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J Clin Invest. 2008;118(3):946-955. <https://doi.org/10.1172/JCI32704>.

Research Article

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Csf3r mutations in mice confer a strong clonal HSC advantage via activation of Stat5

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A fundamental property of leukemic stem cells is clonal dominance of the bone marrow microenvironment. Truncation mutations of CSF3R, which encodes the G-CSF receptor (G-CSFR), are implicated in leukemic progression in patients with severe congenital neutropenia. Here we show that expression of a truncated mutant Csf3r in mice confers a strong clonal advantage at the HSC level that is dependent upon exogenous G-CSF. G-CSF-induced proliferation, phosphorylation of Stat5, and transcription of Stat5 target genes were increased in HSCs isolated from mice expressing the mutant Csf3r. Conversely, the proliferative advantage conferred by the mutant Csf3r was abrogated in myeloid progenitors lacking both Stat5A and Stat5B, and HSC function was reduced in mice expressing a truncated mutant Csf3r engineered to have impaired Stat5 activation. These data indicate that in mice, inappropriate Stat5 activation plays a key role in establishing clonal dominance by stem cells expressing mutant Csf3r.

Introduction

There is accumulating evidence suggesting that most leukemia is initiated by rare cancer stem cells, referred to as leukemic stem cells (LSCs). LSCs are functionally defined as cells able to generate leukemia upon transfer to an appropriate host (1). Two fundamental and interrelated properties of LSCs are the capacity for self-renewal and clonal dominance. Clonal dominance refers to the clonal expansion of LSCs within the bone marrow microenvironment at the expense of normal hematopoietic cells.

The mechanisms by which mutations and/or epigenetic changes that accumulate during leukemogenesis contribute to clonal dominance of LSCs are largely unknown. On a functional level, acquisition of clonal dominance represents a competitive advantage in the bone marrow microenvironment, as suggested by stochastic models (2). A requisite property for clonal dominance is the ability to self-renew. The capacity for self-renewal can be achieved either by transformation of HSCs with inherent self-renewal capacity or by the acquisition of this ability in committed progenitor populations during leukemogenesis (3). The molecular pathways that confer clonal dominance have been largely unexplored, though a recent study utilizing retroviral mutagenesis showed that the majority of retroviral integrations associated with clonal dominance (both malignant and nonmalignant) occurred near genes involved in the regulation of HSC self-renewal, proliferation, or apoptosis (4).

Severe congenital neutropenia (SCN) is a congenital bone marrow failure syndrome characterized by severe neutropenia present from birth, an arrest of myeloid differentiation at the promyelocyte/myelocyte stage, and a marked propensity to develop acute myeloid leukemia (AML) or a myelodysplastic syndrome (MDS) (5, 6). The cumulative risk of MDS/AML in patients with SCN after

10 years is 11%–21% (7, 8). Treatment with G-CSF is effective in increasing neutrophil counts and preventing infections (9); however, the quantity and duration of G-CSF treatment are correlated with an increased risk of MDS/AML (7, 8).

Nonsense mutations of *CSF3R* that truncate the distal cytoplasmic portion of the G-CSF receptor (G-CSFR) are present in approximately 40% of patients with SCN (10). These mutations are acquired and strongly associated with the development of AML/MDS (10). Expression of mutant G-CSFR in myeloid cell lines results in enhanced proliferative and survival signals (11–13). Moreover, transgenic mice carrying truncating mutations of *Csf3r* display a hyperproliferative response to G-CSF (14, 15). However, these mice do not develop MDS/AML; thus, a causal relationship between *CSF3R* mutations and leukemia has not been established.

In the present study, we investigated the mechanisms by which cells containing mutant *CSF3R* gain clonal dominance in vivo. Surprisingly, competitive repopulation studies using HSC from targeted transgenic (knockin) mice carrying the *Csf3r* truncation mutation showed no clonal advantage. However, short-term treatment with G-CSF resulted in a marked competitive advantage of mutant HSC. We utilized this inducible model to characterize the molecular pathways mediating HSC clonal dominance. These studies suggest that accentuated Stat5 activation plays a key role in establishing clonal dominance by HSC-expressing mutant *Csf3R*.

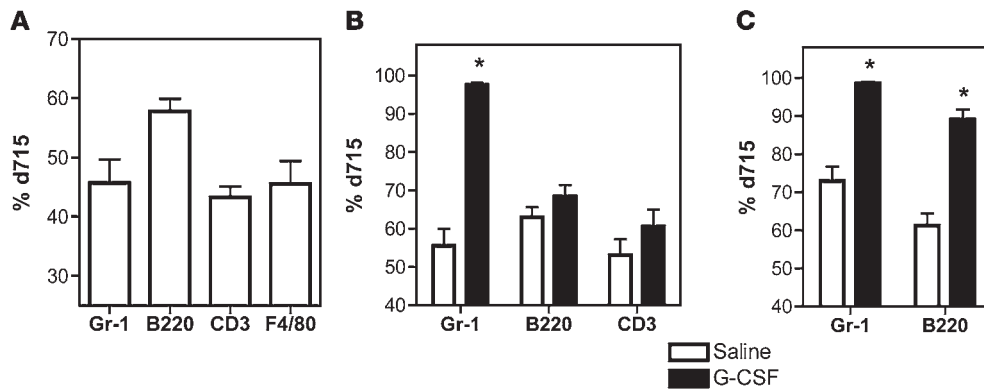
Results

The d715 G-CSFR confers a competitive advantage at the HSC level in a G-CSF-dependent fashion. We previously described the generation of mice carrying a targeted (knockin) mutation of their *Csf3r* gene that is representative of the truncation mutations found in SCN (15). Of note, the targeted transgenic (knockin) approach preserves the genomic organization of the *Csf3r* allele; accordingly, the mutant G-CSFR is expressed in a development- and lineage-specific fashion. These mice, termed “d715 G-CSFR,” have normal basal granulopoiesis but exhibit an exaggerated neutrophil response to exogenous G-CSF. To examine the effect of the d715 G-CSFR on HSC function, a competitive repopulation assay was performed.

Nonstandard abbreviations used: AML, acute myeloid leukemia; G-CSFR, G-CSF receptor; KSL, c-kit⁺Sca¹ lineage⁺; LSC, leukemic stem cell; MDS, myelodysplastic syndrome; SCN, severe congenital neutropenia.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* 118:946–955 (2008). doi:10.1172/JCI32704.

