Inflammation-Mediated Notch Signaling Skews Fanconi Anemia Hematopoietic Stem Cell Differentiation

Wei Du, Surya Amarachintha, Jared Sipple, Jonathan Schick, Kris Steinbrecher and Qishen Pang

*J Immunol* 2013; 191:2806-2817; Prepublished online 7 August 2013;
doi: 10.4049/jimmunol.1203474
http://www.jimmunol.org/content/191/5/2806

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/08/07/jimmunol.1203474.DC1

References
This article cites 52 articles, 17 of which you can access for free at:
http://www.jimmunol.org/content/191/5/2806.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Inflammation-Mediated Notch Signaling Skews Fanconi Anemia Hematopoietic Stem Cell Differentiation

Wei Du,* Surya Amarachintha,* Jared Sipple,* Jonathan Schick,* Kris Steinbrecher,† and Qishen Pang*‡

Hematopoietic stem cells (HSCs) can either self-renew or differentiate into various types of mature blood cells. Delineating the signaling pathways that regulate this choice of self-renewal versus differentiation remains an enduring challenge of foremost importance. One of the key regulators is Notch, an extracellular signal with a critical role in hematopoietic homeostasis (1). Gain or loss of Notch signaling components has been directly linked to multiple human disorders, including hematologic malignancies (2, 3). NF-κB is another known factor that controls cell fate, survival, and differentiation (4, 5). NF-κB is involved in various physiologic processes, including immunity, inflammation, development, and differentiation (4, 6). It has been shown that crossstalk between the canonical NF-κB and Notch signaling pathways can influence tissue homeostasis in certain cell types, including hematopoietic progenitor cells (7, 8).

Fanconi anemia (FA) is an inherited disorder characterized by genome instability and an extremely high cancer predisposition (9–11). FA is genetically heterogeneous, with 15 complementation groups identified thus far. The genes encoding the groups A (FANCA), B (FANCB), C (FANCC), D1 (FANCD1/BRCA2), D2 (FANCD2), E (FANCE), F (FANCF), G (FANCG), I (FANCI/ KIAA1794), J (FANCI/BRIP1), L (FANCL), M (FANCM), N (FANCN/PALB2), O (FANCO/RAD51C), and P (FANCP/SLX4) have been cloned (9–13). The biological function of these FA proteins has been subjected to intensive investigation. The studies demonstrate that eight of the FA proteins (groups A, B, C, E, F, G, L, and M) form a core complex that functions as an ubiquitin ligase. In response to DNA damage or DNA replication stress, the FA core complex monoubiquitinates two downstream FA proteins—FANCD2 and FANCI—which then recruit other downstream FA proteins, including FANCD1 (which is the breast cancer protein BRCA2), FANCJ, FANCN, and other DNA repair factors, to nuclear loci containing damaged DNA and consequently influence important cellular processes such as DNA replication, cell-cycle control, and DNA damage response and repair (12, 13).

Two of the most important clinical hallmarks of FA are bone marrow (BM) failure and progression to leukemia caused by HSC depletion and malignant transformation (14). Indeed, there are fewer HSCs in patients with FA and in knockout mice compared with normal controls (11, 12). In fact, HSCs from patients with FA or from knockout mice display severe defects in in vitro survival and in vivo repopulating (14–17). Although these hematologic phenotypes suggest a specific role of the FA pathway in hematopoiesis, the mechanisms by which FA proteins regulate HSC self-renewal and differentiation is unknown. In this study, we show that FA deficiency enhances Notch signaling in multipotential progenitors (MPPs), which is correlated with decreased phenotypic long-term HSCs and increased formation of MPP1 progenitors. Mechanistically, we show that FA deficiency in MPPs deregulates genes controlled by the NF-κB pathway, leading to enhanced Notch signaling. This study thus identifies a functional crosstalk between the NF-κB pathway and Notch signaling in HSC differentiation and establishes a role of FA proteins in the control of balance between...
renewal and lineage commitment. In addition, our data also lend support to the recent report that several members of FA core complex, including FANCA, Fancc, FANC G, and FANCL, functionally interact with HES1, a key player in the Notch signaling pathway (18).

