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Article

Canonical Notch Signaling Is Dispensable for the Maintenance of Adult Hematopoietic Stem Cells

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SUMMARY

Gain-of-function experiments have demonstrated the potential of Notch signals to expand primitive hematopoietic progenitors, but whether Notch physiologically regulates hematopoietic stem cell (HSC) homeostasis in vivo is unclear. To answer this question, we evaluated the effect of global deficiencies of canonical Notch signaling in rigorous HSC assays. Hematopoietic progenitors expressing dominant-negative Mastermind-like1 (DNMAML), a potent inhibitor of Notch-mediated transcriptional activation, achieved stable long-term reconstitution of irradiated hosts and showed a normal frequency of progenitor fractions enriched for long-term HSCs. Similar results were observed with cells lacking CSL/RBPJ, a DNA-binding factor that is required for canonical Notch signaling. Notch-deprived progenitors provided normal long-term reconstitution after secondary competitive transplantation. Furthermore, Notch target genes were expressed at low levels in primitive hematopoietic progenitors. Taken together, these results rule out an essential physiological role for cell-autonomous canonical Notch signals in HSC maintenance.

INTRODUCTION

Notch is a highly conserved signaling pathway that regulates cell-fate decisions and tissue homeostasis in multiple contexts (Artavanis-Tsakonas et al., 1999). Mammals have four *Notch* genes (*Notch1–4*), which encode cell surface receptors with distinct, but overlapping, patterns of expression. Canonical Notch signaling is initiated by ligands of the Jagged or Delta-like families. Ligand binding initiates several successive proteolytic cleavages, which culminate in intramembrane proteolysis by a gamma-secretase complex and release of the intracellular domain of Notch (ICN) (De Strooper et al., 1999). ICN then exerts its activity in the nucleus as part of a large transcriptional activation complex (Jeffries et al., 2002). Essential components of this complex include ICN, the transcription factor CSL/RBPJ, and transcriptional coactivators of the Mastermind-like (MAML) family (MAML1–3) (Jarriault et al., 1995; Tamura et al., 1995; Wu et al., 2000, 2002). Upon Notch activation, ICN binds to CSL/RBPJ and creates a dual-binding interface for MAML, forming a ternary complex that is essential for transcriptional activation of Notch target genes (Nam et al., 2006; Wilson and Kovall, 2006).

Notch regulates progenitor self-renewal and differentiation in various organs, such as in neural tissue, muscle, skin, and gut (Chiba, 2006). In the hematopoietic system, Notch is essential for the emergence of definitive hematopoietic stem cells (HSCs) during fetal life (Kumano et al., 2003; Hadland et al., 2004). Notch subsequently plays a critical role in splenic marginal zone B (MZB) cell development and at several stages of T cell development (reviewed in Radtke et al., 2004; Maillard et al., 2005; Visan et al., 2006). In addition, it has been widely hypothesized that Notch ligands are a critical component of HSC niches and promote HSC maintenance by activating Notch receptors expressed in HSCs. Gain-of-function experiments suggest that Notch signaling increases self-renewal and decreases differentiation of hematopoietic progenitors. In these experiments, elevated levels of Notch signaling were achieved through exposure of progenitors to Notch ligands in vitro (Varnum-Finney et al., 1998, 2003; Karanu et al., 2001; Suzuki et al., 2006), overexpression of constitutively active *Notch* alleles (Carlesso et al., 1999; Varnum-Finney et al., 2000; Stier et al., 2002; Burns et al., 2005), overexpression of the Notch downstream target *Hes1* (Kunisato et al., 2003; Yu et al., 2006), or activation of *Jagged1* expression through osteoblast stimulation (Calvi et al., 2003). Thus, multiple reports show that experimental manipulations that increase Notch signaling enhance the self-renewal of primitive hematopoietic progenitors.

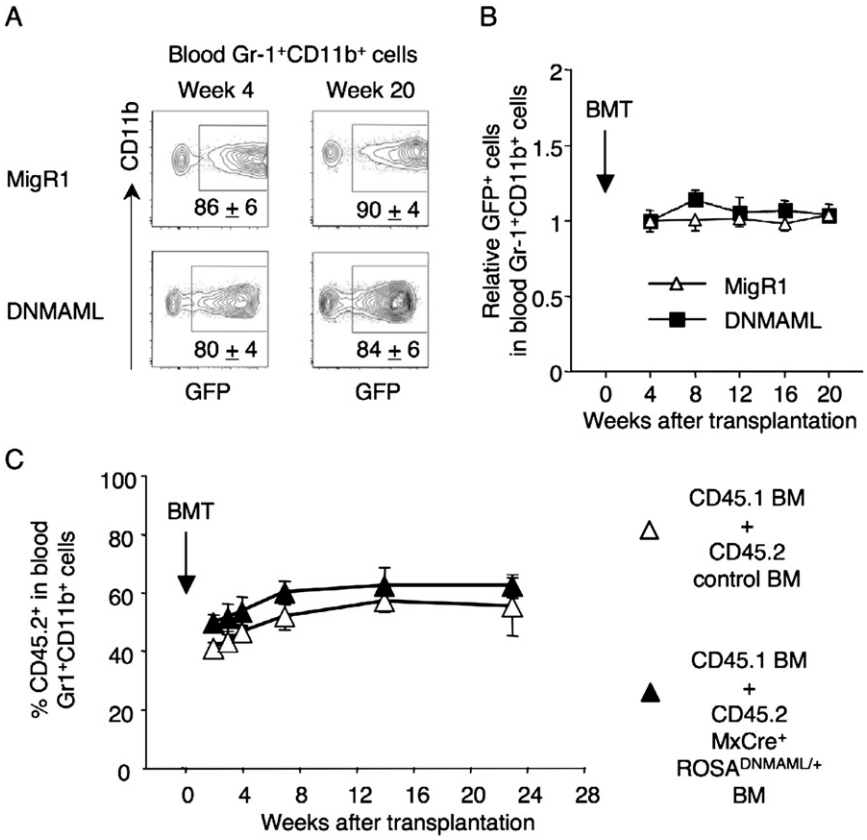

Figure 1. Stable Engraftment of DNMAML-Transduced Progenitors

(A) Lethally irradiated mice were reconstituted with 5-FU-treated BM cells after transduction with the GFP-expressing retrovirus MigR1 or with a retrovirus expressing the GFP-tagged Notch inhibitor DNMAML. Percentage of GFP^+ cells among $Gr1^+CD11b^+$ myeloid cells in the peripheral blood 4 and 20 weeks after transplantation. The percentages are means ± SDs (n = 4 in each group). The experiment is representative of ten similar experiments.

(B) Relative percentage of GFP^+ myeloid cells at various time points after bone marrow transplantation (BMT), with the percentage observed at week 4 normalized to 1. The data represent the normalized means ± SDs (n = 4). White triangles, MigR1; black squares, DNMAML.