**Figure 1**

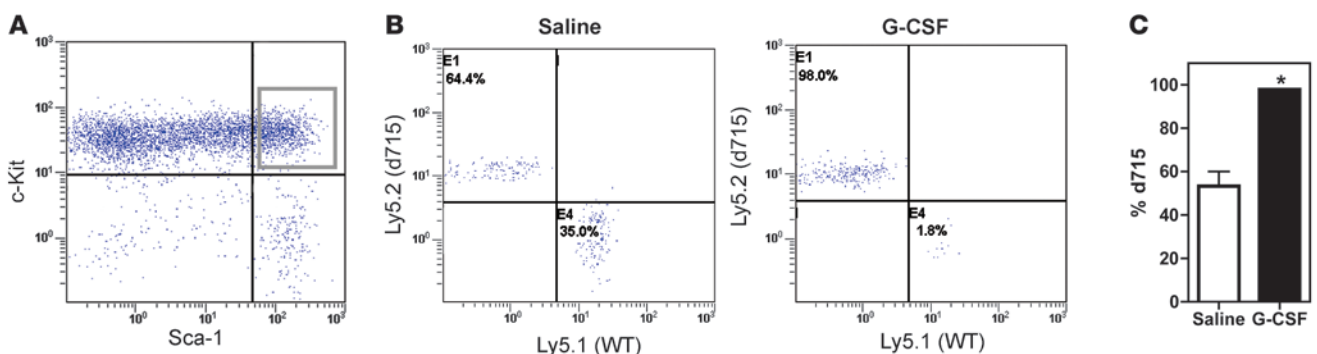
The d715 G-CSFR confers a competitive repopulation advantage that is dependent upon exogenous G-CSF administration. Irradiated syngeneic mice were reconstituted with a 1:1 ratio of d715 G-CSFR (Ly5.2⁺) and wild-type (Ly5.1⁺) bone marrow cells. (A) The percentage of blood neutrophils (Gr-1), B lymphocytes (B220), T lymphocytes (CD3), and monocytes (F4/80) that were derived from d715 G-CSFR (d715) cells is shown ($n = 9$). (B and C) The mice were then treated with G-CSF (10 $\mu\text{g}/\text{kg}/\text{d}$; $n = 5$) or saline alone ($n = 4$) for 21 days and the contributions of d715 G-CSFR cells in the blood (B) and bone marrow (C) were measured. Data represent the mean \pm SEM. * $P < 0.05$.

Recipient mice were reconstituted with a 1:1 ratio of d715 G-CSFR and wild-type bone marrow cells, and the contribution of d715 G-CSFR cells to hematopoiesis was determined by flow cytometry. At 6 months after transplantation, the contribution of d715 G-CSFR cells to the neutrophil, B lymphocyte, T lymphocyte, and monocyte lineages in the blood was near the predicted 50% level, indicating that the d715 G-CSFR does not confer a competitive advantage (Figure 1A).

In patients with SCN, the systemic level of G-CSF is high, either because of increased endogenous production or its pharmacologic administration (16). To simulate this increase in systemic G-CSF, we treated the chimeric mice with G-CSF (10 $\mu\text{g}/\text{kg}/\text{d}$) for 3 weeks and repeated the chimerism analysis (Figure 1, B and C). After G-CSF treatment, nearly all of the circulating neutrophils were derived from d715 G-CSFR cells (saline, 55.5% \pm 4.4% versus G-CSF, 97.6% \pm 1.2%; $P < 0.001$). Likewise, nearly all (98.6% \pm 0.7%) of Gr-1⁺ granulocytic cells in the bone marrow were of d715 G-CSFR origin after G-CSF treatment. A significant increase in the contribution of d715 G-CSFR cells to the B lymphocyte lineage in

the bone marrow also was observed (saline, 61.2% \pm 6.4% versus G-CSF, 89.1% \pm 5.7%; $P < 0.001$); the lack of significant increase in circulating B or T lymphocytes after only 3 weeks of G-CSF treatment likely reflects the long half-life of these cells.

The increase in d715 G-CSFR chimerism in the B lymphocyte lineage raised the possibility that the d715 G-CSFR confers a competitive advantage at the HSC level after G-CSF treatment. To address this possibility, chimerism in c-kit⁺Sca-1^{lineage}⁻ (KSL) cells, a cell population enriched in HSCs, was assessed. In saline-treated mice, the percentage of KSL cells derived from d715 G-CSFR cells was 53.3% \pm 11.5% (Figure 2). After 3 weeks of G-CSF treatment, this percentage increased to 97.8% \pm 0.8% ($P < 0.001$). We next asked whether short-term (3-week) treatment with G-CSF results in durable increases in d715 G-CSFR chimerism. A separate cohort of chimeric mice was generated by transplanting equal numbers of wild-type and d715 G-CSFR bone marrow cells into recipient mice. Five months after transplantation, these mice were treated for 3 weeks with G-CSF or saline and then followed for an additional 3 months. As expected, immediately after G-CSF treatment, virtu-

**Figure 2**

The contribution of d715 G-CSFR cells to the KSL cell compartment is increased after G-CSF treatment. Chimeric mice generated as described in Figure 1 were treated with saline or G-CSF (10 $\mu\text{g}/\text{kg}/\text{d}$) for 21 days, and the percentage of KSL cells in the bone marrow derived from d715 G-CSFR cells was determined. (A) Representative scatter plot showing the strategy to identify KSL cells (boxed); data are gated on lineage⁻ cells (data not shown). (B) Representative scatter plots gated on KSL cells showing the percentage of d715 G-CSFR (Ly5.2⁺) cells. (C) The percentage of KSL cells derived from d715 G-CSFR cells is shown ($n = 4-5$ each). Data represent the mean \pm SEM. * $P < 0.001$.

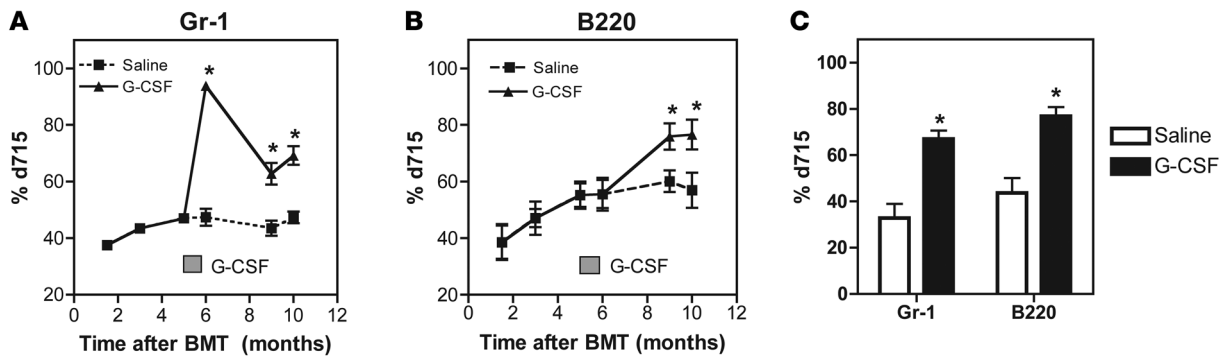


Figure 3

Long-term d715 G-CSFR chimerism following G-CSF treatment is increased. Chimeric mice generated as described in Figure 1 were treated 5 months after bone marrow transplantation (BMT) with saline alone or G-CSF (10 μg/kg/d) for 21 days (indicated by the gray box). The percentage of circulating neutrophils (A) and B lymphocytes (B) derived from d715 G-CSFR cells was determined at the indicated times ($n = 6-7$ for each group). (C). Bone marrow was harvested from chimeric mice after treatment with G-CSF or saline and transplanted into secondary recipients ($n = 5-10$ each cohort of recipient mice). Shown is the percentage of d715 G-CSFR cells in the blood of the secondary recipients 4 months after transplantation. Data represent the mean \pm SEM. * $P < 0.001$.

ally all of the circulating neutrophils were of d715 G-CSFR origin (Figure 3A). This increase persisted 3 months after discontinuing G-CSF treatment; the percentage of circulating neutrophils derived from d715 G-CSFR cells was $69.2 \pm 3.28\%$ versus $47.3 \pm 2.07\%$ for saline-treated mice ($P < 0.001$). Strikingly, G-CSF treatment also significantly increased the contribution of d715 G-CSFR cells to the B lymphocyte lineage. Three months after discontinuing G-CSF, the percentage of circulating B lymphocytes derived from d715 G-CSFR cells was $76.6 \pm 1.98\%$ versus $56.9 \pm 2.35\%$ for saline-treated mice ($P < 0.001$).

