Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells

Barbara Varnum-Finney,[1] Lia M. Halasz,[1] Mingyi Sun,[1] Thomas Gridley,[2] Freddy Radtke,[3] and Irwin D. Bernstein[1]

[1]Pediatric Oncology, Clinical Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA. [2]The Jackson Laboratory, Bar Harbor, Maine, USA. [3]Ludwig Institute for Cancer Research, University of Lausanne, Epalinges, Switzerland.

HSCs either self-renew or differentiate to give rise to multipotent cells whose progeny provide blood cell precursors. However, surprisingly little is known about the factors that regulate this choice of self-renewal versus differentiation. One candidate is the Notch signaling pathway, with ex vivo studies suggesting that Notch regulates HSC differentiation, although a functional role for Notch in HSC self-renewal in vivo remains controversial. Here, we have shown that Notch2, and not Notch1, inhibits myeloid differentiation and enhances generation of primitive Sca-1$^+$c-kit$^+$ progenitors following in vitro culture of enriched HSCs with purified Notch ligands. In mice, Notch2 enhanced the rate of formation of short-term repopulating multipotential progenitor cells (MPPs) as well as long-term repopulating HSCs, while delaying myeloid differentiation in BM following injury. However, consistent with previous reports, once homeostasis was achieved, neither Notch1 nor Notch2 affected repopulating cell self-renewal. These data indicate a Notch2-dependent role in assuring orderly repopulation by HSCs, MPPs, myeloid cells, and lymphoid cells during BM regeneration.

Introduction

HSCs either self-renew or give rise to multipotent cells whose progeny provide precursors committed to the lymphoid and myeloid lineages. Recent studies have identified factors that maintain HSCs in a quiescent state required for long-term maintenance or that induce their proliferation (1, 2). However, little is known about factors that regulate HSC choice of self-renewal versus differentiation. One candidate, the Notch pathway, is known to affect self-renewal and lineage fate of precursor cells in numerous developing systems, including in neural, muscle, and skin precursors (3). Within the hematopoietic system, Notch directs many lineage choices, including Notch1-mediated megakaryocyte specification (4). In the thymus, Notch1 promotes T cell differentiation at the expense of B cell differentiation, while in the spleen, Notch2 mediates mast cell fate and promotes generation of B cell subsets (5–9). Nonetheless, although Notch receptors are expressed by HSCs and their progeny, and Notch ligands are expressed in surrounding niches in BM, studies involving in vivo deletion or inhibition of Notch pathway components have not convincingly demonstrated an in vivo effect of Notch signaling on HSC self-renewal and differentiation (10–12).

Ex vivo studies have shown that overexpression of constitutively active Notch1 intracellular domain or the Notch downstream target Hes1, or incubation of HSCs with cell-expressed or purified recombinant Notch ligands, leads to inhibition of myeloid differentiation while enhancing multipotential progenitor cell (MPP) generation, resulting in increased numbers of MPPs capable of short-term repopulation (13–18). More recently, Butler et al. achieved Notch-dependent self-renewal of HSCs upon coculture with adenovirus-infected endothelial cell lines (18). These results suggest that failure to identify an in vivo role for Notch signaling in HSC/MPP function reflects compensatory effects of other factors, such as hematopoietic growth factors, that ensure appropriate generation of mature blood elements. They also raise the possibility that Notch-driven events are recapitulated in vivo under nonhomeostatic conditions, in which compensatory mechanisms may not mask Notch-induced effects on HSC/MPP growth and differentiation.

Our previous ex vivo studies suggest that alternative cell fates induced by Notch activation result from quantitative differences in Notch signaling (19). Ex vivo culture of BM-derived HSCs/MPPs with different densities of purified Notch ligands revealed that both low and high levels of Notch signaling inhibit myeloid differentiation and enhance generation of MPPs, but only low levels of Notch signaling enhance generation of B220$^+$ cells capable of rapid B cell differentiation after coculture with OP-9 cells. High levels of Notch signaling inhibit generation of these B220$^+$ B cell precursors and instead enhance generation of CD25$^+$Thy1$^+$ T cell precursors.

Although in vivo mechanisms underlying quantitative regulation of Notch signaling are not fully understood, ex vivo studies suggest that individual Notch receptors have different transcriptional activating ability (20); thus, selective paralog usage can determine the extent of Notch target gene activation. This selective activation of specific Notch paralogs can result from interaction with specific ligands, such as selective activation of Notch2 by Jagged family members in the presence of Fringe, a glycosyltransferase that mediates differential receptor activation by unique ligands in both *Drosophila* and mammals (21–25). Overall, these studies have raised questions of distinct function of individual Notch paralogs in HSC function.

Here, we identified distinct roles for Notch1 and Notch2 receptors in modulating HSC/MPP growth and differentiation, both ex vivo during culture with purified ligands and in vivo during

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stress hematopoiesis. Using mice bearing deletions of either *Notch1* or *Notch2*, our ex vivo studies revealed that either Jagged1 or Delta1 selectively activated Notch2 in the HSC-enriched quiescent side population of Lin$^-$Sca-1$^+$c-kit$^+$ (LSK) cells (referred to herein as LSKSP cells) to inhibit myeloid differentiation and enhance generation of MPP. Our in vivo findings supported previous publications showing no effect of Notch signaling on long-term repopulating HSC self-renewal when assessed during homeostasis (12), but also revealed Notch2-dependent inhibition of myeloid differentiation and an enhanced rate of generation of both short- (MPP) and long-term (HSC) repopulating cells during stress hematopoiesis. These results demonstrated a role for Notch signaling in impeding myeloid differentiation and enhancing HSC/MPP formation, assuring orderly development during nonhomeostatic hematopoiesis.

Results

Notch ligand inhibition of myeloid differentiation and enhanced generation of SK$^+$ cells occurs in the quiescent LSKSP BM fraction. We previously showed that immobilized Notch ligand Delta1$^{ext\text{-}IgG}$ inhibits default myeloid differentiation by Lin$^-$Sca-1$^+$c-kit$^+$–enriched (LSK-enriched) BM cells and instead induces a multilog–increased number of progeny coexpressing Sca-1 and c-kit (SK$^+$; ref. 26). To determine whether Notch signaling acts directly on an HSC-enriched population, we further enriched for HSCs based on a Hoechst 33342 dye side population efflux profile. We then cultured the isolated the side population and non–side population fractions of LSK cells with engineered Notch ligands consisting of the extracellular domains of Delta1 or Jagged1 fused to the Fc domain of human IgG1 (Delta1$^{ext\text{-}IgG}$ and Jagged1$^{ext\text{-}IgG}$) immobilized with fibronectin fragments on the plastic surfaces of tissue culture wells and in the presence of soluble cytokines stem cell factor (SCF), IL-6, Flt3-L, and IL-11. As shown in Figure 1, A and B, more than 80% of the progeny generated by LSKSP cells after 3 and 7 days of culture with either ligand were SK$^+$ and did not coexpress myeloid antigens GR1 and F4/80. Furthermore, more than 80% of the progeny were still SK$^+$ after 14 days, when the 1,000 original LSKSP cells generated greater than 10^{10} SK$^+$ progeny (data not shown). In contrast, the non–side population fraction of LSK progenitors failed to undergo SK$^+$ generation with either ligand, and few progeny of the initial 1,000 non–side population LSK cells remained viable after 7 days (data not shown). With control substrate human IgG, LSKSP and non–side population LSK cells generated similar numbers and proportions of myeloid progeny, but generated reduced proportions of SK$^+$ progeny compared with cells incubated with Notch ligand (Figure 1, A and B, and data not shown). Furthermore, SK$^+$ progeny of LSKSP cells after 7 days of culture with human IgG coexpressed myeloid antigen F4/80 (Figure 1B). These results indicated that the BM LSK cells responding to Notch ligands with enhanced generation of SK$^+$ cells are contained in the HSC-enriched SP$^+$ fraction.