(C) Contribution of $CD45.2^+$ cells to blood $Gr1^+CD11b^+$ myeloid cells after competitive transplantation of $CD45.1^+$ BM and poly(I:C)-induced *Mx-Cre*$^-$ *ROSA*$^{DNMAML/+}$ BM (white triangles) or *Mx-Cre*$^+$ *ROSA*$^{DNMAML/+}$ BM (black triangles) (1:1 ratio). Data shown are the means ± SDs for nine control and eight DNMAML recipients.

In contrast, whether Notch signaling has an obligate role in HSC self-renewal is controversial. Duncan et al. showed that a Notch reporter transgene was activated in primitive bone marrow (BM) progenitors, and blockade of Notch signaling with gamma-secretase inhibitors in vitro or with a dominant-negative form of the *Xenopus* CSL/RBPJ homolog in vitro and in vivo increased differentiation and decreased progenitor self-renewal (Duncan et al., 2005). At first glance, these results appear to disagree with previous work in which genetic inactivation of the *Rbpj* gene (encoding CSL/RBPJ) caused a failure of T and MZB cell development, but no other obvious hematopoietic phenotype (Han et al., 2002; Tanigaki et al., 2002). However, stringent assays of HSC function were not performed with CSL/RBPJ-deficient progenitors. In addition, inactivation of *Notch1* and combined inactivation of *Notch1* and *Jagged1* have not revealed defects in HSC function (Radtke et al., 1999; Mancini et al., 2005); however, these studies did not rule out redundant effects from other Notch receptors or ligands. Hence, whether Notch signaling is essential for HSC maintenance under physiological conditions remains unknown.

To resolve this issue, we inhibited all canonical Notch signals in adult HSCs by either expressing a dominant-negative Mastermind-like1 construct fused to GFP (DNMAML) (Weng et al., 2003; Maillard et al., 2004, 2006a, 2006b; Sambandam et al., 2005; Tu et al., 2005) or by conditional deletion of *Rbpj*. In stringent stem cell assays, DNMAML-expressing cells and CSL/RBPJ-deficient cells failed to show any defect when allowed to compete with normal HSCs in vivo. We also noted that, although HSCs respond to Notch ligands ex vivo, the Notch signaling tone of normal HSCs appears to be low in the BM microenvironment. Our results argue strongly against an obligate physiologic role for Notch signaling in adult BM HSCs.

RESULTS

Stable Engraftment of DNMAML-Transduced Cells

A comprehensive assessment of the physiological effects of Notch in vivo requires inhibition of signaling from all four Notch receptors. To this end, we developed a dominant-negative Mastermind-like1 construct (DNMAML), encoding the N-terminal Notch-binding domain of MAML1 fused to GFP (Weng et al., 2003; Maillard et al., 2004). The DNMAML-GFP fusion protein interferes with the Notch transcriptional activation complex, leading to potent inhibition of Notch1–4 signaling in vitro and in vivo, while allowing us to track single cells deprived of Notch signaling (Weng et al., 2003; Maillard et al., 2004, 2006a, 2006b; Sambandam et al., 2005; Tu et al., 2005).

We first transduced adult HSCs with a GFP-expressing retrovirus (MigR1) or a retrovirus encoding DNMAML-GFP (DNMAML), and these cells were used to reconstitute lethally irradiated mice with a mixed population of transduced tester and untransduced competitor progenitors. The degree of chimerism achieved after transplantation of MigR1 and DNMAML-transduced progenitors was high, both at early (4 weeks) and late time points (20 weeks) (Figure 1A). The percentage of GFP^+ myeloid cells remained stable over time and was not significantly different between MigR1 and DNMAML cohorts (Figure 1B). As shown previously, DNMAML expression resulted in profound inhibition of T and

MZB cell development, consistent with inhibition of Notch1 and Notch2, respectively (Maillard et al., 2004; Sambandam et al., 2005).

To exclude any confounding effect of retroviral transduction, we generated *ROSA*$^{DNMAML/+}$ mice in which DNMAML is knocked into the *ROSA26* locus downstream of a floxed stop cassette (Tu et al., 2005; Maillard et al., 2006b). We bred these mice to *Mx-Cre* transgenic mice and induced Cre expression with poly(I:C). This routinely led to DNMAML expression in >98% of BM progenitors (Figure S1 available online). We mixed BM cells from poly(I:C)-induced *Mx-Cre*$^{+}$ × *ROSA*$^{DNMAML/+}$ mice or from control poly(I:C)-treated mice with a fixed dose of CD45.1^{+} competitor cells and used these mixtures to reconstitute lethally irradiated recipients (Figure 1C). We observed similar levels of stable long-term chimerism in animals with Notch-replete and Notch-deficient progenitors. Importantly, as with retroviral transduction, DNMAML expression from the *ROSA26* locus led to efficient Notch inhibition in vivo, as shown by a complete blockade of T and MZB cell development (Figure S2). Overall, these data indicate that wild-type progenitors do not have a competitive advantage over DNMAML-expressing cells in primary transplantation experiments.

Normal Frequency of DNMAML-Expressing Hematopoietic Progenitors

Long-term hematopoietic stem cells (LT-HSCs) are found in a population characterized by no or low-level expression of lineage markers, high Sca-1, and high c-Kit expression (Lin$^{-/lo}$Sca-1^{hi}c-Kit^{hi}, LSK cells) (Kondo et al., 2003). Defects in HSC homeostasis often result in an abnormal LSK frequency (Kondo et al., 2003). Therefore, we assessed the frequency of LSK progenitors in GFP^{+} MigR1 or DNMAML-transduced BM cells late after transplantation. The LSK frequency was similar among MigR1 and DNMAML-transduced BM cell populations (Figure 2A). Furthermore, there was no significant difference in LSK frequency between untransduced (GFP^{-}) and transduced (GFP^{+}) BM in cohorts of MigR1 and DNMAML mice (Figure 2B). Similarly, DNMAML did not alter the frequency of BM LSK progenitors in poly(I:C)-induced *Mx-Cre*$^{+}$ × *ROSA*$^{DNMAML/+}$ mice (Figure 2C).

Normal Frequency of DNMAML-Expressing LT-HSC Progenitors

The LSK population is heterogeneous and contains both LT-HSCs and progenitors with multilineage potential but limited self-renewal capacity (Morrison and Weissman, 1994; Kondo et al., 2003). Therefore, we assessed the effect of DNMAML on BM subpopulations enriched for LT-HSCs, using additional criteria (Figures 2D–2F).