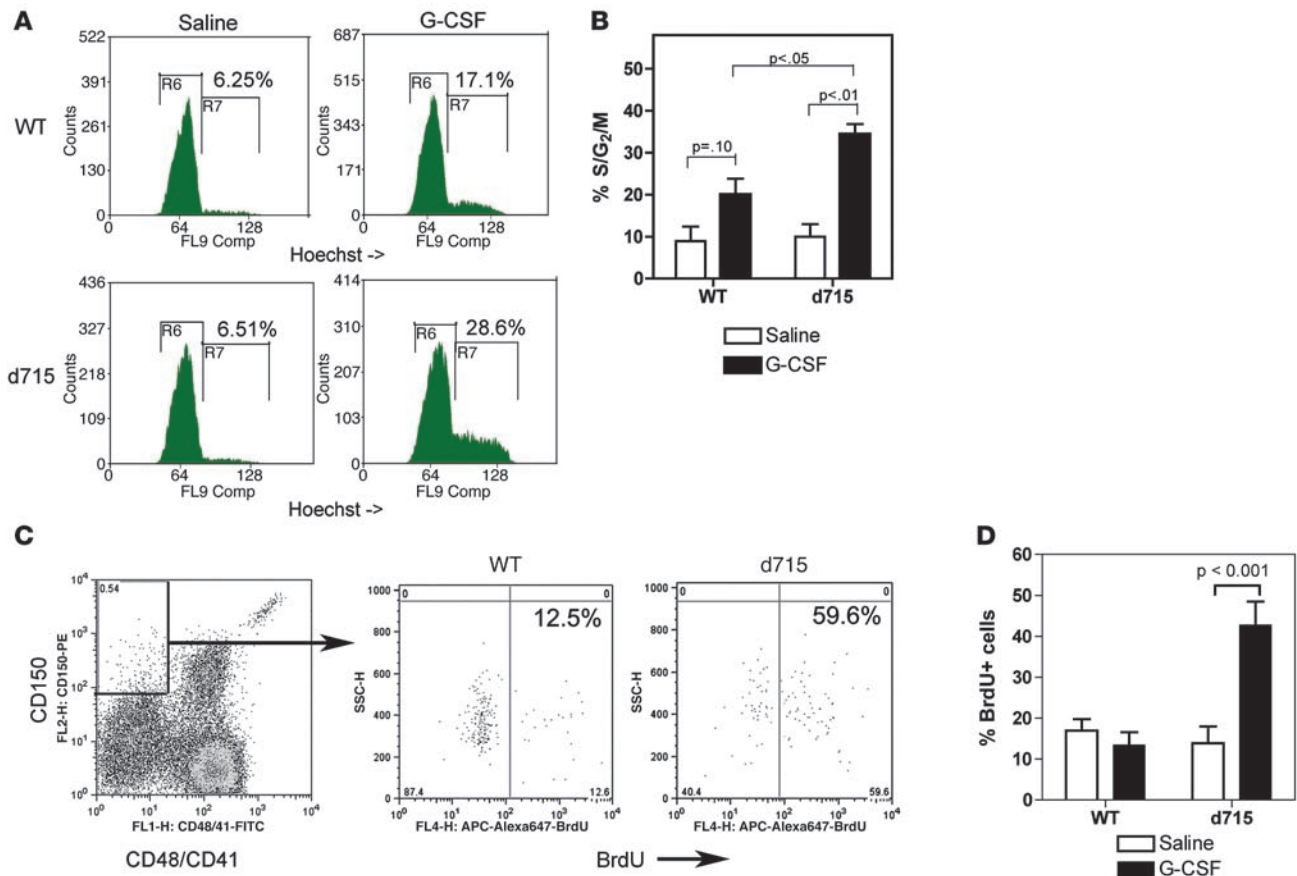
A defining feature of HSC is their ability to support multilineage long-term engraftment after transplantation into recipient mice. Consequently, we transplanted bone marrow from chimeric mice after treatment with G-CSF or saline into secondary recipient mice, and d715 G-CSFR chimerism was assessed 4 months after transplantation. A significant increase in d715 G-CSFR contribution to circulating neutrophils ($66.9 \pm 3.68\%$ versus $32.8 \pm 6.09\%$; $P < 0.001$) and B lymphocytes ($76.8 \pm 3.90\%$ versus $43.7 \pm 6.36\%$; $P < 0.001$) was detected in secondary recipients that had received G-CSF treated bone marrow (Figure 3C). Collectively, these data show that the d715 G-CSFR confers a strong competitive advantage to HSC in a G-CSF-dependent fashion.

G-CSF-induced proliferation is increased in d715 G-CSFR HSCs. The clonal advantage of d715 G-CSFR HSCs suggested that proliferation, survival, and/or self-renewal were enhanced. To characterize the effect on proliferation, wild-type or d715 G-CSFR mice were treated with a single dose of G-CSF, and 24 hours later, the cell cycle status of KSL cells in the bone marrow was measured (Figure 4). At baseline, a similar percentage of KSL were in the S/G₂/M phases of the cell cycle (wild type, $8.9 \pm 2.0\%$ versus d715 G-CSFR, $10.0 \pm 3.0\%$; $P = NS$). After treatment with G-CSF, the percentage of cycling KSL cells increased significantly in d715 G-CSFR mice ($34.4 \pm 2.4\%$), with a trend to increased cycling in wild-type KSL cells ($20.0 \pm 3.8\%$). Of note, G-CSF induced a significantly greater proliferative response in d715 G-CSFR compared with wild-type KSL cells ($P < 0.05$).

The KSL cell population is heterogeneous, containing both HSCs and lineage-committed progenitors. Consequently, we repeated these experiments using more restrictive criteria to identify HSCs.

