7, 37), suggesting that JMML is initiated within a MPP that undergoes a differentiation block during T lymphocyte commitment. Consistent with this hypothesis, case reports suggest that patients with JMML have decreased T cell frequencies in the BM and spleen (38, 39). These findings parallel our Flt3Cre+ KrasG12D model, which has a paucity of T cells, an atrophied thymus, and abnormal T cell differentiation.

An earlier study found similarly skewed T lymphocyte development when KrasG12D expression was restricted to DCs in p53−/− mice (40). Our results advance these findings to show that KrasG12D expression in multipotent progenitors results in widespread tissue infiltration with DCs that are distinct from the concomitantly and CD135 (Supplemental Figure 11). This finding is reminiscent of reports describing DC-like tumor cells in patients with JMML (2, 33, 34) and prompted us to analyze DC populations in Flt3Cre+ KrasG12D animals. We observed a marked increase in the frequency of CD11c+ cells in the BM and spleen (Figure 3C). Strikingly, the CD11c+ cell expansion was particularly prominent in the atrophied thymus, where we saw a concomitant deficit of CD4+CD8− double-positive cells (Figure 3, C–F, and Supplemental Figure 12). We also observed this propensity for preferential DC differentiation in recipients of Flt3Cre+ KrasG12D FL progenitors (Supplemental Figure 13).

We present the first KrasG12D model to our knowledge to unify JMML disease-defining features: an in utero origin; viability at birth followed by a failure to thrive; anemia; thrombocytopenia; monocytosis; hepatosplenomegaly; and infiltration of tissues with histiocytes. All Flt3Cre+ KrasG12D mice succumbed to a myeloid disease that could be recapitulated following FL transplantation. This is in contrast to existing KrasG12D models, whose MPN is exacerbated by nonhematopoietic oncogene expression and by the unpredictable coemergence of T-ALL (15–20).

Flt3Cre has an expression pattern markedly different from that of Mx1Cre, which may explain the observed respective myeloid versus lymphoid disease outcomes. Mx1Cre is used to target adult progenitors, whereas Flt3Cre becomes active in fetal MPPs. As such, Flt3Cre initiates KrasG12D expression within an in utero progenitor that is more proliferative, has greater repopulating ability, and has enhanced myeloid cell production compared with adult progenitors targeted by Mx1Cre (24, 27). This context emulates studies of JMML patients that highlighted the fetal origins of this disease: the causative somatic mutation commonly occurs before birth, and BM cells have a gene expression signature that is characteristic of fetal progenitors (22, 23). Therefore, in contrast to Mx1Cre, Flt3Cre targets KrasG12D expression to hematopoietic progenitors at the appropriate developmental stage to recapitulate the origin of JMML.

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lates underappreciated features of JMML such as a paucity of mature T lymphocytes and an expansion of DCs and thereby hints at potential new therapeutic strategies. Flt3Cre+ KrasG12D mice will prove useful for preclinical drug studies targeting the RAS/MEK/ERK signaling pathway and will help elucidate the developmental origins of JMML and pediatric leukemias.

Methods

Detailed methods, including all flow cytometry antibodies (Supplemental Table 1), are described in the Supplemental Methods.

Study approval. Animal studies were approved by the IACUC of the Indiana University School of Medicine. Animals were genotyped using primers outlined in Supplemental Table 2 of Supplemental Methods.

Statistics. P values comparing mutant and littermate groups were calculated using 2-tailed Student’s t tests, Mantel-Cox log-rank tests, or χ² tests, as indicated in the figure legends. P values of less than 0.05 were considered significant. All error bars represent the SEM.

Author contributions

SPT conceived the study, designed, performed, and analyzed experiments, and wrote the manuscript. MK designed and performed experiments. RJC and MCY conceived the study, designed and analyzed experiments, and wrote the manuscript.

Acknowledgments

The authors thank Momoko Yoshimoto (University of Texas Health Science Center, Houston, Texas, USA) for their helpful discussions. The authors are grateful to Slava Epelman (University of Toronto, Toronto, Canada) for the gift of Flt3Cre+ ROSA26<sup>tmG/tmG</sup> mice.

In summary, we describe what to our knowledge is the first Kras<sup>G12D</sup> mouse model that recapitulates defining features of JMML. Flt3Cre+ Kras<sup>G12D</sup> mice are viable, develop monocytosis, anemia, thrombocytopenia, and hepatosplenomegaly and die from a fully penetrant myeloid disease. This model further expanded neutrophils (Supplemental Figure 13B). Importantly, case reports have equally noted that JMML patients’ tissues are infiltrated by atypical histiocytes (2, 41) and that children with the DC disorder juvenile xanthogranuloma are at increased risk of JMML (42). Additionally, Langerhans cell histiocytosis and Erdheim-Chester disease, two aggressive DC disorders, are also characterized by hyperactive RAS signaling (43). These reports, along with our findings from Flt3Cre+ Kras<sup>G12D</sup> mice, suggest that a more formal investigation of DC involvement in JMML is warranted.

A unique feature of Flt3Cre+ Kras<sup>G12D</sup> mice is that their HSCs do not express the oncogene (Supplemental Figure 10). The full penetrance of a MPN in our model is therefore consistent with the hypothesis that the HSC is the cell of origin for Kras<sup>G12D</sup>-evoked T-ALL (16, 17). Notably, the quiescence of nononcogene-expressing HSCs in Flt3Cre Kras<sup>G12D</sup> mice demonstrates a profound non–cell-autonomous effect of this mutation. Our finding supports the conclusions of Sabnis et al., who noted that residual nonrecombined LSK Flt3+ cells in MxiCre Kras<sup>G12D</sup> mice did not expand to compensate for diminishing oncogene-expressing HSCs (16). Our results suggest that Kras<sup>G12D</sup>-expressing hematopoietic cells induce an aberrant BM microenvironment that stifles the expansion of normal neighboring HSCs. This yields the provocative hypothesis that patients with JMML relapse following allogenic transplantation as a result of an adverse niche that impedes the proliferation of donor HSCs.

In summary, we describe what to our knowledge is the first Kras<sup>G12D</sup> mouse model that recapitulates defining features of JMML. Flt3Cre+ Kras<sup>G12D</sup> mice are viable, develop monocytosis, anemia, thrombocytopenia, and hepatosplenomegaly and die from a fully penetrant myeloid disease. This model further expanded neutrophils (Supplemental Figure 13B). Importantly, case reports have equally noted that JMML patients’ tissues are infiltrated by atypical histiocytes (2, 41) and that children with the DC disorder juvenile xanthogranuloma are at increased risk of JMML (42). Additionally, Langerhans cell histiocytosis and Erdheim-Chester disease, two aggressive DC disorders, are also characterized by hyperactive RAS signaling (43). These reports, along with our findings from Flt3Cre+ Kras<sup>G12D</sup> mice, suggest that a more formal investigation of DC involvement in JMML is warranted.
This work was supported by the Riley Children’s Foundation and the NIH (F30 HL128011, to SPT, and R21 CA202296, to RJC and MCY). We appreciate the technical assistance provided by Karen Pollok and Tony Sinn of the Indiana University In Vivo Therapeutics Core and Susan Rice of the Indiana University Flow Cytometry Resource Facility (supported by NIH grant P30 CA082709). The authors gratefully acknowledge the administrative assistance of Tracy Winkle and Tiffany Lewallen (Indiana University School of Medicine, Indianapolis, Indiana, USA).

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