Expression of the SLAM molecule CD150 in the absence of CD48 defines a small population of cells highly enriched for LT-HSCs (Kiel et al., 2005). We found that ~7%–8% of LSK progenitors had a CD150^{+}CD48^{-} phenotype in both control and DNMAML-expressing BM cells (Figure 2D). Low-level expression of Thy1.1 in B6-Thy1.1 mice and absence of the cytokine receptor Flt3 have also been used to define a population highly enriched in LT-HSCs (Adolfsson et al., 2001; Christensen and Weissman, 2001). We transduced B6-Thy1.1 BM with MigR1 or DNMAML and reconstituted irradiated recipients (Figure 2E). At steady state, Flt3^{-}Thy1.1^{lo} cells represented ~35% of GFP^{+} LSK progenitors in both the MigR1 and DNMAML-transduced progenitors. Active extrusion of supravital dyes is another method to enrich for LT-HSCs. For example, dye efflux generates a characteristic "side population" (SP) appearance when BM cells stained with Hoechst 33342 are examined for a combination of blue and red fluorescence. These SP cells harbor the vast majority of LT-HSCs (Goodell et al., 1996). The frequency of the SP population was not significantly different between GFP^{+} control cells and DNMAML-transduced cells (Figure 2F). Altogether, these data show that inhibiting canonical Notch signaling does not affect the overall frequency of BM LSK progenitors or the frequency of LSK fractions highly enriched for LT-HSCs.

Secondary Competitive Repopulation Assays

The most sensitive measures of HSC function are assays in which test progenitors are serially transplanted into irradiated recipients with normal competitor cells. To assess if Notch inhibition resulted in subtle LT-HSC defects, we performed secondary competitive transplants with MigR1 or DNMAML-transduced LSK cells (Figure 3). BM cells from a CD45.1^{+} B6 donor were transduced with MigR1 or DNMAML and used to reconstitute irradiated CD45.2^{+} B6 recipients (Figure 3A). Twelve weeks after this primary transplantation, we purified CD45.1^{+} GFP^{+} MigR1 or DNMAML-transduced LSK cells and transplanted a mixture of transduced LSK cells (n = 500) and fresh CD45.2^{+} competitor BM cells (n = 2 × 10^{5}) into irradiated secondary CD45.2^{+} B6 recipients. We assessed the contribution of donor CD45.1^{+} GFP^{+} cells to the myeloid, B, and T cell lineages 16 weeks after transplantation (Figure 3B). There was no difference in the contribution of control and DNMAML-transduced cells to the myeloid and B cell lineages at 16 weeks ($p = 0.42$ and $p = 0.26$). In contrast, DNMAML-transduced cells were absolutely deficient in T lineage reconstitution ($p < 0.001$), indicating that the effect of DNMAML persisted throughout the experiment.

Finally, we assessed LT-HSC function in limiting dilution competitive transplantation assays (Table 1) (Szilvassy et al., 1990). Decreasing numbers of MigR1 or DNMAML-transduced LSK cells were transplanted together with normal competitor BM cells. At 16 weeks after reconstitution, we assessed chimerism and scored recipient mice for long-term myeloid engraftment (defined as >1% donor-derived cells). In this assay, we focused on the myeloid compartment to avoid possible biases caused by Notch effects on lymphoid commitment. Poisson statistics showed no significant difference in the frequency of long-term myeloid repopulation among control and DNMAML-transduced cells (Table 1), with a nonsignificant trend toward modestly enhanced activity in Notch-deprived progenitors. Thus, even in the setting of a limiting dilution assay, inhibition of canonical Notch signaling did not impair the long-term repopulation activity of BM progenitors.

Early Hematopoietic Recovery from Notch-Deprived Progenitors

Next, we assessed if Notch deprivation affected early stages of hematopoietic recovery. To this end, we transplanted poly(I:C)-induced *Mx-Cre*$^{+}$ × *ROSA*$^{DNMAML/+}$ mice or control poly(I:C)-treated BM into lethally irradiated recipients, in the absence of competitor cells. In this setting, recipient mice showed >95% reconstitution with DNMAML-GFP-expressing

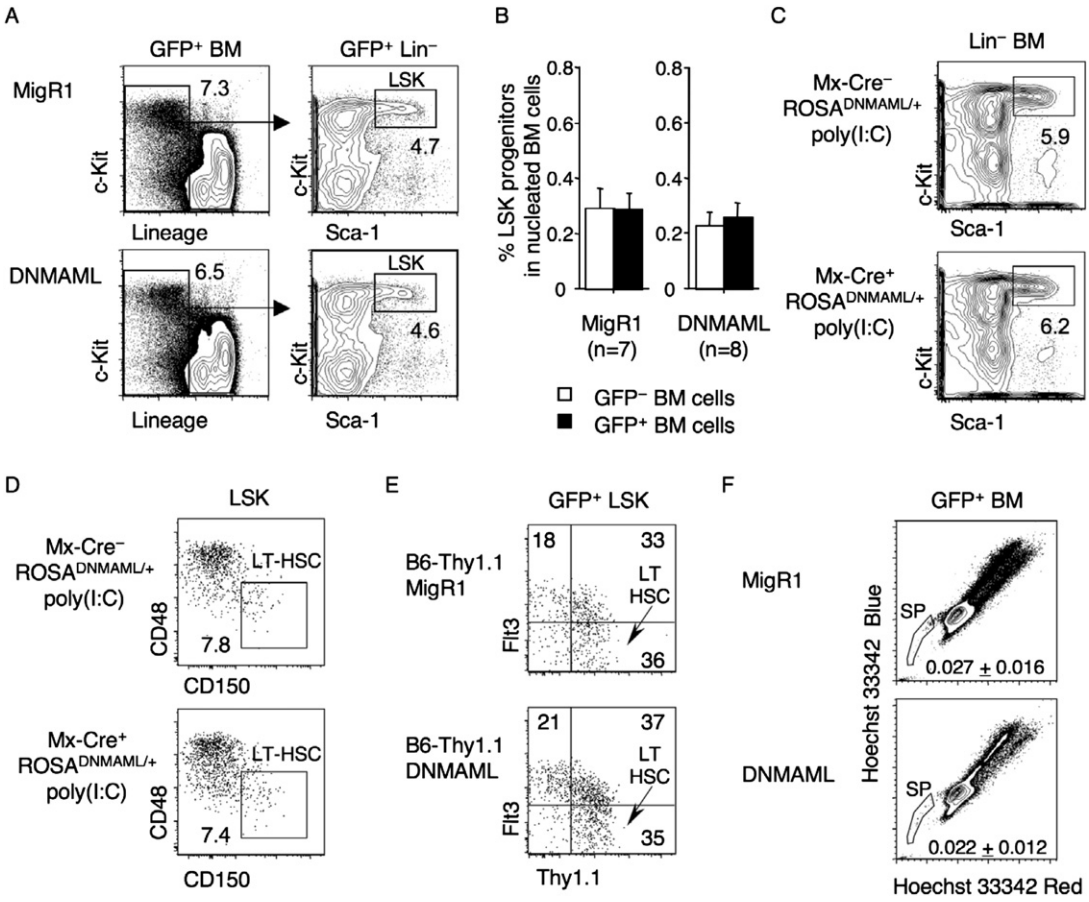

Figure 2. Normal Frequency of Populations Enriched for LT-HSCs in Notch-Deprived BM

(A) Frequency of Lin$^{-/lo}$Sca-1^{hi}c-Kit^{hi} (LSK) progenitors in the control GFP$^+$ fraction of MigR1 BM (n = 7) or in DNMAML-transduced BM (n = 8) 16 weeks after reconstitution. One representative example is shown.

