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A MEK Inhibitor Abrogates Myeloproliferative Disease in *Kras* Mutant Mice

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Abstract

Chronic and juvenile myelomonocytic leukemias (CMML and JMML) are aggressive myeloproliferative neoplasms that are incurable with conventional chemotherapy. Mutations that deregulate Ras signaling play a central pathogenic role in both disorders, and *Mx1-Cre*, *Kras*^{LSL-G12D} mice that express the *Kras* oncogene develop a fatal disease that closely mimics these two leukemias in humans. Activated Ras controls multiple downstream effectors, but the specific pathways that mediate the leukemogenic effects of hyperactive Ras are unknown. We used PD0325901, a highly selective pharmacological inhibitor of mitogen-activated protein kinase (MEK), a downstream component of the Ras signaling network, to address how deregulated Raf/MEK/ERK signaling drives neoplasm formation in *Mx1-Cre*, *Kras*^{LSL-G12D} mice. PD0325901 treatment induced a rapid and sustained reduction in leukocyte counts, enhanced erythropoiesis, prolonged mouse survival, and corrected the aberrant proliferation and differentiation of bone marrow progenitor cells. These responses were due to direct effects of PD0325901 on *Kras* mutant cells rather than to stimulation of normal hematopoietic cell proliferation. Consistent with the *in vivo* response, inhibition of MEK reversed the cytokine hypersensitivity characteristic of *Kras*^{G12D} hematopoietic progenitor cells *in vitro*. Our data demonstrate that deregulated Raf/MEK/ERK signaling is integral to the growth of *Kras*-mediated myeloproliferative neoplasias, and further suggest that MEK inhibition could be a useful way to ameliorate functional hematologic abnormalities in patients with CMML and JMML.

Introduction

Somatic mutations in the *NRAS* or *KRAS* oncogenes are identified in 20–40% of patients with chronic or juvenile myelomonocytic leukemias (CMML or JMML). Mutations in related molecules collectively implicate hyperactive Ras signaling in ~50% of CMML and 90% of JMML (1). The lack of compounds that directly inhibit oncogenic Ras has led to

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Author contributions: N.L. participated in the design and execution of all experiments. M.F.G. designed and performed initial experiments to evaluate the phenotypic effects of PD0325901 in *Mx1-Cre*, *Kras*^{G12D} mice. J.O.L. helped define pharmacokinetic and pharmacodynamic properties of PD0325901 and its toxicity in this mouse strain. W.X.H. designed and performed colony assay experiments. J.K.A. maintained mouse strains, and designed and performed pharmacodynamic studies of PD0325901 using flow cytometry. K.S. was instrumental in obtaining PD0325901, aided in the design and interpretation of experiments, and provided ongoing suggestions and discussions throughout the study. B.S.B. conceived and supervised this study, was involved in the design and evaluation of all experiments, and wrote the manuscript with assistance from N.L. and K.S.

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widespread efforts to find alternative therapeutic targets. We previously developed a genetically engineered mouse model that recapitulates many features of human myeloproliferative neoplasms. In this animal model, *Kras^{LSL-G12D}* mice carry a conditional *Kras* allele that expresses oncogenic K-Ras^{G12D} after an upstream “stop” cassette is removed by Cre recombinase (2). *Mx1-Cre* transgenic mice express Cre in response to polyinosinic-polycytidylic acid (pIpC). Therefore, *Mx1-Cre, Kras^{LSL-G12D}* mice express *Kras^{G12D}* from the endogenous locus after treatment with pIpC. These mice (hereafter designated *Mx1-Cre, Kras^{G12D}*) rapidly develop a progressive myeloproliferative neoplasm that is characterized by leukocytosis, splenomegaly, and severe anemia (3, 4). The similarities of this model to human myeloproliferative neoplasms suggest that it might be useful for studying disease mechanisms and for testing potential therapeutic strategies.