Specifically, we determined the fraction of CD150⁺CD48⁻CD41⁻ lineage⁻ cells that labeled with BrdU in vivo after G-CSF treatment. A previous study showed that 1 in 2.2 of these cells has long-term repopulating activity (17). Consistent with this study, the frequency of CD150⁺CD48⁻CD41⁻ lineage⁻ cells in the bone marrow of wild-type mice was approximately 0.005% (Figure 4C). A similar frequency was observed in d715 G-CSFR mice and did not change with G-CSF treatment (data not shown). In wild-type mice, G-CSF treatment had no effect on BrdU incorporation of CD150⁺CD48⁻CD41⁻ lineage⁻ cells (Figure 4D). In contrast, in d715 G-CSFR mice, G-CSF treatment significantly stimulated BrdU incorporation in these cells (saline, $15.4 \pm 7.6\%$ versus G-CSF, $42.8 \pm 12.9\%$; $P = 0.004$). Together, these data suggest that proliferative signaling in HSC is greatly enhanced by the d715 G-CSFR.

Short-term G-CSF treatment induces the activation of Stat3 and/or Stat5 target genes in KSL cells. To elucidate the molecular mechanisms by which the d715 G-CSFR confers a clonal HSC advantage, RNA expression profiling experiments were performed. Specifically, wild-type or d715 G-CSFR mice were treated with a single dose of G-CSF or saline, and 3 hours later KSL cells were sorted from the bone marrow by flow cytometry. The 3-hour time point was chosen to identify early transcriptional targets of G-CSF signaling in KSL cells. Of note, this brief exposure to G-CSF had no discernible effect on KSL number or flow cytometry characteristics (data not shown). Data from 3 independent experiments were pooled; genes that were consistently upregulated or downregulated by G-CSF treatment are summarized in Supplemental Tables 1 and 2 (supplemental material available online with this article; doi:10.1172/JCI32704DS1). Two criteria were used to identify genes differentially regulated by G-CSF in d715 G-CSFR KSL cells. First, genes were selected in which the magnitude of the change in mRNA expression after G-CSF treatment in d715 G-CSFR KSL cells was at least 2.0-fold greater than that observed with wild-type KSL cells. Second, a significant difference in the absolute gene chip signal between G-CSF-treated wild-type and d715 G-CSFR KSL cells was required. Based on these criteria, 14 genes were identified (Table 1). Strikingly, there is evidence that Stat3 and/or Stat5 regulate the mRNA expression of 6 of these genes, including *Sprr2a* (18), *Tcrg* (19, 20), *Pim2* (21), *Cdkn1a* (22, 23),

**Figure 4**

G-CSF–induced proliferation of d715 G-CSFR HSCs is increased. Wild-type or d715 G-CSFR mice were treated with saline or G-CSF (2 doses of 100 μ g/kg, 12 hours apart); in some cases (C and D), mice also received BrdU (2 doses of 1 mg, 12 hours apart). (A) Representative histograms showing Hoechst staining of KSL cells. (B) Shown is the percentage of KSL cells in the S/G₂/M phases of the cell cycle ($n = 3–4$ per condition). (C) Representative scatter plots showing the gating strategy and BrdU staining of CD150⁺CD48[–]CD41[–] lineage[–] cells. (D) Shown is the percentage of CD150⁺CD48[–]CD41[–] lineage[–] cells that labeled with BrdU ($n = 5–6$ per condition). Data represent the mean \pm SEM.

Cish (24), and *Socs2* (25). These data suggest the hypothesis that accentuated activation of Stat3 and/or Stat5 mediates the HSC clonal advantage of the d715 G-CSFR.

Stat5 but not Stat3 activation by G-CSF is accentuated in d715 G-CSFR KSL cells. A flow cytometric assay to quantify the phosphorylation of Stat3 and Stat5 was used to assess the activation of these proteins in KSL cells (26). We first validated the assay in primary murine myeloid cells (Figure 5, A and B). Consistent with previous studies utilizing gel shift assays (12, 27), Stat5 activation by the d715 G-CSFR in primary myeloid cells was markedly enhanced (AUC: wild type, 148 \pm 28 and d715 G-CSFR, 371 \pm 86; $P < 0.05$). Again, consistent with previous reports (12, 27), Stat3 activation in d715 G-CSFR myeloid cells, though delayed, was comparable with wild-type cells (AUC: wild type, 2,053 \pm 546 and d715 G-CSFR, 2,711 \pm 815; $P = \text{NS}$).

Similar experiments were performed with KSL cells (Figure 5, C and D). Compared with wild-type cells, both the magnitude and duration of Stat5 activation by G-CSF was increased in d715 G-CSFR cells (AUC: wild type, 203 \pm 73 and d715 G-CSFR, 771 \pm 187; $P = 0.05$). In contrast, Stat3 activation by G-CSF in d715 G-CSFR KSL cells was significantly reduced at early time points after G-CSF stimulation (Figure 5D), with a trend to a

decrease in overall Stat3 activation (AUC: wild type, 1,315 \pm 491 and d715 G-CSFR, 1,020 \pm 530; $P = 0.16$). These data show that Stat5 activation by the d715 G-CSFR in KSL cells was enhanced, whereas Stat3 activation was attenuated.

The hyperproliferative response of the d715 G-CSFR is abrogated in Stat5-deficient myeloid progenitors. We next sought to assess the effect of the loss of Stat5 on the clonal dominance conferred by the d715 G-CSFR. However, the engraftment potential of *Stat5A*^{–/–} \times *Stat5B*^{–/–} HSC is markedly impaired (28, 29), precluding the direct assessment of the d715 G-CSFR on HSC function in Stat5-deficient cells. Instead, we determined whether the previously described hyperproliferative phenotype conferred by the d715 G-CSFR on myeloid progenitors is abrogated by Stat5 deficiency. In brief, hematopoietic cells harvested from the fetal liver of wild-type or *Stat5A*^{–/–} \times *Stat5B*^{–/–} mice were transduced with retrovirus expressing wild-type or d715 G-CSFR and colony formation in response to G-CSF measured. As reported previously, expression of the d715 G-CSFR in Stat5-sufficient cells resulted in a significant increase in both colony number and average colony size (Figure 6, A and B). In *Stat5A*^{–/–} \times *Stat5B*^{–/–} cells, G-CSF–induced colony formation was reduced, and the hyperproliferative response conferred by the d715 G-CSFR was abrogated.