To test induction of Notch signaling by ligand, we measured expression of the Notch target *Hes1* and found 3-fold increased expression after a 3-hour incubation of LSKSP cells with either Delta1$^{ext\text{-}IgG}$ or Jagged1$^{ext\text{-}IgG}$ (Figure 1C, control). Moreover, analysis of mRNA expression of *Cebpa*, a key regulator of myeloid differentiation, showed that LSKSP cells cultured for 3 hours with human IgG expressed 3-fold more *Cebpa* mRNA copies than did LSKSP cells cultured for 3 hours with Delta1$^{ext\text{-}IgG}$ or Jagged1$^{ext\text{-}IgG}$ (Figure 1D, control). Thus, even at this very early time point, Notch signaling inhibited induction of a myeloid differentiation program.

Selective activation of Notch2 mediates inhibition of myeloid differentiation and enhanced generation of SK$^+$ cells. To identify specific Notch receptors involved in myeloid inhibition and SK$^+$ generation, we monitored differentiation of *Notch*-deficient LSKSP cells during culture with Notch ligands. Because both *Notch1* and *Notch2* deletion results in embryonic lethality, we induced receptor deletions using mice bearing floxed *Notch1* or *Notch2* genes and an *MxCre* gene (*Notch1$^{fl/fl}$Cre$^+$* or *Notch2$^{fl/fl}$Cre$^+$*). *Notch1* or *Notch2* deletions were induced in vivo in young mice with multiple injections of the interferon inducer poly-I/C. At 1–2 weeks after the last poly-I/C injection, *Notch1* or *Notch2* copy number in genomic DNA from peripheral blood (PB) cells from deficient animals (*Cre$^+$*) was less than 10% of that in PB cells from control animals (*Cre$^-$*), as assessed by quantitative genomic PCR. For culture experiments, *Notch* deletions were also induced ex vivo by infecting sorted LSKSP cells from *Notch1$^{fl/fl}$* or *Notch2$^{fl/fl}$* mice with lentivirus encoding Cre recombinase. Similar results were obtained with either method of gene deletion. *Notch1*-deficient LSKSP cells generated a multi-log increase in number of SK$^+$ progeny in response to Notch ligands, similar to control LSKSP cells, whereas few *Notch1*-deficient LSKSP progeny expressed GR1 or F4/80 (Figure 1E and data not shown), which indicates that Notch1 does not mediate ligand-induced SK$^+$ generation. In striking contrast, *Notch2*-deficient LSKSP cells differentiated and did not undergo SK$^+$ generation, with only 23% of the *Notch2*-deficient progeny being SK$^+$ 7 days after incubation with Notch ligands, similar to wild-type LSKSP cells cultured with human IgG (Figure 1E and data not shown). Furthermore, after 7 days, many *Notch2*-deficient SK$^+$ cells incubated with Notch ligands also expressed F4/80 (Figure 1E), and after 14 days, most *Notch2*-deficient progeny with Notch ligands were no longer SK$^+$, but were GR1$^+$ and/or F4/80$^+$ (Figure 1F).

To determine the basis for the selective function of Notch2 in LSKSP cells, we assessed *Hes1* expression during culture of LSKSP cells with Notch ligand. After 3 hours of culture, increased *Hes1* mRNA expression was observed in *Notch1*-deficient and control cells, but not in *Notch2*-deficient cells (Figure 1C). Similarly, reduced *Cebpa* expression was seen in *Notch1*-deficient cells, but not *Notch2*-deficient cells (Figure 1D and data not shown). As expected, after 7 days of culture, *Notch2*-deficient cells expressed increased levels of *Cebpa* mRNA (data not shown). Within the SK$^+$ precursors, we found 4-fold higher *Cebpa* mRNA levels in *Notch2*-deficient cells than in control cells (Figure 1G), indicative of rapid precursor differentiation along the myeloid lineage. Together, these findings indicate that Notch2 signaling inhibits myeloid differentiation by LSKSP progeny, perhaps by *Hes1*-mediated suppression of *Cebpa* transcriptional activity.

Assessment of whether selective signaling via *Notch2* is dependent on insufficient expression of *Notch1* in LSKSP cells revealed a 40-fold decrease in *Notch1* mRNA expression compared with *Notch2* in freshly isolated LSKSP cells (data not shown). Although these data suggest that lack of Notch1-mediated signaling in LSKSP cells may result from its decreased expression, LSKSP cells cultured with Notch ligand revealed rapid Notch2-dependent upregulation of *Notch1* mRNA (Figure 1, H and I), which suggests that one or more alternative mechanisms, such as selective ligand use, may be required to maintain selective Notch2 activation in developing HSC progeny.