We previously showed that the Raf/MEK/ERK signaling pathway is modestly hyperactive in primary hematopoietic progenitor cells from the bone marrow of *Mx1-Cre, Kras^{G12D}* mice (5). Because numerous pathways are potentially deregulated by oncogenic Ras, the importance of deregulated Raf/MEK/ERK signaling in *Kras^{G12D}*-driven myeloproliferative neoplasia remains unclear (6). To address this question and to evaluate alternative therapeutic strategies for CMML and JMML, we treated *Mx1-Cre, Kras^{G12D}* mice with PD0325901, a potent and highly specific inhibitor that binds to an allosteric site on mitogen-activated protein kinase kinase (MEK) that is not conserved in other protein kinases (7–9). We show that PD0325901 treatment improves multiple hematologic abnormalities in *Mx1-Cre, Kras^{G12D}* mice by direct effects on bone marrow progenitor cells that express oncogenic *Kras*. This demonstrates that deregulated Raf/MEK/ERK signaling is integral to *Kras*-mediated myeloproliferative neoplasia, and suggests that MEK inhibition may be a useful approach for treating patients with CMML and JMML.

RESULTS

PD0325901 inhibits MEK *in vivo*

The ability of PD0325901 to inhibit MEK *in vivo* was validated by measuring ERK phosphorylation induced by GM-CSF stimulation of primary hematopoietic progenitor cells (Fig. 1A). In a flow cytometry based assay, Lin^{−/lo} c-kit⁺ CD34⁺ CD105[−] bone marrow cells were enriched for myeloid progenitors that responded to GM-CSF. We treated *Mx1-Cre, Kras^{G12D}* mice with PD0325901 and measured the ability of GM-CSF to evoke protein phosphorylation in bone marrow harvested at various times after administration. An oral dose of 5 mg/kg suppressed the ability of GM-CSF to phosphorylate ERK in mouse bone marrow cells for 18–24 h (Fig. 1B), which is consistent with previous data in this mouse strain (10). Phosphorylation of STAT5, which is independent of Raf/MEK/ERK activity, was unimpaired (Fig. 1C), consistent with the expected specificity of PD0325901.

PD0325901 controls disease in *Mx1-Cre, Kras^{G12D}* mice

To investigate whether PD0325901 reduces the severity of disease in *Mx1-Cre, Kras^{G12D}* mice, we induced *Kras^{G12D}* expression in 3–4 week old pups and allowed the myeloproliferative neoplasia to progress until the age of 8 weeks. The disease was well-established by this time, as indicated by high blood leukocyte counts (41,000/μL ± 25,000 s.d.) (Fig. 2A) and low hemoglobin concentrations (10.6 g/dL ± 3.8 s.d.) (Fig. 2B), compared with wild-type control mice. *Mx1-Cre, Kras^{G12D}* mice and wild-type littermates were then randomized to receive PD0325901 at a dose of 5 mg/kg/day or vehicle treatment. *Mx1-Cre, Kras^{G12D}* mice that received the PD0325901 MEK inhibitor demonstrated rapid improvements in composition of the peripheral blood, with reduced leukocyte counts (Fig. 2A), disappearance of anemia (Fig. 2B) and reticulocytosis (Fig. 2C), and reduced splenomegaly (Fig. 2D). Daily treatment with PD0325901 also prolonged dramatically the

survival of *Mx1-Cre, Kras^{G12D}* mice compared with vehicle-treated mice (8.1 vs. 2.0 weeks on trial; $p < 0.0001$ by log rank test) (Fig. 2E). Two of three *Mx1-Cre, Kras^{G12D}* mice treated for 12 weeks died with *Kras^{G12D}* T-lineage leukemia/lymphoma (T-ALL), suggesting that some hematopoietic malignancies are not susceptible to MEK inhibition (10). There were no adverse effects of PD0325901 administration observed in wild-type mice.

PD0325901 improves myeloid and erythroid differentiation *in vivo*

To investigate the mechanism of this response to MEK inhibition, we first explored whether PD0325901 treatment eliminated *Kras* mutant cells from the bone marrow. We used PCR genotyping to determine the configuration of the conditional *Kras* allele in myeloid colonies obtained from *Mx1-Cre, Kras^{G12D}* mice treated with PD0325901. Because each colony is derived from a single progenitor cell, termed a granulocyte/macrophage colony forming unit (CFU-GM), this analysis estimates the frequency of progenitor cells that express oncogenic *Kras^{G12D}*. This assay demonstrated that the majority of CFU-GM contained the mutant *Kras* allele in the expressed configuration that lacks the “stop” cassette (Fig. 2F), even after 5 weeks of PD0325901 treatment. The improvement in blood counts without a change in the genotype of progenitor cells suggests that MEK inhibition alters the behavior of hematopoietic cells expressing oncogenic K-Ras^{G12D}.