(B) Frequency of LSK progenitors among GFP$^-$ and GFP$^+$ BM cells in mice reconstituted with MigR1 (n = 7) or DNMAML-transduced BM (n = 8). The data are shown as mean ± two SEM.

(C) Analysis of BM LSK progenitors in *Mx-Cre*$^-$ *ROSA*$^{DNMAML/+}$ or *Mx-Cre*$^+$ *ROSA*$^{DNMAML/+}$ mice, after induction of Cre expression with poly(I:C). More than 98% of the cells in the LSK population had achieved DNMAML activation (Figure S1). One representative example is shown (n = 5).

(D) CD150 and CD48 expression in LSK cells of *Mx-Cre*$^-$ *ROSA*$^{DNMAML/+}$ (n = 6) or *Mx-Cre*$^+$ *ROSA*$^{DNMAML/+}$ (n = 4) mice after poly(I:C) induction. One representative example is shown.

(E) Flt3 and Thy1.1 expression in GFP$^+$ BM LSK cells of mice reconstituted with MigR1 or DNMAML-transduced B6-Thy1.1 BM. One representative example is shown (n = 5).

(F) Side population (SP) analysis after labeling BM cells with Hoechst 33342 and gating on GFP$^+$ BM. One representative example is shown (n = 5). Numeric data displayed in the contour plot represent the means ± two SEM.

progenitors. Eight weeks after transplantation, mice were challenged with a single injection of 5-fluorouracil (5-FU) and assessed with weekly complete blood counts during BM ablation and recovery. We observed no significant difference in the recovery of white blood cell, platelet, and red cell counts in the two groups of mice (Figure S3). This similarity extended to the transient elevation of platelet and white cell counts observed 2–3 weeks after 5-FU challenge. We also did not detect any defect in blood count recovery at early time points after transplantation of *Mx-Cre*$^+$ × *ROSA*$^{DNMAML/+}$ BM into lethally irradiated recipients or any change in blood chimerism after 5-FU challenge of the CD45.1/CD45.2 *Mx-Cre ROSA*$^{DNMAML/+}$ mixed chimeras described in Figure 1C (data not shown). Altogether, these findings suggest that canonical Notch signaling is not required for early hematopoietic recovery after BM ablation.

Normal HSC Numbers and Function in the Absence of CSL/RBPJ

To assess the impact of Notch deprivation by another genetic approach, we studied mice carrying a conditional *Rbpj* gene (Han et al., 2002; Tanigaki et al., 2002, 2004), which encodes a DNA-binding factor that is essential for signaling from all Notch receptors. After breeding to *Mx-Cre* transgenic mice, we induced Cre expression with poly(I:C) and harvested BM cells for competitive transplantation experiments. *Mx-Cre*$^+$ *Rbpj*$^{f/f}$ BM and control *Rbpj*$^{f/f}$ BM produced similar levels of stable long-term chimerism in blood myeloid cells (Figure 4A) and B cells

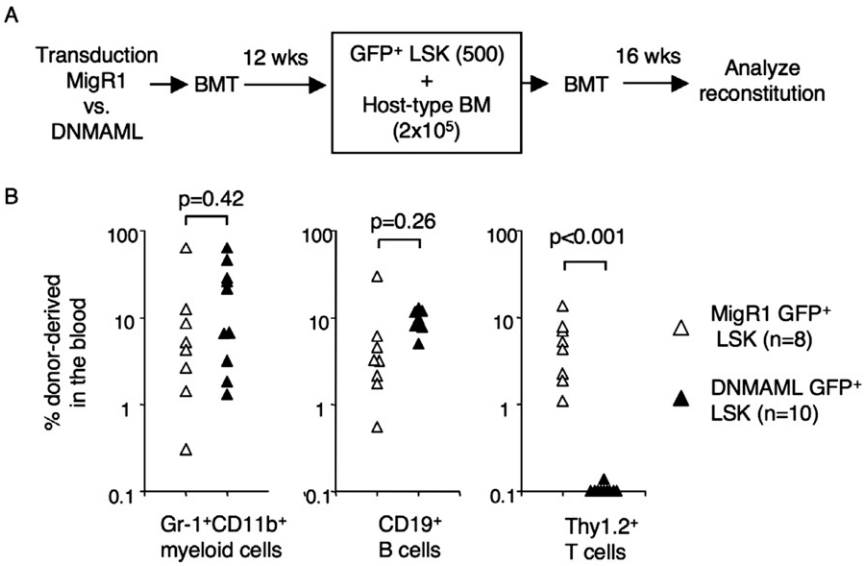

Figure 3. Competitive Repopulation Potential of Notch-Deprived LSK Progenitors in Secondary Recipients

(A) Experimental design. MigR1 or DNMAML-transduced progenitors were used to repopulate primary lethally irradiated recipients. After 12 weeks, purified GFP$^+$ LSK cells were used in a secondary transplantation together with host-type competitor BM cells (500 and 2 × 10^5 cells, respectively).

(B) Blood of the secondary recipients analyzed 16 weeks after transplantation for donor-derived myeloid (Gr1$^+$CD11b$^+$), B (CD19$^+$), and T (Thy1.2$^+$) lineage cells (MigR1 LSK, n = 8; DNMAML LSK, n = 10). There was no significant difference between the ability of control and DNMAML-transduced LSK cells to repopulate the myeloid and B cell lineages ($p = 0.42$ and $p = 0.26$, respectively, Student's t test). DNMAML LSK cells were deficient at repopulating the T cell compartment ($p < 0.001$).

(data not shown). When analyzed 18 weeks after transplantation, as compared to control CD45.2$^+$ cells, we found a similar or even slightly higher contribution of CSL/RBPJ-deficient cells to the BM LSK, myeloid, and B lineage progenitor populations (Figure 4B). In contrast, there was virtually no contribution of *Mx-Cre*$^+$ *Rbpj*$^{f/f}$ CD45.2$^+$ cells to the T cell and MZB cell lineages (Figure 4B and Figure S4A). This was consistent with efficient *Rbpj* inactivation and little or no selection for rare cells that might have escaped Cre-mediated excision (Figure S4B). Finally, we assessed the frequency of LSK progenitors, as well as LSK subsets further enriched for LT-HSCs, in engrafted CD45.2$^+$ BM cells (Figure 4C). There was no significant difference in LSK or LT-HSC frequency in control and CSL/RBPJ-deficient BM cell populations. These results were consistent with our observations using the DNMAML system and reinforce our conclusion that canonical Notch signaling has no physiologic role in the maintenance of adult BM HSCs.