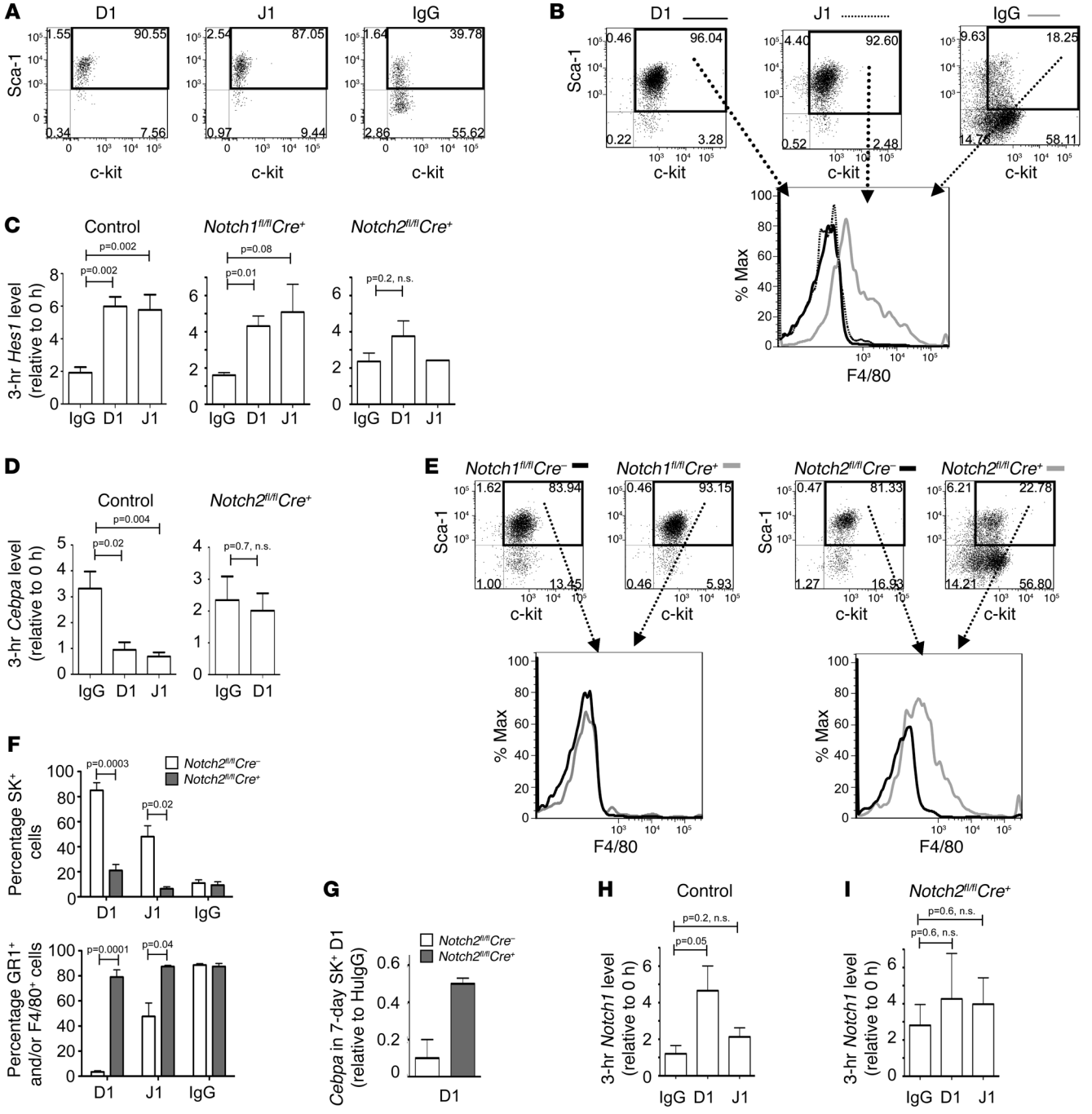

Figure 1

Notch2, but not Notch1, mediates SK+ self-renewal and inhibition of myeloid differentiation with Delta1$^{ext\text{-}IgG}$ (D1) and Jagged1$^{ext\text{-}IgG}$ (J1). (**A** and **B**) Representative dot plots after 3 (**A**) and 7 (**B**) days of LSKSP culture with Notch ligand. (**C**) *Hes1* mRNA levels after 3 hours of LSKSP culture with Delta1$^{ext\text{-}IgG}$, Jagged1$^{ext\text{-}IgG}$, and human IgG. Data represent mean ± SEM for 3–5 independent experiments, except *Notch2$^{fl/fl}$Cre+* with Jagged1$^{ext\text{-}IgG}$ (mean ± range for 2 independent experiments). *P* values were derived with 2-tailed Student's *t* test. (**D**) *Cebpa* mRNA levels after 3 hours of LSKSP culture. Data are mean ± SEM from 5 and 3 independent experiments for control and *Notch2$^{fl/fl}$Cre+*, respectively. *P* values were derived with 2-tailed Student's *t* test. (**E**) Representative dot plot after 7 days of LSKSP culture with Delta1$^{ext\text{-}IgG}$ with gated SK+ cell expression of F4/80 (histograms). (**F**) Percent SK+ or GR1+F4/80+ cells after 14 days of LSKSP culture. Data represent mean ± SEM from 4 independent experiments. *P* values were derived with 2-tailed paired Student's *t* test. (**G**) *Cebpa* mRNA levels in SK+ sorted cells after 7 days of LSKSP culture. Data represent mean ± range from 2 independent experiments. (**H** and **I**) *Notch1* mRNA levels after 3 hours of LSKSP culture with Delta1$^{ext\text{-}IgG}$. Data represent mean ± SEM from 3–4 independent experiments. *P* values were derived with 2-tailed Student's *t* test. Numbers within dot plots denote percentage of events within the respective quadrants.

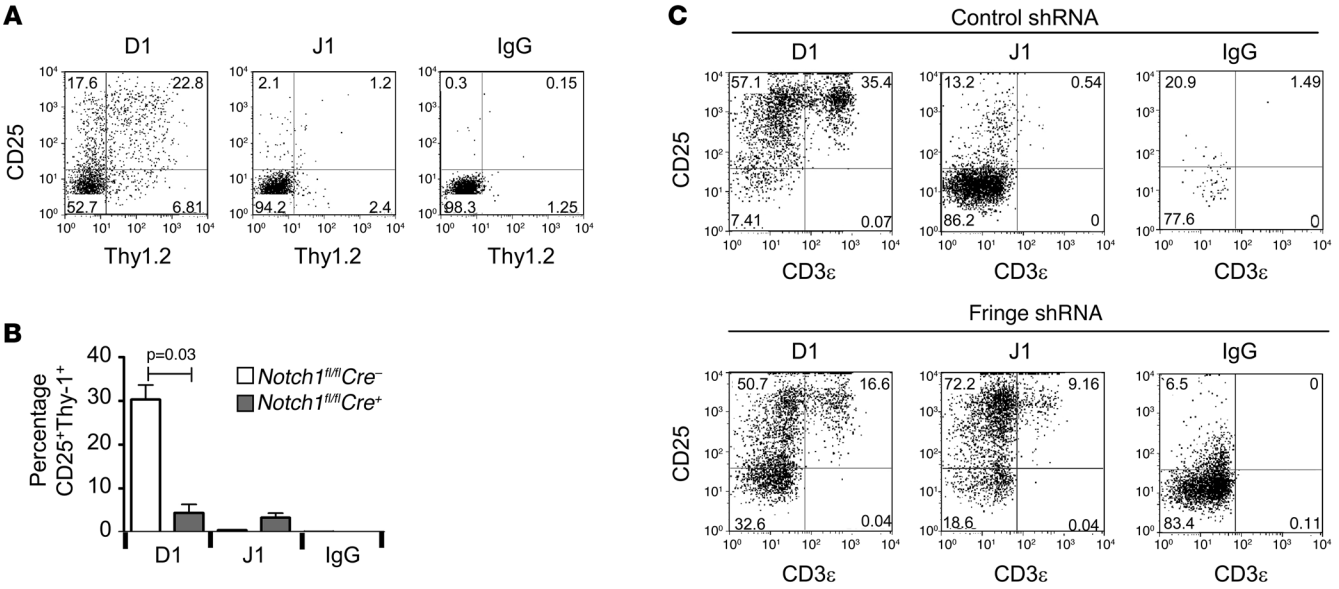

Figure 2

Notch1 mediates generation of CD25$^+$Thy1.2$^+$ progenitors, and Fringe mediates differential Notch1/Notch2 activation. LSKSP cells were cultured with Delta1$^{ext\text{-}IgG}$, Jagged1$^{ext\text{-}IgG}$, and human IgG. (**A**) Representative dot plot after 14 days of culture. (**B**) Percent CD25$^+$Thy1.2$^+$ progeny generated after 14 days of culture. Data are mean ± SEM from 3–4 experiments. *P* values were calculated using 2-tailed paired Student's *t* test. (**C**) Representative dot plots from 1 of 2 separate experiments after 18 days of culture of LSKSP cells infected with shRNA. Numbers within dot plots denote percentage of events within the respective quadrants.

Selective activation of Notch2 by Jagged is mediated by Fringe. Hematopoietic differentiation toward the T cell lineage is known to be dependent upon Notch1 signaling. Previous studies from our group (19, 26) and others (27, 28) have shown that the differentiation of hematopoietic precursors toward the T cell lineage is promoted in the presence of *Delta* family members. Here we showed that culture of control cells with Delta1$^{ext\text{-}IgG}$, but not Jagged1$^{ext\text{-}IgG}$, induced differentiation toward the T lineage, as indicated by generation of CD25$^+$Thy1.2$^+$ progeny and increased expression of *Ptcra* mRNA (Figure 2A and data not shown). In addition, Delta-induced differentiation toward the T lineage was not observed upon culture of *Notch1*-deficient cells (Figure 2B). Together, these results are consistent with the known requirement for Notch1 in inducing T cell differentiation in response to Delta and suggest that subsequent to Notch1 message upregulation in LSKSP cells, Jagged activates Notch2 but not Notch1. *Notch2*-deficient LSKSP cells failed to generate CD25$^+$Thy1.2$^+$ progeny in the presence of either Notch ligand, perhaps because insufficient Notch signaling leads to rapid myeloid differentiation with loss of competence for T cell differentiation prior to acquisition of Notch1 signaling.