Bone marrow contains discrete populations of multi-potent progenitors with the potential to generate granulocytes and macrophages, megakaryocytes and erythrocytes, or all four myeloid lineages. We used flow cytometry to ascertain whether improved blood composition reflected changes in the abundance of these populations (Fig. 3A) (11). Vehicle-treated *Mx1-Cre, Kras^{G12D}* mice demonstrated a bias toward granulocyte and macrophage progenitors at the expense of megakaryocyte and erythroid progenitors and progenitors of the four myeloid lineages; conversely, PD0325901 treatment restored the distribution seen in wild-type control mice, in which these populations are approximately equal in size (Fig. 3A,B). Analysis after only three daily doses of PD0325901 showed that changes in progenitor cell distributions precede any improvement in the peripheral blood (Fig. 3B,C). We did not find a significant difference in the numbers of cells expressing hematopoietic stem cell markers in the bone marrow of treated versus control mice (Fig. S1). Together, these data suggest that MEK inhibition reduces myeloproliferative neoplasia severity by modulating the behavior of hematopoietic progenitor cells, but not stem cells, in the bone marrow.

Treatment with PD0325901 also normalized the distributions of differentiating myeloid cells. *Mx1-Cre, Kras^{G12D}* mice demonstrated a relative increase in the percentage of monocytes and a decrease in the percentage of intermediate granulocytes in the bone marrow and the spleen, but treatment with PD0325901 restored percentages of these populations to near normal levels (Fig. 3D, 3E) (4–10% monocytes and 20–30% intermediate granulocytes in bone marrow, and <1% of each in spleen). Similarly, untreated *Mx1-Cre, Kras^{G12D}* mice progressively lose the TER119+ erythroid population in the marrow, and accumulate immature erythroid cells in the spleen (12). Treatment with PD0325901 partially corrected the distribution of differentiating cells in the bone marrow (Fig. 3F), and eliminated the massive accumulation of splenic erythroblasts (Fig. 3G). Collectively, these studies demonstrate that MEK inhibition alleviates aberrant myelopoiesis and ineffective erythropoiesis in *Mx1-Cre, Kras^{G12D}* mice. PD0325901 treatment had no adverse hematological effects, such as anemia or leukopenia, in wild-type mice, although we observed an increase in splenic erythroid cells, suggesting that MEK inhibition may impose some stress on normal erythropoiesis. (Fig. 3G).

PD0325901 corrects the hypersensitive growth pattern of *Mx1-Cre, Kras^{G12D}* myeloid progenitors *in vitro*

Bone marrow cells from patients with JMML and CMML and from *Mx1-Cre, Kras^{G12D}* mice display a characteristic hypersensitive growth pattern, resulting in greater production of granulocyte-macrophage colonies in response to GM-CSF (3, 13). CFU-GM progenitors from *Mx1-Cre, Kras^{G12D}* mice treated with PD0325901 remained hypersensitive to GM-CSF *in vitro* (Fig. 4A), which is consistent with retention of the activated *Kras^{G12D}* allele (Fig. 2F). Similarly, we did not observe selective inhibition of *Kras* mutant CFU-GM progenitor colony growth over a range of PD0325901 concentrations in the presence of a saturating concentration of GM-CSF (Fig 4B). Nevertheless, some specificity was observed in cultures containing a low concentration of PD0325901 (0.1 μ M). Whereas this had only modest effects on total number of colonies when GM-CSF was present at saturating levels (Fig. 4C), PD0325901 0.1 μ M both abrogated growth of cytokine-independent myeloid colonies and restored a normal response to increasing concentrations of GM-CSF (Fig. 4D). At saturating doses of GM-CSF, a low concentration of PD0325901 (0.1 μ M) was sufficient to normalize the numbers and types of cells within the colonies (Fig. 4E,F). Together, these data show that a low concentration of PD0325901 is sufficient to overcome the dominant effects of oncogenic *Kras^{G12D}* expression and impart a normal program of proliferation and differentiation in primary myeloid progenitors. A structurally distinct MEK inhibitor, PD98059, similarly inhibited the growth of cytokine-independent colonies, confirming that MEK is the physiological target of PD0325901 in this assay (Fig. S2) (14).