Low Abundance of Notch Target Gene Transcripts in Primitive BM Progenitors

Whereas induction of Notch signaling can enhance the self-renewal of primitive hematopoietic progenitors, inhibition of endogenous Notch signals with DNMAML did not impair HSC numbers or function, suggesting that under basal physiologic conditions there might be little ongoing Notch signaling in HSCs. Thus, we studied the expression of Notch-regulated genes in HSCs and other progenitors (Figure 5). Transcript levels were compared between BM LSK cells and populations of T cell progenitors that experience high (ETP, early T lineage progenitors; DN3, CD44$^-$CD25$^+$ CD4$^-$CD8$^-$ double-negative thymocytes) or low levels of Notch signaling (DP, CD4$^+$CD8$^+$ double-positive thymocytes) (Figure 5A). The amount of *Hes1* mRNA in LSK progenitors was low, similar to the amount found in DP thymocytes, and ~10- to 20-fold lower than in ETP and DN3 cells. We then studied the expression of *Dtx1*, a gene whose expression correlates with Notch signaling intensity (Deftos et al., 2000; Saito et al., 2003; Sambandam et al., 2005; Maillard et al., 2006a). *Dtx1* mRNA was undetectable in LSK cells, present at low levels in DP thymocytes, and markedly upregulated in ETP and DN3 cells. Critical components of the Notch signaling pathway were expressed in LSK cells, as noted previously (Milner et al., 1994; Calvi et al., 2003; Maillard et al., 2006a). Specifically, *Notch1*, *Notch2*, and *Maml1* mRNAs were readily detected (Figure 5A), as were *Maml2* and *Maml3* mRNAs (data not shown). Therefore, LSK progenitors express core components of Notch signaling but low levels of Notch target gene mRNA.

Table 1. Long-Term Myeloid Repopulation Potential of Control (MigR1-Transduced) and Notch-Deprived (DNMAML-Transduced) LSK Progenitors

LSK Number	MigR1 GFP$^+$ LSK Positive for Donor Engraftment/Total Mice	DNMAML GFP$^+$ LSK Positive For Donor Engraftment/Total Mice
500	7/8	10/10
150	5/9	7/9
50	3/7	5/10
Reconstitution frequency (95% confidence interval)	1:182 (1:100–1:332)	1:86 (1:48–1:154)

Cohorts of lethally irradiated recipient mice were transplanted with decreasing numbers of GFP$^+$ MigR1 or DNMAML-transduced LSK cells (500, 150, 50), together with a fixed dose of host-type BM (2 × 10^5). Blood was analyzed 16 weeks after reconstitution for the percentage of donor-derived myeloid cells. Positive engraftment was defined as >1% donor-derived cells. Poisson statistics were used to calculate the frequency of long-term repopulation units. Data from the cohort of mice receiving 500 LSK cells are also included in Figure 3.

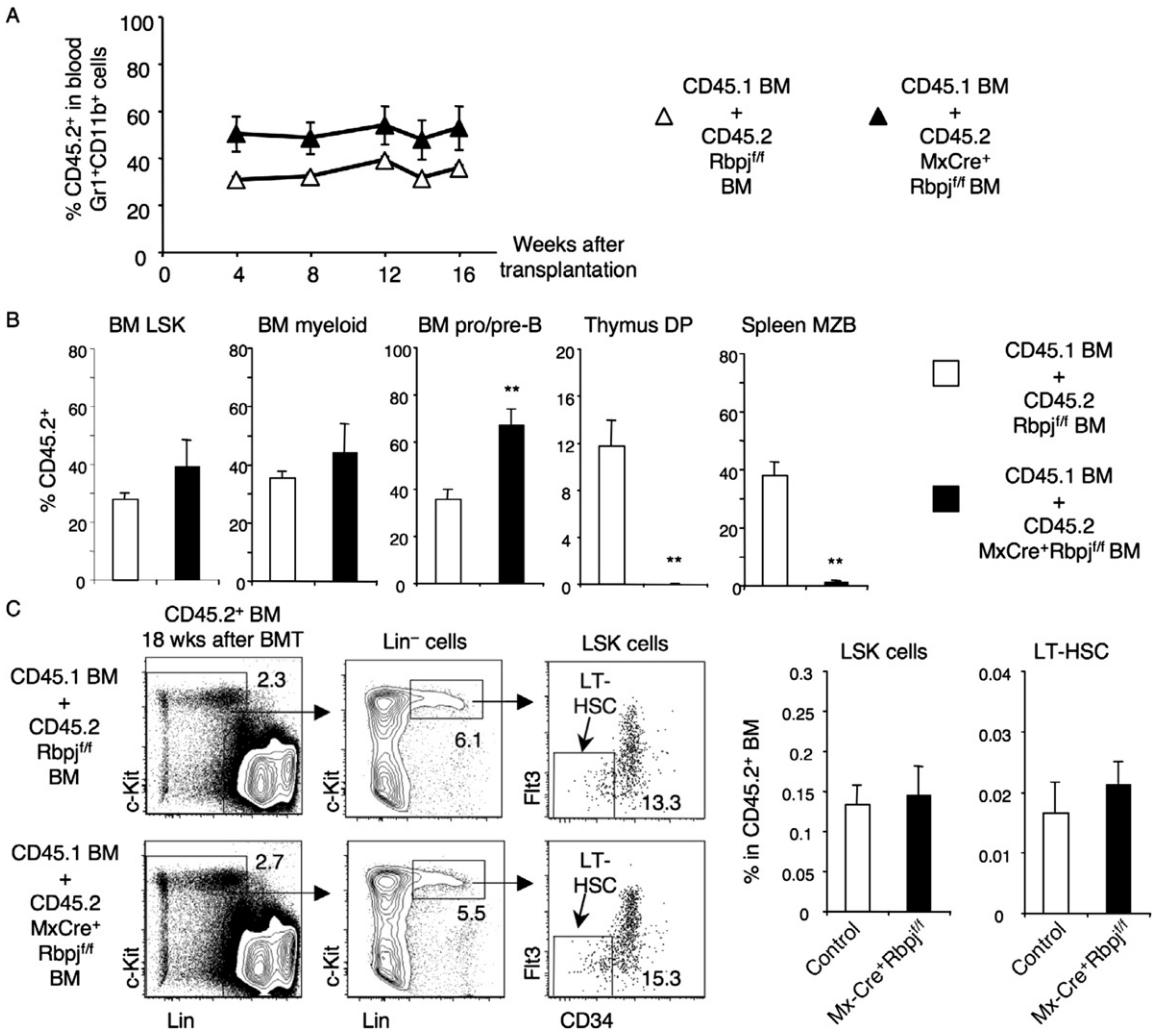

Figure 4. LT-HSC Numbers and Function in the Absence of CSL/RBPJ

(A) Stable CD45.2$^+$ chimerism in lethally irradiated recipients of BM cells from poly(I:C)-treated *Mx-Cre*$^+$ *Rbpj*$^{f/f}$ (black triangles) or control *Rbpj*$^{f/f}$ CD45.2$^+$ mice (white triangles) (n = 5 in each group). Chimerism was assessed by analysis of blood Gr1$^+$CD11b$^+$ myeloid cells. Data are the means ± SEM.