Previous studies indicated that selective Notch activation in response to Delta versus Jagged is mediated via Fringe (21). To test whether the failure of Jagged1$^{ext\text{-}IgG}$ to induce T cell differentiation was caused by Fringe-mediated inhibition of Notch1 activation, we reduced Fringe levels in LSKSP cells by infection with a GFP lentivirus encoding shRNA designed to inhibit expression of all 3 Fringe homologs: lunatic, manic, and radical (encoded by *Lfng*, *Mfng*, and *Rfng*, respectively). After 10 days in culture, mRNA levels of *Lfng* and *Mfng* were reduced 2- to 3-fold, and *Rfng* mRNA was reduced 4-fold, in sorted GFP$^+$ cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43868DS1). Reduced Fringe expression did not affect the percentage of CD25$^+$Thy1.2$^+$ progeny generated in cultures incubated with Delta1$^{ext\text{-}IgG}$, but did increase the percentage of cells expressing cytoplasmic CD3ε and/or CD25 in cultures with Jagged1$^{ext\text{-}IgG}$ (Figure 2C). Thus, within the LSKSP progeny, the selective effect of Delta1$^{ext\text{-}IgG}$ on T cell progenitor generation rests in Fringe-mediated inhibition of Notch1 activation by Jagged1$^{ext\text{-}IgG}$. These findings are consistent with the known requirement for Delta ligands in thymus for induction of T cell differentiation (25) and suggest a role for the known presence of Jagged in BM for enhancing precursor generation (29).

Neither Notch1 nor Notch2 is required for HSC function in vivo during homeostasis. To assess the role of Notch signaling in stem/progenitor cells within the adult BM, we compared the frequency and number of different BM subsets from mice bearing in vivo–induced *Notch1* or *Notch2* deletions with those of similarly treated littermates lacking the *MxCre* gene. Analysis of BM in homeostasis from control, *Notch1*-deficient, and *Notch2*-deficient mice revealed similar numbers of cells as well as normal proportions and numbers of SK$^+$ progenitors, cells expressing myeloid markers GR1 and F4/80, and cells expressing the B cell marker B220 (data not shown). Limiting-dilution competitive repopulation transplant analysis using BM in homeostasis indicated similar HSC frequencies from control, *Notch1*-, and *Notch2*-deficient mice (Supplemental Tables 1 and 2); furthermore, secondary transplants with mice initially transplanted with *Notch2*-deficient BM, performed after the primary transplanted BM reached homeostasis (40 weeks after transplant), showed no differences (Supplemental Table 3). These results are consistent with previous studies of homeostatic hematopoiesis showing that ablation of canonical Notch signaling does not affect frequency of functional BM HSC (12).

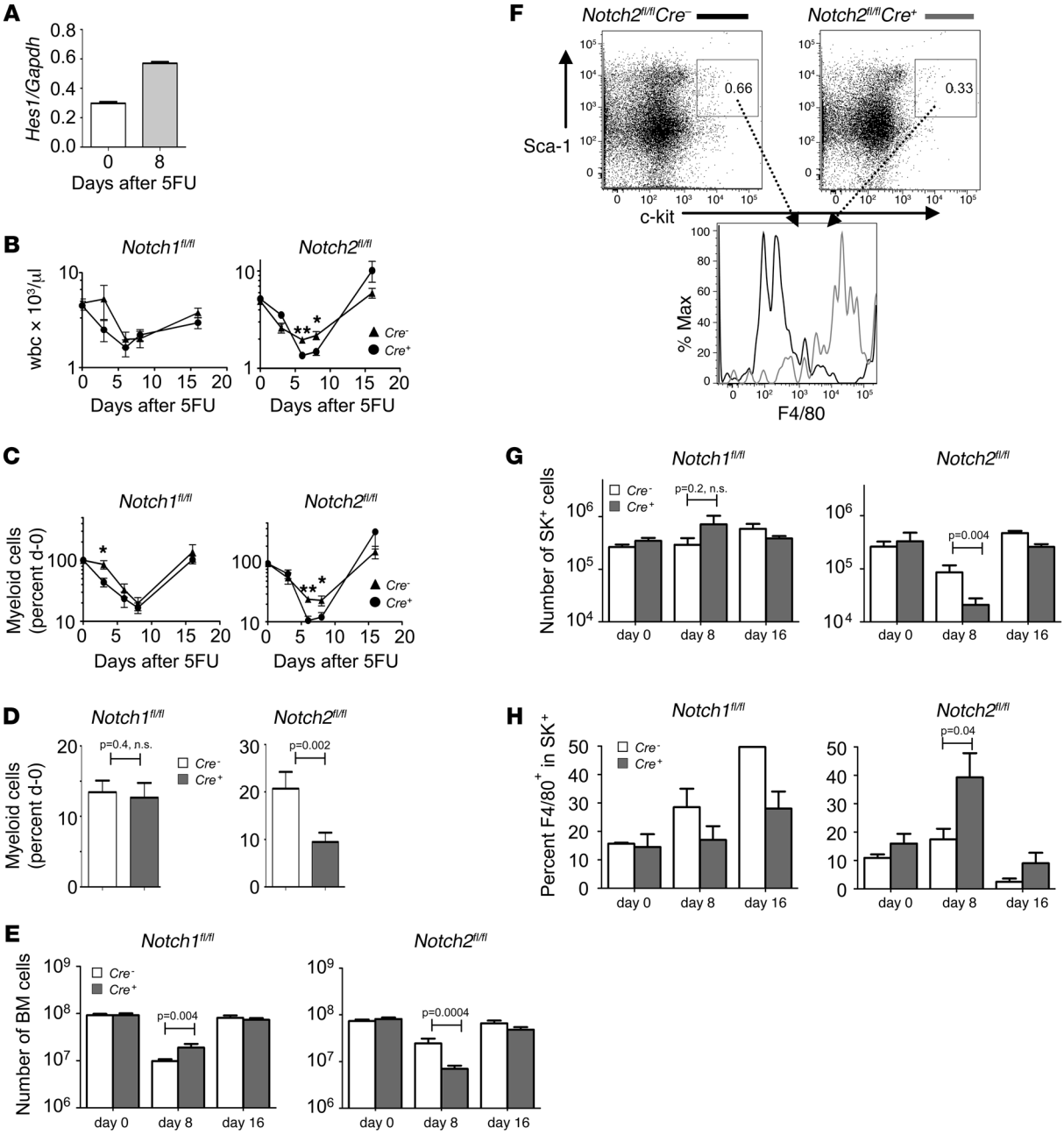

Figure 3

Notch2 modulates BM stress recovery from 5FU. (**A**) *Hes1* mRNA levels in SK⁺ sorted BM cells 8 days after 5FU treatment. Data are mean ± SD of 3 replicates. (**B**–**D**) Recovery of PB wbc number and myeloid cell numbers (GR1⁺ and/or F4/80⁺; shown relative to day-0 levels) in mice following 5FU treatment. (**B** and **C**) Data are mean ± SEM from 6 (days 3 and 16) or 12 (days 6 and 8) mice from a representative experiment (*$P < 0.05$, **$P < 0.01$). (**D**) Data are mean ± SEM percent day-0 levels 8 days after 5FU treatment from 3 independent Notch1 experiments with 30–32 mice per group and 2 independent Notch2 experiments with 17 mice per group. (**E**) BM cell number at 0, 8, and 16 days after 5FU. (**F**) Representative dot plots generated from BM 8 days after 5FU. Gated SK⁺ cell expression of F4/80 is shown in the histogram. Numbers represent the proportion of cells within the respective gate. (**G** and **H**). Number and percentage of SK⁺ gated cells expressing F4/80 at 0, 8, and 16 days after 5FU. (**E**, **G**, and **H**) Notch1 data represent mean ± SEM from 3 independent experiments with 3 or 6 mice per group (total 17 *Notch1*$^{fl/fl}$*Cre*⁺ and 17 *Notch1*$^{fl/fl}$*Cre*⁻ individuals). Notch2 data represent mean ± SEM from 2 independent experiments with 5 or 6 mice per group (total 11 *Notch2*$^{fl/fl}$*Cre*⁺ and 11 *Notch2*$^{fl/fl}$*Cre*⁻ individuals). *P* values derived with Mann-Whitney test.