Table 1
RNA expression profiling

Gene	WT			d715 G-CSFR			Ratio ^A	P value ^B
	Saline	G-CSF	Fold change	Saline	G-CSF	Fold change		
<i>Tcrg</i>	318 ± 91	202 ± 88	0.64	266 ± 24	2,492 ± 438	9.37	14.7	<0.005
<i>Sprr2a</i>	810 ± 124	2,098 ± 903	2.59	599 ± 122	21,072 ± 6,628	35.2	13.6	<0.05
<i>Tnfsf11</i>	435 ± 116	251 ± 95	0.58	232 ± 108	1,116 ± 74	4.81	8.34	<0.005
<i>Cish</i>	2,730 ± 525	3,005 ± 110	1.10	2,829 ± 801	13,794 ± 556	4.88	4.43	<0.0005
<i>Pim2</i>	1,382 ± 293	1,981 ± 530	1.43	1,040 ± 326	6,337 ± 1,523	60.09	4.25	<0.05
<i>Enah</i>	230 ± 83	1,495 ± 127	6.50	628 ± 203	17,320 ± 2,265	27.6	4.24	<0.005
<i>Zfpn1a4</i>	167 ± 68	181 ± 68	10.08	248 ± 92	1,105 ± 76	4.46	4.11	<0.0005
<i>Socs2</i>	903 ± 62	1,315 ± 335	1.46	2,210 ± 868	11,020 ± 1,488	4.99	3.42	<0.005
<i>Cdkn1a</i>	987 ± 171	1,541 ± 206	1.56	791 ± 135	4,057 ± 568	5.13	3.29	<0.01
<i>Serpina3g</i>	22,519 ± 5,363	31,260 ± 4,761	1.39	20,302 ± 5,034	83,742 ± 13,422	4.12	2.97	<0.05
<i>Bcat1</i>	137 ± 49	572 ± 95	4.18	75 ± 34	883 ± 46	11.8	2.82	<0.05
<i>Cacnb2</i>	769 ± 769	1,621 ± 77	2.11	585 ± 277	3,018 ± 595	5.16	2.45	<0.05
<i>Ltb4r1</i>	1,025 ± 259	730 ± 198	0.71	3,231 ± 2,069	276 ± 34	0.09	0.12	<0.05
<i>Bhlhb2</i>	127 ± 52	1,267 ± 179	9.98	723 ± 583	2,501 ± 133	3.46	0.35	<0.005

The mean ± SEM raw signal of experiments performed in triplicate is shown. ^ARatio of the fold change after G-CSF treatment between wild-type and d715 G-CSFR cells. ^BComparing G-CSF-treated wild-type KSL cells with G-CSF-treated d715 G-CSFR KSL cells.

Loss of Stat3 and Stat5 activation by the d715F G-CSFR is associated with decreased HSC function. To further test the hypothesis that Stat3 and/or Stat5 activation mediates the clonal HSC advantage of the d715 G-CSFR, we next studied d715F G-CSFR-targeted transgenic (knockin) mice. In the d715F G-CSFR mutant, the sole remaining tyrosine (Y704) of d715 G-CSFR is mutated to phenylalanine. We previously showed that Stat3 and Stat5 activation by the d715F G-CSFR in primary murine myeloid cells is markedly impaired (27). Similar results were obtained with KSL cells (Figure 7, A and B). In fact, no detectable Stat5 or Stat3 activation was observed in d715F KSL cells following G-CSF stimulation.

To determine whether the loss of Stat3 and Stat5 activation by the d715F G-CSFR affected HSC function, we repeated the competitive repopulation assay using a 1:1 ratio of d715F G-CSFR and wild-type bone marrow cells. Consistent with the known defect in granulopoiesis in d715F G-CSFR mice (27), the contribution of d715F G-CSFR cells to the neutrophil lineage was consistently less than that observed in the B or T lymphocyte lineages (Figure 7, C-E). Chimeric mice were treated with G-CSF for 3 weeks starting 4 months after transplantation. Despite the loss of Stat3 and Stat5 activation, a significant increase in d715F G-CSFR chimerism in the neutrophil lineage was observed. However, this increase was transitory, returning to control levels after stopping the G-CSF. In fact, a gradual decrease in d715F G-CSFR contribution to neutrophils, B lymphocytes, and T lymphocytes was observed over time, irrespective of whether G-CSF was given. These data suggest that the d715F G-CSFR confers a competitive disadvantage to HSC.

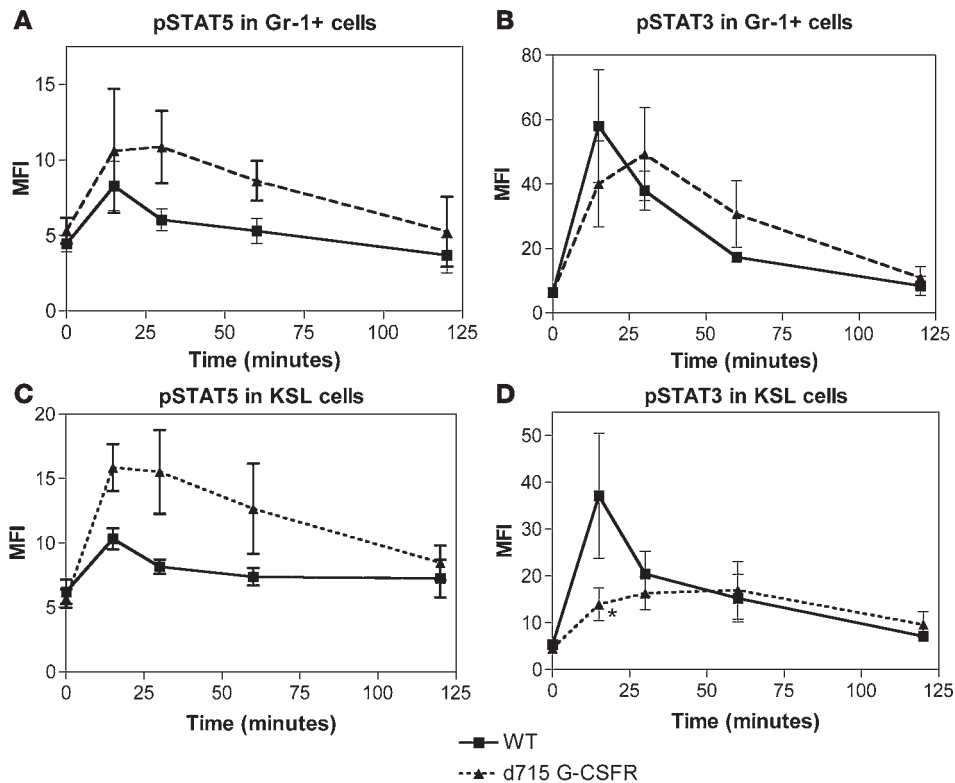
Discussion

Truncation mutations of *CSF3R* are strongly and uniquely associated with the development of MDS/AML in patients with SCN. Whereas truncation mutations of *CSF3R* are present in 78% of cases of MDS/AML in patients with SCN (10), they are observed in only 1%–2% of de novo AML cases (10, 30, 31). These mutations are acquired and achieve clonal dominance in the human bone marrow microenvironment. In the present study, we show that expression of a representative G-CSFR truncation mutant confers a strong clonal advantage at the HSC level. However,

the clonal dominance is dependent upon exogenous G-CSF administration; similar results were observed after treating mice for 3 weeks with 2 different doses of G-CSF: 10 µg/kg/d and 100 µg/kg/d (data not shown). The requirement for high systemic levels of G-CSF may explain the unique association of *CSF3R* mutations with SCN, since in SCN, systemic levels of G-CSF are high due to increased endogenous production or pharmacologic administration (16). A novel feature of this model is the inducibility of the HSC clonal dominance, providing a tool to explore molecular mechanisms for this process.