DISCUSSION

Patients with well-differentiated forms of cancer may derive substantial clinical benefit from therapies that modify the aberrant behavior of the malignant clone, without damaging normal tissues, even if the tumor is not eradicated. In this case, the value of therapy lies in reducing the symptoms caused by the neoplasm for a meaningful time period. A classic example of this paradigm is the use of anti-proliferative agents, such as hydroxyurea, in the treatment of chronic myelogenous leukemia (CML), a myeloproliferative neoplasm driven by the aberrant fusion kinase Bcr-Abl (15). Even as highly effective small-molecule-targeted inhibitors of the Bcr-Abl kinase have markedly prolonged the survival of patients with CML, the causative mutation often remains detectable at low levels, and clinical experience supports the idea that a highly beneficial treatment does not always eliminate all mutant stem cells (16). In our studies, *Mx1-Cre, Kras^{G12D}* mice treated with PD0325901 demonstrated a rapid and sustained improvement in hematopoietic abnormalities, even though the *Kras^{G12D}* oncogene persisted at high levels in the bone marrow. This is similar to patients with myeloproliferative neoplasms that undergo clinical, but not molecular, remissions. An implication of this idea is that a reduction in oncogene ‘allele burden,’ which is frequently used as a surrogate of disease response in clinical trials, may not always be required for a therapeutic agent to demonstrate clinical utility.

Our data indicate that the dramatic hematological responses observed in *Mx1-Cre, Kras^{G12D}* mice treated with the MEK inhibitor PD0325901 are not due to a purely anti-proliferative effect, as observed with conventional chemotherapy. The effects of MEK inhibition in *Mx1-Cre, Kras^{G12D}* mice also contrast with those of Abl kinase inhibitors in patients with CML, where treatment provides a selective advantage to normal myeloid progenitor populations (17). Instead, the resolution of both leukocytosis and anemia indicates that PD0325901 rebalances the output of the hematopoietic system, despite continued *Kras^{G12D}* expression. This “rebalancing” might indicate that MEK activity regulates the expression of genes controlling lineage choice during hematopoietic differentiation. Such a model would be consistent with studies showing that cytokine stimulation instructs lineage choice in multipotent progenitors (18), and that Ras activation promotes monocytic over granulocytic

differentiation (19). Further experiments to identify genes that respond to MEK activity in multipotent cells, and to test their influence on cell fate, are required to address this hypothesis.

Curative therapy for myeloproliferative neoplasia will need to eliminate neoplastic stem cells. Whether or not *Kras* mutant alleles could be eliminated by MEK inhibition was not fully addressed in these experiments. Because recombination in the bone marrow of *Mx1-Cre* mice is usually incomplete, *Mx1-Cre, Kras^{G12D}* mice can be expected to harbor a small pool of stem cells in which the conditional *Kras^{LSL-G12D}* allele remains in the germline, non-expressed configuration. We have demonstrated such genetic chimerism in the bone marrow of young *Mx1-Cre, Kras^{G12D}* mice (20). However, we did not determine if this persisted until the initiation of PD0325901 treatment. Interestingly, a similar question applies to patients with myeloproliferative neoplasia; some patients might lose their normal hematopoietic elements over the course of their disease. Although we did not observe emergence of cells lacking oncogenic *Kras* in these studies, it is possible that longer or more intense MEK inhibition might be more effective. Alternatively, inhibition of other signals evoked by mutant *Kras* may be required for elimination of mutant stem cells. The retention of *Kras^{G12D}* stem cells in the bone marrow likely contributed to the development of T-ALL in some mice after prolonged drug treatment, because *Kras^{G12D}* stem cells can create a reservoir of lymphoid progenitors that are susceptible to undergoing leukemic transformation (20). The observation that these acute leukemias arise during ongoing treatment suggests they are less dependent than myeloproliferative neoplasia on hyperactive Raf/MEK/ERK signaling.