Notch2 modulates hematopoietic recovery following 5FU challenge by retarding myeloid differentiation and enhancing generation of early precursor cells. BM recovery after stress induced by the chemotherapeutic agent 5-fluorouracil (5FU) requires rapid generation of MPPs to efficiently replenish depleted cycling progenitors and cells differentiating along various hematopoietic lineages. A role for Notch signaling in recovery after 5FU treatment was suggested by the 2-fold increase in *Hes1* mRNA in sorted LSK BM cells from mice

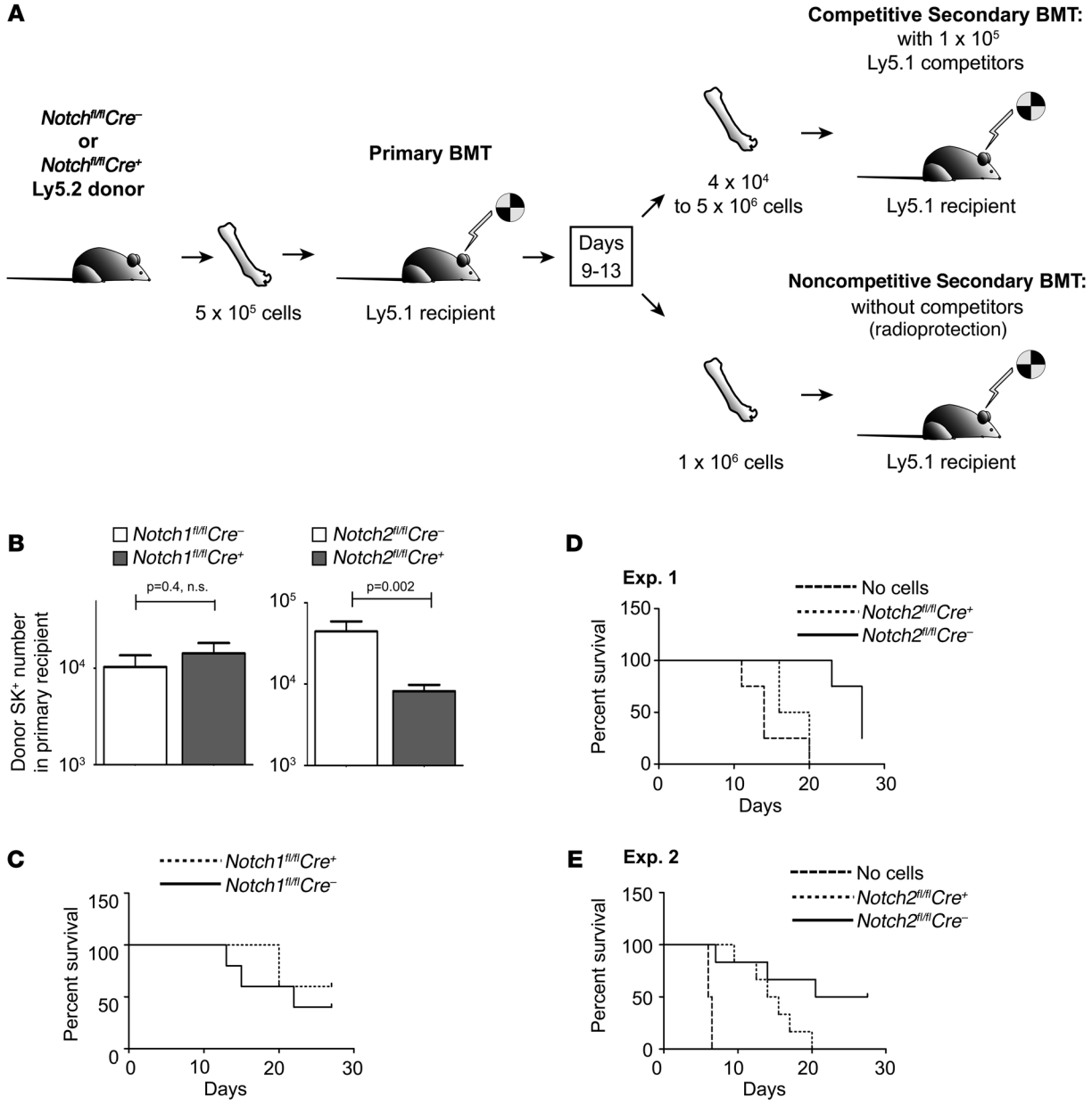

Figure 4

Notch2 enhances the tempo of HSC and MPP recovery after transplant into irradiated recipients. (**A**) Measurement of HSC and MPP recovery after transplant into irradiated recipients. BMT, BM transplantation. (**B**) Number of donor (Ly5.2$^+$) and SK$^+$ gated cells generated 13 days after transplant into primary recipients. Data are mean ± SEM from 2 independent experiments with 5 mice per group per experiment (total 10 *Notch2*$^{fl/fl}$*Cre*$^+$ and 10 *Notch2*$^{fl/fl}$*Cre*$^–$). (**C**) Survival outcome after transplant into lethally irradiated recipients of 1 × 10^6 cells from primary recipients transplanted 11 days previously with 5 × 10^5 *Notch1*$^{fl/fl}$*Cre*$^–$ or *Notch1*$^{fl/fl}$*Cre*$^+$ cells. Results were analyzed with a log-rank nonparametric test and expressed as Kaplan-Meier survival curves (n = 4, P = 0.45). (**D**) Survival outcome after transplant into lethally irradiated recipients of 1 × 10^6 cells from primary recipients transplanted 11 days previously with 5 × 10^5 *Notch2*$^{fl/fl}$*Cre*$^–$ or *Notch2*$^{fl/fl}$*Cre*$^+$ cells or with no cells. Results were analyzed with a log-rank nonparametric test and expressed as Kaplan-Meier survival curves (n = 4, P = 0.01). (**E**) Survival outcome after transplant into lethally irradiated recipients of 1 × 10^6 cells from primary recipients transplanted 13 days previously with 5 × 10^5 *Notch2*$^{fl/fl}$*Cre*$^–$ or *Notch2*$^{fl/fl}$*Cre*$^+$ cells or with no cells. Results were analyzed with a log-rank nonparametric test and expressed as Kaplan-Meier Survival curves (n = 6, P = 0.05).

that received a single dose of 5FU 8 days previously (Figure 3A). This corroborates previous studies showing reduced Hes1 in BM LSK progenitors following loss of Notch signaling (11).