(B) Mixed BM recipients analyzed 18 weeks after BMT for the contribution of CD45.2$^+$ cells to BM LSK progenitors (Lin$^{-/lo}$Sca-1^{hi}c-Kit^{hi}), myeloid cells (Gr1$^+$CD11b$^+$), pro- and pre-B cells (B220$^+$BP-1$^+$sIgM$^-$), double-positive (DP) thymocytes (CD4$^+$CD8$^+$), and spleen MZB cells (B220$^+$CD21^{hi}CD23^{lo}). Data are means ± SEMs (n = 5 in each group). Double asterisk (**) indicates statistical significance (Student's t test, $p < 0.01$).

(C) Similar frequency of LSK progenitors and CD34$^-$Flt3$^-$ LSK cells (enriched for LT-HSC) in engrafted control and CSL/RBPJ-deficient BM 18 weeks after competitive transplantation. Plots show a representative example; bar graphs indicate means ± SDs.

We next asked whether the expression of Notch target genes in LSK cells might result from low but detectable levels of canonical Notch signaling by determining if gene expression was affected by DNMAML (Figure 5B). *Hes1* transcript levels were not different in control and DNMAML-transduced LSK progenitors, whereas *Dtx1* transcripts were undetectable in both. We also measured c-*myc* mRNA, because c-*myc* is regulated by Notch signaling in certain contexts and plays a role in HSC homeostasis (Wilson et al., 2004; Klinakis et al., 2006; Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). The abundance of c-*myc* transcripts was similar in control and DNMAML-transduced LSK cells. Finally, we assessed *Runx1* mRNA, because Notch signaling has been reported to upregulate *Runx1* in zebrafish hematopoietic progenitors (Burns et al., 2005). DNMAML expression did not affect *Runx1* expression in LSK progenitors. Therefore, regulatory inputs other than canonical Notch signaling must contribute to basal levels of *Hes1*, c-*myc*, and *Runx1* expression in adult mouse hematopoietic progenitors. Altogether, these data suggest that LSK progenitors have low levels of Notch signaling under basal conditions in vivo.

Rapid Inducibility of Notch Signaling in Primitive BM Progenitors

Next, we asked if Notch signaling could be induced by exposure of LSK cells to Notch ligands ex vivo (Figure 6). We cultured control or DNMAML LSK cells with OP9-DL1 stromal cells expressing the Notch ligand Delta-like-1 (Schmitt and Zuniga-Pflucker, 2002) or control OP9 cells. Six hours of coculture with OP9-DL1

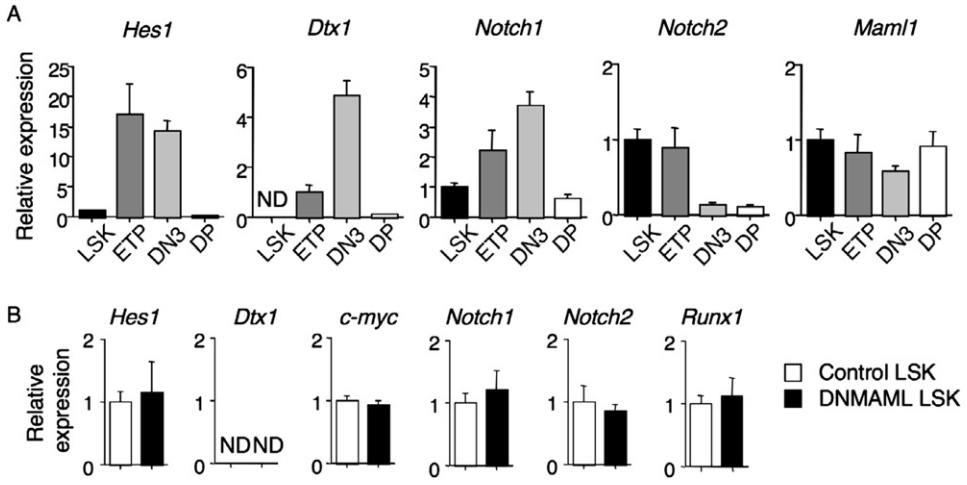

Figure 5. LSK Progenitors Are Physiologically Exposed to a Low Intensity of Notch Signaling

(A) LSK progenitors were assessed by real-time RT-PCR in comparison to early T lineage progenitors (ETP), DN3, and $CD4^+CD8^+$ DP thymocytes. The amount of the Notch target gene transcripts *Hes1* and *Dtx1* (normalized to *Hprt*) was very low in LSK cells and DP thymocytes but high in ETP and DN3 cells. Results are given as the mean ± SEM. ND, not detectable.

(B) *Hes1*, *Dtx1*, *c-myc Notch1*, *Notch2*, and *Runx1* transcripts in control and DNMAML-expressing LSK progenitors (normalized to *Hprt*). The measurements were performed in triplicates for four to five independent samples. Results are given as the mean ± SEM. ND, not detectable.

was sufficient to induce *Dtx1* mRNA (Figure 6A). This upregulation was abolished in DNMAML-expressing LSK cells. When the coculture was continued for 48 hr (Figure 6B), exposure to OP9-DL1 resulted in both *Hes1* and *Dtx1* upregulation, and this again was completely inhibited by DNMAML. Other investigators have reported that the rapid upregulation of *Hes1* and *Dtx1* mRNA in cocultures of progenitors with OP9-DL1 cells occurs before induction of T lineage specification (Taghon et al., 2005). Our findings confirm that DNMAML efficiently blocks Notch signaling in the context of primitive hematopoietic progenitors and show that, although LSK progenitors are poised to transduce Notch signals, they do so at only low levels within their physiological niche in vivo.