In this study, we have provided direct evidence *in vivo* that aberrant hematopoiesis caused by hyperactive Ras signaling is mediated by the Raf/MEK/ERK pathway. These results are consistent with a previous investigation, which showed that constitutive activation of MEK could block erythroid differentiation *in vitro*, (21). However, the requirement for MEK in myeloproliferative neoplasia has been called into question by our previous biochemical studies, which revealed that endogenous levels of *Kras^{G12D}* do not strongly activate Raf/MEK/ERK signaling (3, 5). This implied that aberrant hematopoiesis might be mediated by other effector pathways regulated by Ras (22). Therefore, the importance of Raf/MEK/ERK signaling in mediating the myeloproliferative phenotype provides insight into the mechanism by which excessive Ras activity causes hematologic malignancies.

Our preclinical data imply that MEK inhibitors might be useful for treating patients with JMML and CMML, by alleviating the burdens of anemia, splenomegaly, and complications from myeloid cell overproduction such as tissue infiltration. In particular, treating children with JMML prior to hematopoietic stem cell transplantation might improve their clinical status by reducing the morbidity caused by infiltration of organs with leukemic cells (1). Many adults with CMML suffer substantial morbidity owing to chronic anemia (23), and a treatment that both reduces myeloproliferation and enhances erythropoiesis, as seen with MEK inhibitors, could prove clinically beneficial.

Materials and Methods

In Vivo MEK Inhibition

All procedures were approved by the institutional animal research committee. F1 (129Sv/Jae x C57BL/6) *Mx1-Cre, Kras^{LSL-G12D}* mice and littermates were injected intraperitoneally with 250 µg of pIpC (Sigma-Aldrich) at 4 weeks of age. Mice were randomly assigned to cohorts that received either 5 mg/kg PD0325901 (Pfizer) or hydroxypropylmethylcellulose (Sigma-Aldrich) vehicle by gavage once daily. Blood cell and reticulocyte counts were measured weekly using a Hemavet blood analyzer (Drew Scientific) and Retic-Count

reagent (BD Biosciences). Mice were removed from study after 5 weeks of PD0325901 treatment, or when Hb <6 g/dL.

Flow Cytometry

Nucleated bone marrow and spleen cells were stained with the following antibodies (eBiosciences, Biolegend, AbCam and BD Biosciences): lineage markers (PE-Cy7–conjugated CD3, CD4, CD5, CD8, B220, TER119, CD11b, or Gr1), FITC-CD34, PE-CD16/32, Pacific Blue-Sca1, APC-750–C-kit for stem cells and progenitors, PE-Cy7-CD11b, FITC-Gr1, PE-Ly6C for myeloid and monocytic precursor maturation (24) and FITC-CD71, PE-Ter119, PE-Cy7-C-kit, Pacific Blue-CD105 for erythroid precursor maturation (25, 26). Intracellular phosphoproteins were analyzed as described (5), with the addition of staining for CD34 and CD105. Antibodies were titrated individually. Data were collected on a LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo (TreeStar).

Colony Assays

Nucleated bone marrow and spleen cells (5×10^4 cells per culture) were suspended in M3231 methylcellulose medium (StemCell) supplemented with recombinant murine GM-CSF (PeproTech) 0.01–10 ng/ml, and/or PD0325901 0.01–25 μ M, PD98059 (Cell Signaling Technology) or DMSO 0.2%. CFU-GM colonies were counted on day 7. To evaluate recombination of the *Kras*^{LSL-G12D} allele, colonies were individually harvested into 10 μ L distilled water, and 2 μ L was analyzed by PCR (2).

Polymerase chain reaction (PCR)

Genotyping was performed using primers SD5' (AGCTAGCCACCATGGCTTGAGTAAGTCTGCA), DT5' IO (GTCGACAAGCTCATGCGGGTG), and LJ3' (CCTTTACAAGCGCACGCAGACTGTAGA), with 35 cycles of 94°C (30 s), 65°C (90 s), and 72°C (60 s). This reaction yields amplicons of 500 bp for the wild-type *Kras* allele and 550 bp for the *Kras*^{LSL-G12D} allele. Recombination of the *Kras*^{LSL-G12D} allele was determined by PCR with the Advantage-GC kit (Clontech) using primers 5'-1 (GGGTAGGTGTTGGGATAGCTG) and 3'-3 (TCCGAATTCAGTGACTACAGATGTACAGAG), with 35 cycles of 95°C (30 s), 58°C (30 s), and 72°C (30 s); the wild-type *Kras* allele yields a 285-bp amplicon, and the recombined *Kras*^{LSL-G12D} allele yields a 315-bp amplicon. The non-recombined *Kras*^{LSL-G12D} allele does not amplify under these conditions.