To assess the role of increased Notch signaling in recovery from 5FU stress and to distinguish the involved receptor, we first compared recovery in PB from mice with *Notch1* or *Notch2* deletion. At 7–9 days after 5FU, wbc counts were 2-fold less in *Notch2*-deficient mice, but were similar or tending toward an increase in *Notch1*-deficient mice, compared with control littermates (Figure 3B). Moreover, the percent of myeloid (GR1$^+$ and/or F480$^+$)

Table 1
HSC/MPP generation and frequency 2 and 9 weeks after transplant of lethally irradiated recipients

	2 weeks (short-term MPPs)[A]		9 weeks (long-term HSCs)[B]	
	Notch1$^{fl/fl}$*Cre*$^{-}$	*Notch1*$^{fl/fl}$*Cre*$^{+}$	*Notch1*$^{fl/fl}$*Cre*$^{-}$	*Notch1*$^{fl/fl}$*Cre*$^{+}$
HSC/MPP generation after transplant (positive mice/total mice)				
5×10^6 BM cells	5/5	5/5	5/5	4/5
1×10^6 BM cells	4/4	4/4	0/4	1/4
2×10^5 BM cells	0/4	0/4	0/4	0/4
HSCs/MPPs per 1×10^6 BM cells (no.)	1.8	1.8	0.4	0.3
Donor cells per primary recipient (no.)	3.9×10^6	5.2×10^6	3.9×10^6	5.2×10^6
HSCs/MPPs per primary recipient (no.)	7.0	9.3	1.4	1.5

Lethally irradiated secondary recipients were transplanted with decreasing numbers of BM cells from primary recipients 11 days after transplant with *Notch1*$^{fl/fl}$*Cre*$^{-}$ or *Notch1*$^{fl/fl}$*Cre*$^{+}$ cells. PB was analyzed 2 and 9 weeks after secondary transplant for the percentage of donor-derived cells. Animals were considered positive for HSCs/MPPs if engraftment in PB was more than 2.0% donor-derived cells with both lymphoid and myeloid contribution. Poisson statistics were used to calculate HSC/MPP frequency. [A]$P = 0.6$ between groups at the 2-week time point. [B]$P = 0.7$ between groups at the 9-week time point.

cells in PB after 5FU injection relative to myeloid cell number before injection was reproducibly and significantly lower in injected *Notch2*-deficient mice, but not significantly different in *Notch1*-deficient PB, compared with controls (Figure 3, C and D). Platelet counts and rbc counts did not differ between *Notch1*- or *Notch2*-deficient mice compared with control littermates during recovery from 5FU (data not shown). Because differences were not seen with *Notch1*-deficient mice, we are convinced effects seen with *Notch2*-deficient mice were due to the loss of Notch2 and not due to Cre expression.

Analysis of BM regeneration in injected mice 8 days after 5FU revealed 4-fold fewer cells in *Notch2*-deficient BM, but similar numbers of cells in *Notch1*-deficient and control BM (Figure 3E). *Notch2*-deficient cells also consistently had lower proportions of SK^{+} progenitors, whereas *Notch1*-deficient BM contained proportions similar to those of control BM (Figure 3F and data not shown). We also found 4-fold fewer SK^{+} progenitors in *Notch2*-deficient BM, whereas SK^{+} progenitor numbers in *Notch1*-deficient and control BM were similar (Figure 3G). A higher proportion of SK^{+} *Notch2*-deficient BM cells expressed the myeloid antigen F4/80 than did normal or *Notch1*-deficient SK^{+} cells (Figure 3H), suggestive of rapid differentiation to the myeloid lineage in the absence of Notch2. This evidence of rapid myeloid differentiation of *Notch2*-deficient precursor cells in vivo is analogous to the above results from ex vivo culture of *Notch2*-deficient cells with Delta1$^{ext\text{-}IgG}$.

By 16 days after 5FU treatment, absolute numbers of BM and SK^{+} cells were similar to those seen before 5FU treatment in *Notch1*- and *Notch2*-deficient as well as wild-type mice (Figure 3, E and G). Limiting-dilution competitive repopulation transplant experiments were used to measure HSC numbers during recovery from 5FU. Prior to 5FU treatment, the absolute number of HSCs was similar in BM of control mice (903 HSCs; range, ±363) and *Notch2*-deficient littermates (655 HSCs; range, ±36), which suggests that the differences observed during recovery did not originate from differences in the initial number of cells capable of long-term repopulation. Furthermore, at 8 days after 5FU treatment, the absolute number of HSCs did not differ in control (805 HSCs; range, ±70) and *Notch2*-deficient mice (524 HSCs; range, ±187). These data indicate during the initial 8 days of BM recovery after treatment with 5FU, Notch 2 inhibits myeloid differentiation and promotes generation of short-term repopulating MPPs. However, although we did not detect differences in long-term repopulating HSCs in *Notch2*-deficient compared with control mice, we suspect that a single dose of 5FU failed to deplete HSCs; to a great extent, they remained quiescent. Hence, we chose to quantify differences in HSC/MPP recovery after transplantation of control and *Notch2*-deficient BM into lethally irradiated mice. We harvested BM from transplanted mice at early time points prior to reaching homeostasis, assayed MPP generation using secondary radioprotection assays, and quantified MPP/HSC numbers using limiting dilution secondary transplantation assays.

Notch2 enhances the tempo of HSC and MPP recovery after transplant of lethally irradiated recipients with a limiting number of BM cells. To further address whether Notch2 is required for HSC or MPP self-renewal during nonhomeostasis, we monitored BM recovery in lethally irradiated recipients transplanted with control *Notch1*- and *Notch2*-deficient BM cells at a limiting dose of 5×10^5 BM cells (Figure 4A). Upon sacrifice after primary transplant, we enumerated stem cell phenotypes as well as HSC numbers in BM of primary recipients. Recovery was evident in mice transplanted with control cells between 11 and 13 days after transplant. Prior to 11 days, BM from mice transplanted with control cells contained a baseline number of $9.1 \pm 0.7 \times 10^6$ (SEM) cells, whereas by 13 days, BM from similarly transplanted mice contained significantly increased numbers of cells ($13.0 \pm 1.5 \times 10^6$; $P = 0.01$), indicative of recovery. We therefore compared BM from mice 11 days or later and when control BM numbers were higher than the threshold level of 9.1×10^6 cells, to ensure that primary recipients were in recovery. After 13 days, recipients of control cells generated significantly more donor SK^{+} cell numbers than did recipients of *Notch2*-deficient BM, whereas recipients of control cells and *Notch1*-deficient cells generated similar SK^{+} cell numbers (Figure 4B). To quantitate long-term repopulating HSCs and short-term repopulating MPPs generated in the recovering BM, competitive repopulating units (CRU) were enumerated in competitive secondary transplants in which BM cells were injected into secondary recipients in limiting dilutions (Figure 4A). Long-term repopulating HSCs contributed to the donor long after 3 weeks, whereas short-term MPPs contributed to PB only in early weeks, and their contribution was minimal after 6 weeks. HSC numbers were similar in control and *Notch1*-deficient transplanted primary recipients; however, short-term repopulating MPPs (measured after 2 weeks in secondary recipients) and long-term repopulating HSCs (measured after 15 weeks in secondary recipients) were significantly reduced in primary recipients transplanted with *Notch2*-deficient cells (Tables 1 and 2). Furthermore, lethally irradiated mice receiving 1×10^6 BM cells from primary recipients previously transplanted with 5×10^5 or 2×10^6 control cells or *Notch1*-deficient cells survived significantly longer than

Table 2
HSC/MPP generation and frequency 2 and 15 weeks after transplant of lethally irradiated recipients