There is increasing evidence that HSC quiescence is associated with long-term repopulating activity. Transplantation studies of phenotypically defined HSC fractionated based on cell cycle have shown that the quiescent fraction has the highest repopulating activity (32, 33). Moreover, loss-of-function mutations for *Cdkn1a* (p21^{waf1}) (34) or *Pten* (35, 36) that result in excessive HSC proliferation are associated with loss of long-term repopulating ability. In contrast, in the present study, we show that mutant *Csf3r* confers increased repopulating activity despite inducing greater proliferation of HSC (as defined by KSL or CD150⁺CD41⁺CD48⁻lineage⁻cells). These observations suggest the possibility that the nature of the proliferative signals generated by mutant *Csf3r* may preserve the long-term repopulating activity of HSC. In this regard, it is interesting to note that *Cdkn1a* (p21^{waf}) expression is strongly induced by G-CSF in *Csf3r* mutant HSC (Table 1). As noted above, *Cdkn1a* is a negative regulator of HSC proliferation and helps maintain HSC quiescence. Thus it is possible that simultaneous induction of proliferative signals and *Cdkn1a* expression may prevent the loss of long-term repopulating activity associated with uncontrolled HSC proliferation.

There is considerable evidence linking Stat5 to the regulation of HSC function. Stat5 is activated by several cytokine receptors implicated in the regulation of HSC, including *MPL* and *FLT3* (37, 38). Moreover, loss of function mutations for both Stat5A and Stat5B lead to a profound defect in hematopoietic repopulating activity (28, 29, 39). Conversely, expression of a constitutively active form of Stat5 in both murine and human hematopoietic progenitors promoted the self-renewal and expansion of multi-

**Figure 5**

Stat5 but not Stat3 activation by G-CSF is enhanced in d715 G-CSFR cells. Wild-type or d715 G-CSFR bone marrow cells were treated in vitro for the indicated times with G-CSF (100 ng/ml), and the cells were then incubated with antibodies specific for phosphorylated Stat5 or Stat3. (A and B) The MFI of Gr-1⁺ (myeloid) cells for pStat5 (A) and pStat3 (B) is shown. (C and D) The MFI of KSL cells for pStat5 (C) and pStat3 (D) is shown. Data represent the mean \pm SEM ($n = 4$ per cohort). * $P < 0.05$ compared with wild-type cells at the same time point.

potential progenitors ex vivo (37, 40). Herein, we show that Stat5 but not Stat3 phosphorylation by G-CSF is accentuated in *Csf3R* mutant KSL cells. Accordingly, several well-known Stat5 target genes, including *PIM2* and *Cdkn1a*, are induced to a greater degree by G-CSF in *Csf3R* mutant compared with wild-type KSL cells. Moreover, though the severe repopulating defect of Stat5A^{-/-} \times Stat5B^{-/-} cells precluded a direct measure of HSC activity, we show that the proliferative advantage of myeloid progenitors expressing mutant (d715) *Csf3R* is dependent upon Stat5. Finally, to directly assess the importance of Stat5 activation by G-CSF in the regulation of HSC function, we characterized the long-term repopulating activity of HSCs expressing a mutant (d715F) *Csf3R* engineered to have impaired Stat5 activation. In fact, the competitive repopulating activity of d715F HSCs was reduced compared with wild-type HSCs. A caveat of this experiment is that other signaling pathways besides Stat5 (and Stat3) may be disrupted by the d715F mutation. Collectively, these data suggest that accentuated Stat5 activation by mutant *Csf3r* mediates the clonal HSC advantage.

Stat5 has been implicated in leukemogenesis. Constitutive Stat5 activation has been detected in approximately 25% of leukemias (41), and it is specifically associated with leukemias carrying internal tandem duplications of *FLT3* (*FLT3* ITD) (42). Expression of constitutively active Stat5 in hematopoietic cells results in a rapidly fatal myeloproliferative disease in mice (37, 41, 43). Studies

of Stat5A \times Stat5B doubly deficient mice have suggested an obligatory role for Stat5 in leukemogenesis induced by BCR-ABL and TEL-JAK2 (43, 44). Moreover, a recent study showed that RNAi-mediated inhibition of STAT5 expression in human leukemic progenitors inhibited their long-term growth in vitro (45). Our data provide further support for a key role for Stat5 in leukemogenesis. Specifically, our data suggest that Stat5 activation may contribute to the acquisition of clonal dominance by LSCs. However, Stat5 activation alone does not appear to be sufficient to induce leukemia, since we previously showed that d715 G-CSFR mice did not develop leukemia despite chronic (6-month) treatment with G-CSF (15).

The Stat5 target genes that regulate HSC function and confer clonal dominance have not yet been determined. A number of genes have been implicated in the regulation of HSC self-renewal, including *Bmi1* (46), *HoxB4* (47), *Mef* (48), *Tie2* (49), *Catnb* (50), *CEBPA* (51), *Cdkn2c* (p18^{INK4c}) (52), *Notch1*, *Notch4* (53), *Gfi1* (54, 55), and *NfyA* (56). However, none of these genes were significantly upregulated in vivo in KSL cells after 3 hours of G-CSF stimulation (data not

shown). Studies are underway to see whether any of these genes are activated at later time points after G-CSF stimulation. Of the genes differentially regulated by G-CSF in *Csf3r* mutant KSL cells (Table 1), several have been implicated in the regulation of HSC function or leukemogenesis. As already discussed, *Cdkn1a* has been shown to regulate HSC self-renewal (34). *Pim2* is a serine-threonine kinase that has been shown to play a pivotal role in the transmission of proliferative and survival signals by *FLT3* ITD (47, 57). The importance of these genes is currently being investigated through the use of the relevant loss-of-function transgenic mice.

The high rate of leukemic transformation in SCN, and the dismal prognosis once MDS or AML develops, has led to considerable debate over the timing of allogeneic HSC transplantation (HSCT) in patients with SCN. Unfortunately, there are no randomized clinical trials comparing HSCT with standard therapy in SCN. However, based on its strong association with MDS/AML, the presence of *CSF3R* mutations has been proposed as an indication for HSCT, even in the absence of MDS/AML (10). The present study supports this approach. Though none of the *Csf3r* mice developed MDS/AML, our data showing that the mutant *Csf3r* confers a clonal HSC advantage increases the likelihood that *CSF3R* mutations contribute to leukemic transformation. Alternatively, the dependence of the HSC clonal advantage on high systemic levels of G-CSF suggests that discontinuing G-CSF administration may result in the