Statistics

Data were analyzed using Prism 4.0 software (GraphPad). Student's *t* tests, 2-tailed, unpaired, were used to compare blood cell counts and spleen weights after treatment. WBC data were log transformed to correct heteroscedasticity; otherwise, Welch's correction was applied when variances were unequal. For bone marrow and spleen population frequencies, and for colony counts, effects of genotype/treatment cohort and populations were analyzed by 2-way ANOVA, and Bonferroni post-test comparisons were performed within each population against the wild-type/vehicle cohort. Kaplan Meier survival analysis was analyzed by the log rank test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

Inhibiting the Raf/MEK/ERK pathway reverses the harmful effects of oncogenic Kras on hematopoietic differentiation, suggesting a strategy for treating myeloproliferative neoplasms.

Fig. 1. PD0325901 inhibits MEK *in vivo*

(A) Flow cytometry was used to measure ERK and STAT5 phosphorylation in primary myeloid progenitor cells from the bone marrow of *Mx1-Cre, Kras^{G12D}* and wild-type mice in the presence or absence of 10 ng/mL GM-CSF. After gating for surface-marker expression, phosphoprotein staining was analyzed as a histogram (open) in comparison with an unstimulated control samples (gray). (B) *Mx1-Cre, Kras^{G12D}* mice were treated with a single dose of the MEK inhibitor PD0325901 (PD) or vehicle, and bone marrow and spleens were harvested 12 h, 18 h, and 24 h later. Bone marrow and spleen cells were stimulated with GM-CSF and analyzed for phosphorylated ERK (pERK). (C) Bone marrow and spleen cells were analyzed for phosphorylation of STAT5 (pSTAT5), 12 h after treatment with PD0325901. The phosphorylation of STAT5 in cells that do not phosphorylate ERK indicates that some signals induced by GM-CSF are intact after exposure to PD0325901, demonstrating specific inhibition of the Raf/MEK/ERK pathway.

Fig. 2. PD0325901 controls myeloproliferative neoplasia in *Mx1-Cre, Kras*^{G12D} mice
(A) Total leukocyte (WBC) count, **(B)** hemoglobin (Hb) concentration, and **(C)** reticulocyte frequency in peripheral blood of *Mx1-Cre, Kras*^{G12D} (red) or wild-type control (blue) mice treated with PD0325901 (PD; solid line) or vehicle (dashed line). Error bars show s.e.m. (n=5–7 per group). Significance by Student *t* test comparing PD0325901 treated vs. vehicle treated *Mx1-Cre, Kras*^{G12D} mice at the 4 week time point: **p*<0.05; ***p*<0.01; ****p*<0.001. **(D)** Mouse spleen weight after 5 weeks of therapy. **(E)** Kaplan-Meier survival estimates indicating a survival benefit to PD0325901 treatment in *Mx1-Cre, Kras*^{G12D} mice with significance of *p*<0.0001 by the log rank test (n=6 and 7, respectively, for wild-type mice treated with PD0325901 and vehicle; n=8 and 9 for *Mx1-Cre, Kras*^{G12D} mice treated with PD0325901 and vehicle). **(F)** Genotypes of individual granulocyte/macrophage colonies (lanes 1–5) derived from the bone marrow of a representative *Mx1-Cre, Kras*^{G12D} mouse treated daily with PD0325901 for five weeks. Each colony was derived from a single myeloid progenitor, indicating a predominance of cells with a heterozygous *Kras*^{G12D/+} genotype in the progenitor population. Lanes (+) and (–) show controls for *Kras*^{G12D/+} and wild-type genotypes, respectively. A total of 44 colonies grown from three independent *Kras*^{G12D} mice that received PD0325901 were analyzed, and all demonstrated Cre-mediated activation of the *Kras*^{G12D} allele.