	2 weeks (short-term MPPs)[A]		15 weeks (long-term HSCs)[B]	
	Notch2$^{fl/fl}$*Cre*$^{-}$	*Notch2*$^{fl/fl}$*Cre*$^{+}$	*Notch2*$^{fl/fl}$*Cre*$^{-}$	*Notch2*$^{fl/fl}$*Cre*$^{+}$
HSC/MPP generation after transplant (positive mice/total mice)				
5×10^6 BM cells	3/3	3/3	3/3	2/3
1×10^6 BM cells	7/7	5/7	4/7	0/7
2×10^5 BM cells	5/6	1/7	0/6	0/7
4×10^4 BM cells	2/7	1/7	0/7	0/7
HSCs/MPPs per 1×10^6 BM cells (no.)	8.8	1.3	0.7	0.1
Donor cells per primary recipient (no.)	12.0×10^6	7.4×10^6	12.0×10^6	7.4×10^6
HSCs/MPPs per primary recipient (no.)	105.6	9.6	8.4	0.7

Lethally irradiated secondary recipients were transplanted with decreasing numbers of BM cells from primary recipients 11 days after transplant with *Notch2*$^{fl/fl}$*Cre*$^{-}$ or *Notch2*$^{fl/fl}$*Cre*$^{+}$ cells. PB was analyzed 2 and 15 weeks after secondary transplant for the percentage of donor-derived cells. Animals were considered positive for HSCs/MPPs if engraftment in PB was more than 2.0% donor-derived cells with both lymphoid and myeloid contribution. Poisson statistics were used to calculate HSC/MPP frequency. [A]$P = 0.0004$ between groups at the 2-week time point. [B]$P = 0.02$ between groups at the 15-week time point.

did mice receiving 1×10^6 BM cells from primary recipients previously transplanted with equal numbers of *Notch2*-deficient cells without competitors. In one experiment, mice receiving *Notch2*-deficient cells survived no longer than irradiated mice receiving no cells (Figure 4, C–E, and Supplemental Figure 2), which indicates that Notch2 is required to generate sufficient numbers of cells, including short-term and possibly long-term repopulating cells, to provide radioprotection.

Discussion

Our present data revealed a role for Notch2 signaling in the orderly reconstitution of hematopoiesis within the BM space after injury induced by chemotherapy or radiation. Here we show that Notch signaling enhanced the self-renewal of HSCs and MPPs by preventing their depletion caused by rapid differentiation that might result from environmental pressures, such as the presence of high cytokine levels. Hence, Notch signaling assured the rapid establishment of an adequate pool of stem/progenitor cells after BM injury. Although recovery of HSCs and MPPs during nonhomeostasis occurred more rapidly as a result of Notch regulation, no effect of Notch on HSC/MPP numbers during homeostasis occurred, presumably because environmental space constraints in the HSC niche obviate the effects of Notch signaling. These data are consistent with the Notch-dependent increase in HSC numbers observed during coculture of BM-derived LSK cells with endothelial cell layers (18).

Early during HSC culture with Notch ligands, we observed Notch2-mediated molecular changes, such as increased *Hes1* and suppressed *Cebpa* mRNA expression in association with rapid suppression of a myeloid differentiation program and enhanced MPP generation. Furthermore, Notch1 was unable to compensate for Notch2 in *Notch2*-deficient mice. This activation of Notch2, but not Notch1, in HSCs presumably occurs because of reduced expression of Notch1 and/or factors such as Fringe, which suppress Notch1-mediated signaling in the presence of Jagged (21, 24). It is also possible that leukemia/lymphoma-related factor (LRF), a transcriptional repressor recently found to suppress Notch activity, selectively suppresses Notch1-induced signaling (30).

The importance of limiting signaling via Notch1 in HSCs is suggested by the loss of HSCs in LRF-deficient mice or in mice transplanted with HSCs that overexpress activated Notch1, presumably as a result of increased T cell differentiation (31). This effect of the LRF deletion is reversed, however, upon concomitant Notch1 deletion, further suggesting the importance of mechanisms reducing Notch1 signaling in HSCs (32).

Our ex vivo data were consistent with previous studies demonstrating that induction of unique cell fates by Notch ligands is caused by differential activation of Notch receptors by specific ligands (27, 28, 33). Both Delta1$^{ext\text{-}IgG}$ and Jagged1$^{ext\text{-}IgG}$ induce generation of SK^{+} precursors via activation of Notch2, but, as seen previously with stromal cells expressing individual ligands, only Delta1$^{ext\text{-}IgG}$ induces generation of CD25^{+}Thy1^{+} progenitors. We found that these progenitors were generated with Delta1$^{ext\text{-}IgG}$ via Notch1, consistent with in vivo gain- and loss-of-function studies that demonstrate a requirement for Notch1 in T cell development as well as with the notion that exposure of BM HSCs and MPPs to Jagged contributes to enhanced progenitor generation, whereas exposure to Delta family members richly expressed in thymus leads to T cell development (5, 6, 34). Finally, the inability of LSKSP cells lacking *Notch2* but expressing *Notch1* to generate CD25^{+}Thy1^{+} progenitors ex vivo indicates the requirement for Notch2 signaling in less mature progenitors before the Notch1 receptor is functional. This may be a result of rapid differentiation toward the myeloid lineage before the increase in *Notch1* mRNA levels detected 3 hours after Notch2 activation with Delta1$^{ext\text{-}IgG}$ or Jagged1$^{ext\text{-}IgG}$. Although HSC progeny in vivo gained *Notch1* function in the absence of *Notch2* (i.e., *Notch2* deletions did not impair T cell development), it is nonetheless possible that the rate of generation of Notch1-expressing cells with T cell potential is impaired as a result of *Notch2* deficiency.

Overall, our results indicate that Notch2 signaling imposes a choice in HSC progeny between differentiation and self-renewal in recovering BM. Augmented self-renewal in the realm of Notch2 signaling is needed to assure generation of increased numbers of lymphomyeloid precursors prior to the formation of differentiated cells that subsequently reconstitute the BM and PB. Hence, the rapid differentiation toward the myeloid lineage in the absence of Notch2 generates mature progeny and fewer HSCs and MPPs. Our present findings, together with our previous data suggesting that quantitative differences in Notch signaling account for retardation of myeloid differentiation with generation of MPPs or promotion of differentiation toward the T cell lineage, lead us to hypothesize that Notch2 induces expression of genes that impede myeloid differentiation, whereas Notch1 produces increased levels of the same genes as well as genes with a higher activation threshold required for initiation of the T cell program. Whether this is the result of differences in receptor amounts and/or stability or of differences in transcriptional activity

induced by Notch1- or Notch2-activated intracellular domains remains to be determined. Alternatively, our in vivo results could be explained by a reduced capacity for *Notch2*-deficient HSCs to efficiently home in secondary transplants. However, we believe this is unlikely, since *Notch2*-deficient HSCs homed equivalently to wild-type cells — leading to BM recovery — when they were obtained during homeostasis, as indicated by similar frequencies in transplant studies (Supplemental Tables 1 and 3).

Finally, our results point to a requirement for assessing the function of genes in the hematopoietic or other organ systems under nonhomeostatic as well as homeostatic conditions. Genes that are shown to regulate developmental processes but are not required for establishment or maintenance of homeostatic states may also be of practical importance for ex vivo manipulation of stem cell systems, as exemplified by the Notch-mediated expansion of human cord blood hematopoietic progenitor cells capable of rapidly reconstituting BM in patients undergoing HSC transplantation (35).