DISCUSSION

The homeostasis of tissue stem cells is a carefully regulated process that balances long-term self-renewal and differentiation into downstream progeny. Like other kinds of tissue stem cells, HSCs are maintained in a stem cell niche, a specialized microenvironment that provides critical signals to support HSC activity (Moore and Lemischka, 2006; Scadden, 2006; Wilson and Trumpp, 2006). Along with other pathways, Notch signaling induced by ligands expressed on support cells in the niche has been hypothesized to contribute to HSC homeostasis. This hypothesis is based on the ability of Notch signals to regulate progenitor self-renewal and differentiation in certain contexts and observations showing that experimentally enhanced Notch signals increase the self-renewal of hematopoietic progenitors in vitro and in vivo. In contrast, the importance of endogenous Notch signaling in HSCs under physiologic conditions has been unclear and controversial. Here, we addressed this question by interfering with Notch-mediated transcriptional activation using DNMAML, a potent and specific pan-inhibitor of Notch signaling, and through disruption of the *Rbpj* gene. This is the first report, to the best of our knowledge, describing stringent

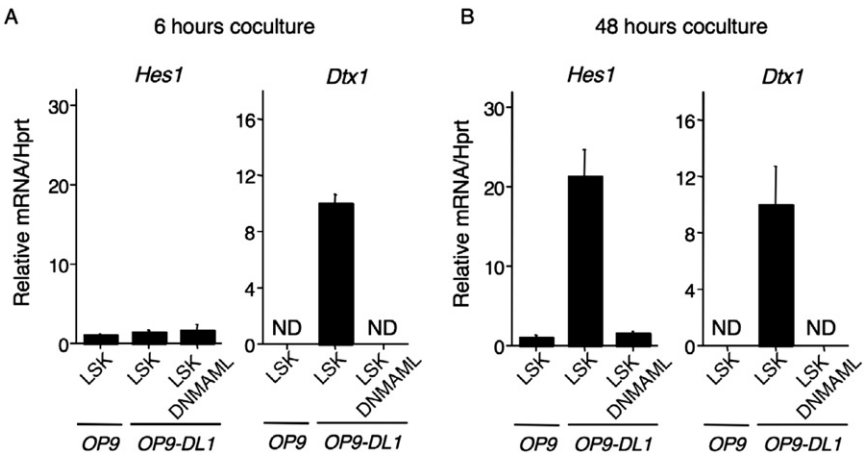

Figure 6. DNMAML Inhibits the Rapid Induction of Notch Signaling after Exposure of LSK Progenitors to Notch Ligands In Vitro

Freshly purified LSK progenitors from control or DNMAML-expressing BM were cultured with stromal monolayers of OP9 cells or OP9-DL1 cells expressing the Notch ligand Delta-like1. The amount of *Hes1* and *Dtx1* mRNA (normalized to *Hprt*) was assessed by real-time RT-PCR in LSK progenitors after 6 (A) or 48 hr (B) of coculture. The data are the means ± SEM. ND, not detectable.

in vivo assays of LT-HSC activity in the absence of canonical Notch signals from all four mammalian Notch receptors. Our observations rule out a significant effect of cell-autonomous canonical Notch signals on LT-HSC maintenance in vivo. In addition, they indicate that primitive hematopoietic progenitors in the BM are exposed to low levels of Notch signaling under basal physiologic conditions.

Our findings are based on an examination of HSC function in the normal mouse BM environment as well as upon serial HSC transplantation in radiation chimeras. Our results clearly show that, in these situations, canonical Notch signaling is not a significant regulator of HSC self-renewal. However, our observations leave open the possibility that Notch might play a role in HSC function if Notch ligand expression is induced in the BM microenvironment. For example, parathyroid hormone (PTH) regulates HSC self-renewal though the activation of osteoblasts, a phenomenon that correlates with increased expression of the Notch ligand Jagged1 (Calvi et al., 2003). It is conceivable that, upon Jagged1 upregulation, HSCs might be exposed to elevated levels of Notch signaling in the osteoblastic stem cell niche. This is consistent with the finding that inhibition of Notch signaling in cocultures of HSC and PTH-treated stroma abolishes the incremental effect of PTH on HSC self-renewal but does not influence basal HSC activity (Calvi et al., 2003). In future work, it will be important to evaluate if Notch signaling is a relevant regulator of HSC homeostasis in situations of hematopoietic stress other than radiation, such as inflammatory states, as well as in primary hematopoietic disorders.

DNMAML is a highly specific inhibitor of canonical Notch signaling through the CSL/RBPJ-ICN-MAML complex. Our studies demonstrate that this canonical pathway has no detectable role in HSC self-renewal but do not rule out Notch effects mediated through noncanonical pathways. Noncanonical pathways have been proposed based on genetic observations in flies that suggest the existence of CSL/RBPJ-independent Notch phenotypes (Martinez Arias et al., 2002) and results obtained with cultured mammalian cells (Shawber et al., 1996; Nofziger et al., 1999). At present, the biochemical details of the noncanonical pathways remain unknown. Importantly, all phenotypes produced to date through genetic deficiencies of individual Notch receptors in mice have been phenocopied by *Rbpj* deletion or DNMAML expression, indicating that they are due to defects in canonical Notch signaling.

The low abundance of Notch target gene transcripts in hematopoietic progenitors suggests that HSCs experience minimal to no Notch signaling in vivo. Because Notch signaling was rapidly induced after short-term exposure of LSK progenitors to Notch ligands ex vivo, it appears paradoxical that these cells fail to respond to Notch ligands expressed in the BM microenvironment. However, the concentration of Notch ligands might be insufficient to induce significant levels of Notch signaling in the HSC niche in vivo. Another nonmutually exclusive possibility is that LSK progenitors use intrinsic mechanisms to downregulate their responsiveness to Notch ligands, so that they only respond when exposed to high concentrations of ligands or to specific Notch ligand families (such as Delta-like ligands). For example, Fringe glycosylases could specifically downregulate the responsiveness of Notch receptors to Jagged in the BM HSC niche (Visan et al., 2006). Another intrinsic factor that negatively influences Notch signaling in the HSC compartment is LRF/Pokemon, loss of which leads to cell-autonomous derepression of Notch signaling in BM LSK progenitors (Maeda et al., 2007). Such putative mechanisms likely serve to prevent ectopic Notch-mediated commitment to the T cell lineage and suppression of B cell development in the BM.

Our findings indicate that low-level expression of certain Notch target genes, such as *Hes1*, can occur independently of canonical Notch signals. Thus, studies using *Hes1* expression as a sole indicator of ongoing Notch signaling should be interpreted with caution (Harman et al., 2005; Yu et al., 2006). Indeed, E2A transcription factors can upregulate *Hes1* expression independently of Notch (Ikawa et al., 2006), and these and other factors could account for the Notch-independent expression of *Hes1* in BM LSK progenitors. Furthermore, modulation of *Hes1* expression might be relevant to HSC function independently of Notch, because *Hes1* overexpression increases LT-HSC self-renewal (Kunisato et al., 2003; Yu et al., 2006).

Our conclusions differ from those of Reya and coworkers, who postulated an essential role for canonical Notch signals downstream of *Wnt* in HSC homeostasis (Duncan et al., 2005). Using a transgenic GFP reporter based on multimerized CSL/RBPJ binding sites, Duncan et al. described that a high proportion of BM LSK progenitors expressed GFP, suggesting that they were exposed to Notch signals in vivo. In fact, the percentage of GFP^+ LSK cells in the BM was in the same range as the percentage of GFP^+ cells among DN thymocytes, which experience high levels of Notch activation. This is in direct contrast to our findings, which showed that Notch target gene transcripts were present at much lower levels in BM LSK cells than in DN thymocytes. It is possible that the transgenic reporter gene used by these investigators was expressed in a Notch-independent fashion in BM LSK cells. Duncan et al. also reported defective LT-HSC competitive repopulation of LSK cells transduced in vitro with dnXSu(H) (a dominant-negative inhibitor of the *Xenopus* CSL/RBPJ homolog), a finding that differs from our observations with DNMAML-expressing and CSL/RBPJ-deficient cells. Unlike DNMAML, whose in vivo effects correlate with Notch loss-of-function phenotypes, the in vivo activity of dnXSu(H) has not been fully described. In any case, the normal self-renewal of DNMAML-expressing and CSL/RBPJ-deficient LT-HSCs, two conditions that completely inhibit Notch signaling, definitively shows that canonical Notch signaling is not required for LT-HSC maintenance in vivo.