Fig. 3. PD0325901 improves myeloid and erythroid differentiation in vivo

Frequencies of precursor populations were determined by flow cytometry (A) Typical distributions of myeloid progenitors in bone marrow of wild-type and *Mx1-Cre, Kras*^{G12D} mice treated with vehicle or PD0325901 (PD) for five weeks. Cells gated for lineage negative/low (*Lin*^{-/lo}), c-kit⁺, *Sca1*⁻ are shown. GMP: granulocyte/macrophage progenitors, MEP: megakaryocyte/erythroid progenitors, CMP: common myeloid progenitors that populate all four myeloid lineages (B) Ratios of GMP to CMP in bone marrow of wild-type control (white bars) or *Mx1-Cre, Kras*^{G12D} (black bars) mice treated with vehicle (solid bar) or PD0325901 (striped bar) for 3 days or 5 weeks, as indicated. All error bars show s.e.m. (n=3–9 per group). In *Mx1-Cre, Kras*^{G12D} mice, differences in treatment with vehicle vs. PD are significant according to Welch's corrected t test at 3 days (*p<0.05) and 5 weeks (**p<0.01). (C) Peripheral blood concentrations of leukocytes (WBC) and hemoglobin (Hb) in *Mx1-Cre, Kras*^{G12D} mice after 3 days of PD0325901 treatment. Differences are not statistically significant (n=4–6 per group). (D) Bone marrow

and **(E)** spleen populations of monocytes (M; Mac1+ Ly6C+), immature granulocytes (G1; Mac1+ Ly6C^{med} Gr1^{med}), intermediate granulocytes (G2; Mac1^{med} Ly6C^{med} Gr1^{high}), and mature granulocytes (G3; Mac1^{high} Gr1^{high}). Differences from the vehicle-treated wild-type cohort are indicated (*p<0.05, **p< 0.01, ***p< 0.001), as determined by Bonferroni testing after 2-way ANOVA (n=3–4 mice per group). **(F)** Bone marrow and **(G)** spleen distributions of erythroid populations, with erythroid precursors at sequential stages of maturation labeled as E1 (c-kit+ CD105+), E2 (CD71^{high} TER119+), or E3 (CD71^{med} TER119+). Statistical significance is indicated as in Fig. 3D.

Fig. 4. PD0325901 improves function of *Mx1-Cre, Kras^{G12D}* myeloid progenitors in vitro
(A) Bone marrow was harvested from wild-type and *Mx1-Cre, Kras^{G12D}* mice after 5 weeks of treatment with PD0325901 (PD) or vehicle and plated in methylcellulose with a range of GM-CSF concentrations. CFU-GM from *Mx1-Cre, Kras^{G12D}* mice treated with PD0325901 remain hypersensitive to GM-CSF. Error bars for all graphs show s.e.m., which is smaller than the plotting symbol for some data points. **(B)** Bone marrow cells from pIpC-treated wild-type or *Mx1-Cre, Kras^{G12D}* mice were plated in methylcellulose medium containing a high concentration of GM-CSF (10 ng/mL) and varying doses of PD0325901. The DMSO concentration was constant. Colonies were enumerated after 8 days, and counts were normalized to the number arising without PD0325901. CFU-GM from wild-type and *Mx1-Cre, Kras^{G12D}* mice have similar sensitivities to PD0325901 at saturating concentrations of GM-CSF. **(C)** A low concentration of PD0325901 (0.1 μ M) causes a modest reduction in the number of colonies derived in the presence of 10 ng/mL GM-CSF. A DMSO control is shown for comparison. To account for variation, the number of colonies for each sample was normalized by dividing colony number by the average number of colonies arising from a control sample in (wild-type cells in DMSO) in the same experiment. ** $p < 0.01$ vs. wild-type DMSO control by Bonferroni testing after 2-way ANOVA. **(D)** Bone marrow cells from pIpC-treated wild-type or *Mx1-Cre, Kras^{G12D}* mice were plated with a range of GM-CSF concentrations and a fixed concentration of PD0325901 (0.1 μ M) or DMSO control. **(E)** CFU-GM from pIpC-treated wild-type or *Mx1-Cre, Kras^{G12D}* mice were harvested from methylcellulose culture after 7 days with 10 ng/mL GM-CSF and either 0.1 μ M PD0325901 or DMSO. Average colony size was calculated as total cell number per plate divided by colony number ($n = 3-5$). Statistical significance is denoted as in Fig. 4C. **(F)** Harvested cells were stained for myeloid differentiation antigens F4/80 and Gr1. All colony assays were performed in triplicate from independent mice.