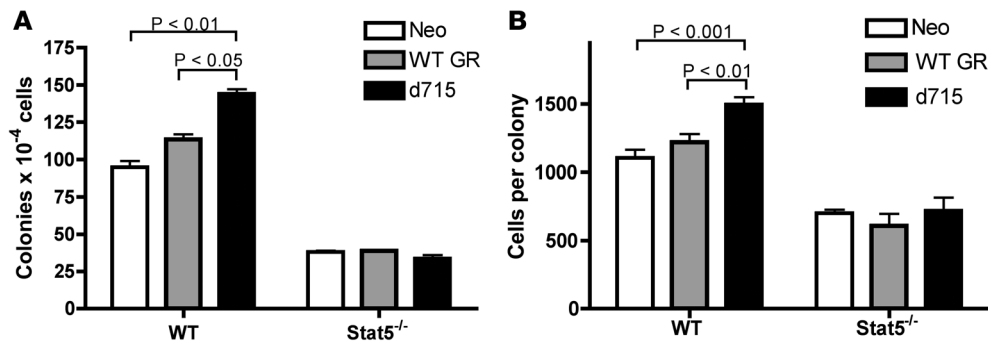


Figure 6

Stat5 deficiency abrogates the hyperproliferative response of the d715 G-CSFR in myeloid progenitors. E12–E15 fetal liver cells from wild-type or Stat5A^{-/-} × Stat5B^{-/-} (Stat5^{-/-}) mice were transduced with retrovirus expressing neomycin phosphotransferase alone (Neo) or this gene and either wild-type G-CSFR (WT GR) or d715 G-CSFR. Cells were cultured in 10 ng/ml of G-CSF and geneticin to select for transduced cells. Shown is the number of colonies (A) and average colony size (B) observed after 7–10 days. Similar results were observed in cultures stimulated with 1 ng/ml of G-CSF (data not shown). No colonies formed in the absence of G-CSF (data not shown). Data represent the mean ± SEM of 3 independent experiments.

loss (or at least stabilization) of the CSF3R mutant clone. Indeed, there is a case report in which a patient with a CSF3R mutation and AML had a spontaneous remission of AML after stopping G-CSF (58). However, treatment with G-CSF is proven to improve surviv-

al in patients with SCN, and there currently is no other effective therapy to increase neutrophil counts in patients with SCN. In the absence of such a therapy, it may be prudent to consider allogeneic HSCT in all patients with SCN who acquire a CSF3R mutation.

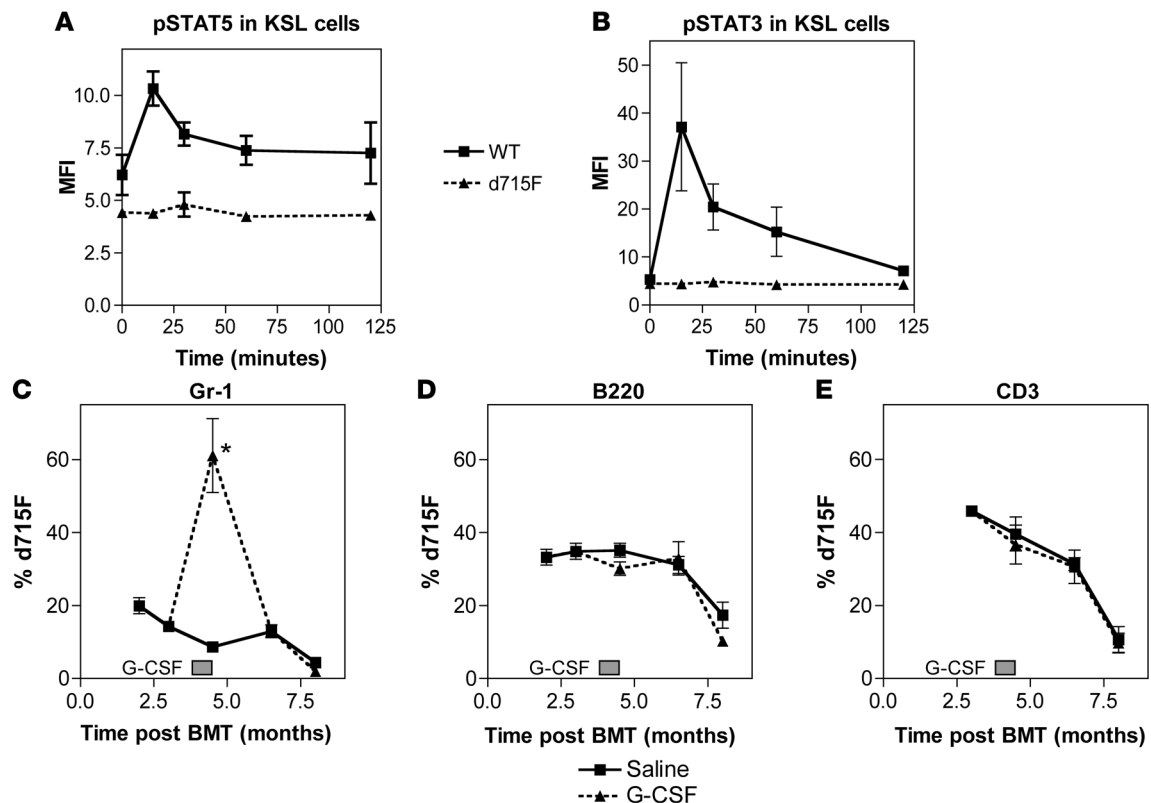


Figure 7

Competitive repopulation by HSC expressing the d715F G-CSFR is impaired. (A and B) Wild-type or d715F G-CSFR KSL cells were treated in vitro for the indicated times with G-CSF (100 ng/ml) and then incubated with antibodies specific for pStat5 (A) or pStat3 (B). The MFI of triplicate experiments is shown. (C–E) Irradiated syngeneic mice were reconstituted with a 1:1 ratio of d715F G-CSFR and wild-type bone marrow cells. The resulting chimeric mice were treated 4 months after bone marrow transplantation with saline alone or G-CSF (10 μg/kg/d) for 21 days. The percentage of circulating neutrophils (C), B lymphocytes (D), and T lymphocytes (E) derived from d715F G-CSFR cells was determined at the indicated times (n = 3–4 for each group). Data represent the mean ± SEM. *P < 0.05 compared with saline-treated mice.



Methods

Mice. d715 G-CSFR and d715F G-CSFR mice backcrossed 10 generations onto a C57BL/6 background were generated as described previously (15, 27). C57BL/6 mice (B6.SJL-Ptprc^{*/Pep3^b/BoyJ}) that have the Ly5.1 gene were obtained from The Jackson Laboratory. Stat5a^{+/-} × Stat5b^{+/-} mice outbred on a NIH Black Swiss × 129SvEv × C57BL/6 background were generated as previously described (59). These mice were intercrossed to generate Stat5a^{-/-} × Stat5b^{-/-} fetuses. Mice were housed in a specific pathogen-free environment. All experiments were approved by the Washington University Animal Studies Committee.

Competitive repopulation assay. Wild-type (Ly5.1), d715, or d715F G-CSFR (Ly5.2) bone marrow cells were harvested from strain- and sex-matched mice. A total of 5 million bone marrow cells were mixed at a 1:1 ratio and injected into the tail vein of lethally irradiated wild-type mice (Ly5.1). Recipient mice were conditioned with 1,000 cGy from a ¹³⁷Cesium source at a rate of approximately 95 cGy/min 24 hours prior to transplantation. Prophylactic antibiotics (trimethoprim-sulfamethoxazole; Alpharma) were given during the initial 2 weeks following transplantation. Mice were analyzed 3–12 months after transplantation. Where indicated, mice were treated with human recombinant G-CSF (Amgen) in PBS with 0.1% low-endotoxin BSA at a dose of 10 µg/kg/d for 21 days. Secondary bone marrow transplantation was performed immediately following treatment with G-CSF or saline alone. Bone marrow cells (5 × 10⁶) from the primary recipient mice were transplanted into secondary recipient mice.