Combinations of gain and loss-of-function approaches have provided important insights into the effects of Notch in different contexts. For example, inhibition of Notch signaling leads to defective T cell development and abnormal B cell expansion in the thymus, whereas induction of Notch signals at supraphysiological levels results in T cell development and suppression of B cell development at extrathymic sites (Pui et al., 1999; Radtke et al., 1999). Such symmetrical phenotypes in cell-fate decisions are expected if the intensity of Notch signaling is high enough to support a physiological effect. In adult HSCs, the intensity of Notch signaling appears too low to translate into a detectable physiological function. We speculate that avoiding high levels of Notch signaling in primitive hematopoietic progenitors is a carefully regulated phenomenon that has an important physiological role, perhaps to prevent ectopic development of T cells and

suppression of B lineage development in the BM. Moreover, it may be important to actively suppress Notch signals in HSCs, as these cells may otherwise be exposed to high-intensity Notch signaling as a consequence of the requirement for the Notch pathway to maintain other elements of the BM microenvironment (Engin et al., 2008; Hilton et al., 2008).

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (B6, CD45.2$^+$) were from Taconic (Germantown, NY). C57BL/6.Ly5.2 (B6-SJL, CD45.1$^+$) were from the NCI (Frederick, MD) or bred locally (EPFL–ISREC, Switzerland). C57BL/6-Thy1^a (B6-Thy1.1, Thy1.1$^+$) were from Jackson Laboratories (Bar Harbor, ME). *ROSA*$^{DNMAML/+}$ mice were generated as described (Tu et al., 2005; Maillard et al., 2006b) and crossed to *Mx-Cre* transgenic mice (Jackson Laboratories). The *Mx-Cre* transgene was also bred to the *Rbpj*$^{f/f}$ background (Han et al., 2002; Tanigaki et al., 2002). Cre expression was induced with poly(I:C) (Sigma, St. Louis, MO) (500 μg i.p. every 2 days for 10 days, repeated once). DNMAML induction was monitored through GFP expression in blood Gr1$^+$CD11b$^+$ cells (routinely >95%). The efficiency of *Rbpj* excision was assessed by Southern blotting, as described in the Supplemental Experimental Procedures. Experimental protocols were approved by the University of Pennsylvania's Office of Regulatory Affairs and by the veterinary authorities of the Canton de Vaud, Switzerland (authorization 1099.3).

Constructs and Retroviruses

The GFP-expressing MigR1 and the dominant-negative DNMAML-GFP MSCV constructs (DNMAML) have been described previously (Weng et al., 2003; Maillard et al., 2004). High-titer retroviral supernatant was produced by transient transfection of 293T cells (Pui et al., 1999).

Flow Cytometry and Cell Sorting

A list of antibodies is provided in the Supplemental Experimental Procedures. Cells were sorted with a FACSAria (Becton Dickinson) or MoFlo (Cytomation, Fort Collins, CO). Analysis was performed on FACSCanto, LSR II (Becton Dickinson), or CyAn (Dako). Files were analyzed in FlowJo (Tree Star, San Carlos, CA). SP analysis was performed on an LSR II equipped with an ultraviolet laser, after incubation with Hoechst 33342 (Sigma), as described (Goodell et al., 1996).

BM Transduction and Transplantation

Retroviral transduction of 5-FU-treated BM cells and transplantation into irradiated (900 rads) recipients was performed as described (Pui et al., 1999).

Competitive HSC Transplantation

CD45.2$^+$ BM from poly(I:C)-induced *Mx-Cre*$^+$ × *ROSA*$^{DNMAML/+}$ or control *Mx-Cre*$^-$ littermates was mixed at a 1:1 ratio with competitor B6-SJL CD45.1$^+$ BM (10^6 cells each) and transplanted into lethally irradiated B6-SJL recipients (900 rads). For experiments involving *Rbpj*$^{f/f}$ mice, donor BM was T cell depleted prior to transplantation by using an anti-Thy1.2 (AT83) monoclonal antibody and rabbit complement (Saxon, UK). Mixed BM chimeras were prepared with irradiated B6-SJL recipients (1000 rads) treated 48 hr previously with 100 μg anti-NK1.1 monoclonal antibodies i.p. B6-SJL recipients were reconstituted with a 1:1 mixture (3×10^6 cells each) of poly(I:C)-induced B6-SJL CD45.1$^+$ BM and either poly(I:C)-induced CD45.2$^+$ *Mx-Cre*$^+$ RBPJ$^{f/f}$ or control MxCre$^-$ BM. For secondary transplantation, BM GFP$^+$ LSK cells were purified from recipients of MigR1 or DNMAML-transduced B6-SJL (CD45.1$^+$) BM at least 12 weeks after transplantation. Sorted LSK cells (500) were transplanted into lethally irradiated B6 recipients together with 2×10^5 competitor B6 BM cells (CD45.2$^+$). In limiting dilution analyses, decreasing numbers of tester LSK cells were used (500, 150, 50). Blood was analyzed 16 weeks after secondary transplantation. Engraftment was defined as >1% tester cells in Gr1$^+$CD11b$^+$ blood myeloid cells. The progenitor frequency was calculated with L-Calc software (StemCell Technologies).

OP9 Cultures

OP9-MigR1 (OP9) and OP9-DL1 cells were kindly provided by J.C. Zuniga-Pflucker (University of Toronto, Canada) and used as described (Schmitt and Zuniga-Pflucker, 2002). LSK progenitors (10^4) were seeded into 24-well plates containing a confluent stromal monolayer in the presence of mSCF (100 ng/ml), hFlt3L (5 ng/ml), and mIL-7 (1 ng/ml) (Peprotech, Rocky Hill, NJ). After 6–48 hr, progenitors were isolated on the basis of CD45 expression.

Quantitative RT-PCR

RNA was purified with the RNEasy Micro Kit (QIAGEN, Valencia, CA). cDNA was prepared with the Superscript II Kit (Invitrogen, Carlsbad, CA). Real-time PCR was performed with SYBRGreen or TaqMan PCR Master Mix and analyzed on ABI Prism 7900 system (Applied Biosystems, Foster City, CA). Primers and probes are listed in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and four figures and can be found with this article online at http://www.cellstemcell.com/cgi/content/full/2/4/356/DC1/.

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