Methods

Generation and immobilization of Delta1$^{ext\text{-}IgG}$ and Jagged1$^{ext\text{-}IgG}$ and HSC culture. Jagged1$^{ext\text{-}IgG}$ was generated as previously described for preparation of Delta1$^{ext\text{-}IgG}$ (36). A PCR product was generated using primers encoding appropriate restriction enzyme recognition sequences and the Fc domain of human IgG_1. Appropriately digested PCR product replaced the myc tags in the previously generated Jagged1$^{ext\text{-}myc}$ (37). Jagged1$^{ext\text{-}IgG}$ protein was produced using NSO cells as previously described for Delta1$^{ext\text{-}IgG}$ (36). Wells of non–tissue culture–treated culture plates (Falcon, BD) were incubated with Delta1$^{ext\text{-}IgG}$ at 5 μg/ml, Jagged1$^{ext\text{-}IgG}$ at 20 μg/ml, or human IgG_1 (Sigma-Aldrich) diluted in PBS together with 5 μg/ml fibronectin fragment CH-296 (Takara Shuzo Co.), incubated overnight at 4°C, washed extensively, and further incubated with 2% bovine serum albumin (Sigma-Aldrich) dissolved in PBS. Equivalence of the concentration of Jagged1$^{ext\text{-}IgG}$ and Delta1$^{ext\text{-}IgG}$ was determined by ELISA using an HRP-conjugated Fc specific anti–human IgG antibody (Sigma-Aldrich). Cells were cultured as described previously (26).

Cell isolation and immunofluorescence studies. Cell immunophenotype was analyzed by multicolor flow cytometry using an LSR cytometer (BD). Antibodies were purchased from BD Biosciences unless otherwise indicated. Cultured cells were prepared as previously described (37); stained with (a) PE-conjugated monoclonal antibodies against CD25 and F4/80 (eBiosciences), (b) APC-conjugated monoclonal antibodies against c-kit, GR1, and Thy1, and (c) biotinylated antibodies against Sca-1, GR1; and secondarily stained with streptavidin-PerCP. LSKSP cells express levels of Sca-1 and c-Kit and have side population characteristics when stained with Hoechst 33342 (Calbiochem) on BM depleted of the following lineage markers: CD2, CD3, CD8a, CD5, CD11b, B220, GR1, and TER-119. LSKSP cells were obtained using fluorescence-activated cell sorting (FACS) on a Vantage Cell Sorter (BD), and gating was as described previously (38). After each sort, we analyzed sorted populations to confirm that percent SK^+SP^+ cells exceeded 90%.

Mice and generation of Notch1- and Notch2-deficient cells. Mice bearing floxed *Notch1* and *Notch2* genes were maintained and bred with transgenic mice bearing the interferon-inducible *Mx1-Cre* gene at Fred Hutchinson Cancer Research Center. To induce a deletion in mice bearing *Mx1-Cre* mutations and *Notch1$^{fl/fl}$* and *Notch2$^{fl/fl}$* mutations, 2- to 3-week-old mice were injected i.p. with 25 mg/kg solution of poly-I/C (Invivogen). Mice were injected over a 3-week period in a series of 5 injections every 2–3 days, followed by a 7-day break and a second series of 5 injections every 2–3 days. To perform ex vivo deletions, LSKSP cells from *Notch1$^{fl/fl}$* or *Notch2$^{fl/fl}$* mice were infected with lentivirus encoding Cre recombinase and GFP (Cre-GFP-lenti) at a multiplicity of infection of 500. GFP^+ cells were isolated after a 3-day culture and replaced. To construct lentivirus encoding Cre and GFP, PCR-amplified Cre with appropriate restriction sites was subcloned into lentivirus. All animal studies were conducted in strict accordance with the NIH guidelines for humane treatment of animals and were approved by the Institutional Animal Care and Use Committee at the Fred Hutchinson Cancer Research Center.

DNA isolation and PCR of genomic DNA. 1 week after the last poly-I/C injection, DNA was extracted from PB of mice using DNA extraction kits according to the manufacturer's instructions (Zymo Research). Genomic *Notch1* and *Notch2* PCR product amounts were normalized to gene *Gja5*. Primer sequences for *Notch1* (forward, TCATTGTACTGGAACTCTGCACTTT; reverse, TCCACGGTCACCCTTCTCA), *Notch2* (forward, CACAGGAGAGGACTGCCAATACT; reverse, TGTCCCGGCTGAGCATGT), and *Gja5* (forward, ACCATGGAGGTGGCCTTCA; reverse, CATGCAGGGTATCCAGGAAGA) were used to determine copy number of genomic DNA using quantitative Power SYBR-green MasterMix and PCR. To verify that genomic PCR with PBL cells reflected the amount of deletion seen in LSKSP cells, we also measured the amount of deletion in progeny derived from LSK clones sorted from BM cells. LSK clones were placed in individual wells, cultured for 14 days, and genotyped using standard PCR. We found progeny from these clones were 100% deleted.

RNA isolation and real-time RT-PCR. Total RNA was extracted with TRIzol. Single-stranded cDNA was synthesized with oligo-dT primer (ThermoScript RT-PCR System; Invitrogen) for 45 minutes at 50°C. Quantitative PCR was performed using Taqman PCR Master Mix on an ABI PRSIM 7700 sequence detection system (Applied Biosystems). Transcript quantification was performed in triplicate or duplicate for every sample, and expression of each gene was normalized to *Gapdh* for Taqman PCR. The following Taqman primers (Applied Biosystems) were used: *Hes1* (Mm00468601_m1), *Cebpa* (Mm00514283_s1), *Notch1* (Mm00435245_m1), *Notch2* (Mm00803077_m1), and housekeeping gene *Gapdh* (Mm99999915_g1). Sequences of SYBR green primers to measure Fringe expression were as follows: *Lfng* forward, 5′-CTGCACCATTGGCTACATTG-3′; *Lfng* reverse, 5′-ATGGGTCAGCTTCCACAGAG-3′; *Mfng* forward, 5′-GCTCCCACTTTGTGGACACT-3′; *Mfng* reverse, 5′-TCCCCTCAAAGACACCGTAG-3′; *Rfng* forward, 5′-ACACCAATTGCTCTGCTGTG-3′; *Rfng* reverse, 5′-CTAGGTCGCCCCAGGTAGAT-3′.

Transplant and 5FU injection. Mice were injected i.p. with a single 150-mg/kg dose of 5FU (source). For transplant experiments, BM cells were collected from Ly5.2 *Notch1*- and *Notch2*-deficient mice and injected into lethally irradiated (900–1,000 cGy using a Cesium source) Ly5.1 recipients via the tail vein. FACS analysis of PB was performed at 3-week intervals after BM transplants. A mouse was considered reconstituted if 2% or more of PB was of donor origin and donor myeloid ($GR1^+$ and/or $F4/80^+$) and donor lymphoid ($CD19^+$ and $CD3^+$) was detected by 9 weeks after transplant. CRU frequency was estimated using Poisson statistics, and 2-tailed ratio of proportions tests with limit dilution transplants were calculated using L-Calc software (StemCell Technologies).

Statistics. Statistical significance of differences was assessed using 2-tailed paired Student's *t* test, 2-tailed Student's *t* test, or Mann-Whitney test as appropriate. A *P* value of 0.05 or less was considered significant.

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Address correspondence to: Irwin D. Bernstein, 1100 Fairview Ave. N., PO Box 19024, Seattle, Washington 98109-1024, USA. Phone: 206.667.4886; Fax: 206.667.6084; E-mail: ibernste@fhcrc.org.

Lia M. Halasz's present address is: Harvard Radiation Oncology Program, Boston, Massachusetts, USA.

Mingyi Sun's present address is: Beijing Novo Nordisk Pharmaceuticals Science and Technology Co., Beijing, China.

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