Flow cytometry. Bone marrow and blood leukocytes were processed for flow cytometry as previously described (60). For chimerism analysis, cells were incubated with FITC-conjugated Ly5.1, Cy5-PE-conjugated Ly5.2, and one of the following PE-conjugated lineage markers: Gr-1 (myeloid), B220 (B lymphocytes), CD3 (T lymphocytes), or F4/80 (monocytes; Caltag Laboratories). Isotype-matched antibodies were used as negative controls. Cells were analyzed on a 2-laser, 5-color FACScan flow cytometer (BD Biosciences and Cytex Development). Unless otherwise indicated, all antibodies were obtained from BD Biosciences.

To analyze KSL cells, lineage⁺ cells were first depleted. In brief, cells were incubated with the following PE-conjugated lineage markers: CD3, B220, Gr-1, and Ter-119. Following incubation with anti-PE colloid, lineage⁺ cells were depleted using the AutoMacs system (Miltenyi Biotec). The lineage-depleted cells were then incubated with FITC-conjugated Sca-1, APC-conjugated c-kit, Cy5-PE-conjugated Ly5.2, and Cy7-APC-conjugated Ly5.1.

Cell cycle analysis of KSL cells. Following lineage depletion and incubation with FITC-conjugated Sca1 and APC-conjugated c-kit, bone marrow cells were incubated with 10 µg/ml Hoechst 33342 (Molecular Probes) in PBS containing 0.2% BSA at 37°C for 60 minutes. Hoechst staining of the KSL cells was assessed using a MoFlo high-speed cell sorter (Dako).

BrdU labeling. Mice (*n* = 5 per condition) were injected with BrdU (2 doses of 1 mg given 12 hours apart; BD Biosciences) and either saline alone or G-CSF (2 doses of 100 µg/kg given with the BrdU). Bone marrow cells were harvested 12 hours after the final dose of BrdU and incubated with FITC-conjugated CD48 (eBioscience) and CD41, biotinylated CD150 (clone number TC15-12F12.1; Biolegend), and the following panel of Cy5-PE-conjugated lineage markers: CD3, B220, Gr-1, and Ter-119. Following incubation with streptavidin-PE (eBioscience), cells were fixed, permeabilized, and incubated with PE-conjugated anti-BrdU antibody per the manufacturer's recommendations (BrdU flow kit; BD Biosciences). Cells were analyzed using a MoFlo high-speed cell sorter.

Expression profiling and data analysis. Groups of 8–10 wild-type, d715 G-CSFR, or d715F G-CSFR mice were treated with a single subcutaneous injection of G-CSF (100 µg/kg) or saline alone. Bone marrow cells were harvested 3 hours later and KSL cells sorted. RNA was prepared from 20,000–100,000

KSL cells using Trizol reagent (Invitrogen). Total cellular RNA samples of 100 ng were subjected to linear amplification, biotin labeling, fragmentation, and hybridization to Affymetrix MOE430 v2.0 GeneChip microarrays per protocols of the Siteman Cancer Center Multiplexed Gene Analysis Core Facility (<http://pathology.wustl.edu/research/multiplexed.php>). Experiments were performed in triplicate.

Visualization and statistical analysis of data were performed using Decision Site (Spotfire). Signal values corresponding to the probesets were normalized to adjust for minor differences in hybridization intensities across the 12 microarrays analyzed. After normalization, mean signal intensities for each probeset were calculated across the 3 experiments. Probesets upregulated or downregulated by G-CSF compared with saline control were selected based upon the following selection criteria: (a) present detection call in at least 2 of 3 probesets corresponding to the upregulated condition; (b) absolute signal intensity > 500 in at least 2 of 3 probesets corresponding to the upregulated condition; and (c) greater than 2-fold change in mean signal intensity (hereafter referred to as *fold-induction ratio*) between G-CSF and saline. In cases where multiple probesets corresponded to the same gene, the probeset with the highest average signal intensity and greatest number of present calls was used.

Assessment of Stat5 and Stat3 phosphorylation by flow cytometry. Bone marrow cells were either incubated with FITC-conjugated Gr-1 or lineage depleted and then incubated with FITC-Sca1 and APC-c-kit. Cells were then suspended in Opti-MEM (Invitrogen) with 10% FBS and stimulated with G-CSF (100 ng/ml) for the indicated time at 37°C. As a control, duplicate samples were cultured without G-CSF. Following incubation, cells were incubated in BD Phosphoflow Lyse/fix buffer (BD Biosciences) for 10 minutes, permeabilized in 100% methanol at 4°C for 20 minutes, and then incubated with anti-pStat5 (pY694) or anti-pStat3 (pY705) antibody (BD Biosciences) at room temperature for 30 minutes.

Retroviral transduction of Stat5a^{-/-} × Stat5b^{-/-} fetal liver progenitors. Stable clones of the ecotropic packaging cell line GP+E86 containing the retroviral viral vector pMPncrdleno alone or this vector with cDNAs for wild-type murine G-CSFR or the d715 G-CSFR have been previously described (61). Cultures of fetal liver cells from 12- to 15-dpc Stat5a^{-/-} × Stat5b^{-/-} fetuses were generated as previously described (62). Cells were cultured for 24 hours in RPMI containing 10% FBS, 50 ng/ml of murine thrombopoietin, 100 ng/ml of murine stem cell factor, 10 ng/ml of murine interleukin-3, and 50 ng/ml of human flt3 ligand at 37°C in 5% CO₂ (all cytokines were obtained from Peprotech). Cells were then cocultured in the same media with irradiated (1,500 cGy) GP+E86 cells for 48 hours. Viable cells (0.5–1 × 10⁵ cells) recovered from these cultures were plated in MethoCult M3231 (Stem Cell Technologies) supplemented with 1 or 10 ng/ml of human G-CSF and 1 mg/ml of geneticin (Invitrogen). Colonies containing at least 50 cells were scored on days 7–10 of culture. To determine transduction efficiency, 1–2 × 10⁴ cultured fetal liver cells were placed in MethoCult M3434 with or without 1 mg/ml of geneticin and the percentage of colonies that were resistant to geneticin measured. A similar transduction efficiency (range 30%–52%) for all 3 retroviruses with both wild-type and Stat5a^{-/-} × Stat5b^{-/-} cells was observed (data not shown).

Statistics. Statistical significance was determined either by a 2-way ANOVA analysis or by a 2-sided Student *t* test. All data represent the mean ± SEM, unless otherwise stated.

Acknowledgments

We thank Amgen for their generous gift of G-CSF. We also thank Timothy A. Graubert for his critical review of this manuscript. This work was supported by NIH grant CA101937 and by the intramural program of the NIDDK/NIH.



Received for publication May 16, 2007, and accepted in revised form December 19, 2007.

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