Materials and Methods

Mice and treatment

Notch-eGFP/Fanca<sup>+/−</sup> or Notch-eGFP/Fancc<sup>−/−</sup> mice were generated by interbreeding the heterozygous Fanca<sup>+/−</sup> or Fancc<sup>−/−</sup> mice (19, 20) with Notch-eGFP transgenic mice (21). Notch-eGFP/Fanca<sup>+/−</sup>/p65<sup>−/−</sup> mice were generated by interbreeding the Rela/p65 (22) with Notch-eGFP, Fanca<sup>+/−</sup>/p65<sup>−/−</sup> gene deletion was accomplished by Cre-mediated deletion of floxed alleles by crossing the Notch-eGFP/Fanca<sup>+/−</sup>/p65<sup>−/−</sup> mice with a CreERT2 strain (23) and constituting 100 mg/ml tamoxifen i.p. (20 mg/ml; Sigma-Aldrich, St. Louis, MO) daily for 3 d. Animals were maintained in the animal barrier facility at Cincinnati Children’s Hospital Medical Center.

For in vivo treatment, mice were injected s.c. with the γ-secretase inhibitor DAPT in a range of doses (0, 10, 50, 100, and 200 mg/kg; Tocris Biosciences, Minneapolis, MN) daily for 2 d followed by BM cell isolation and mixed in BD Cytofix/Cytoperm Buffer (BD Biosciences) and then stained with Notch1 Ab (mN1A) according to the manufacturer’s instructions.

Flow cytometry analysis and cell cycle analysis

The lineage marker (Lin) mixture (BD Biosciences, San Jose, CA) for BM cells from Notch-eGFP-WT and Notch-eGFP-Fanca<sup>+/−</sup> or Notch-eGFP-Fancc<sup>−/−</sup> mice included the following biotinylated Abs: CD3e (145-2C11), CD11b (M1/70), CD45R-B220 (RA3-6B2), mouse erythrocyte cells Ly-76 (Ter119), Ly6G, and Ly-6C (RB6-8C5). Other conjugated Abs (BD Biosciences) were used for surface staining included: CD45.1 (A20), CD45.2 (A104), Scal (D7), c-kit (2B8), CD34 (RM34), Flt3 (A2F10.1), CD48 (HM48-1), CD150 (9D1), IL-7Rα (HIL-7R-M21). Biotinylated primary Abs were detected by incubation of Ab coated cells with streptavidin-PerCP Cy5.5 (BD Biosciences) in a two-step staining procedure.

Intracellular staining for the active Notch1 (NICD) was conducted as previously described (29). Surface marker–stained CD150<sup>−</sup>CD48<sup>−</sup>Lin<sup>−</sup>Scal<sup>−</sup>c-kit<sup>−</sup> (LSK) cells (LT-HSCs) from Notch-eGFP/Fanca<sup>+/−</sup> or Notch-eGFP-Fancc<sup>−/−</sup> mice pretreated with different doses of DAPT were first fixed and permeabilized with a Phosflow kit (BD Pharmingen, San Jose, CA) and then stained with 5 μg/ml Hoechst 33342 and 150 ng/ml Pyronin Y (Sigma-Aldrich). Cells were then subjected to Flow Cytometric analysis (BD Biosciences).

In vitro T cell differentiation assay

Four thousand LSK cells isolated from Notch-eGFP-Fanca<sup>+/−</sup> or Notch-eGFP-Fancc<sup>−/−</sup> mice were seeded to a 24-well plate precoated with OP9-DL1 conditioned media at a density of 4 × 10<sup>5</sup> cells/well and treated with indicated doses of DAPT were washed with ice-cold PBS and resuspended in ice-cold lysis buffer containing 50 mM Tris-HCL (pH 7.4), 10% FBS, and 1% NP40, and 1 M NaCl supplemented with protease and phosphatase.
inhibitors (10 μg/ml aprotinin, 25 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM PMSF, 0.1 M NaP-O4, 25 mM NaF, and 2 mM sodium orthovanadate) for 30 min on ice. Cell lysates were resolved on SDS-PAGE, and immunoblots were analyzed with Abs for the active form of Notch1 (NICD), Stat1 (Cell Signaling, Boston, MA), or β-actin (Sigma-Aldrich). Each lane contains protein from 50,000 Lineage-depleted cells (Miltenyi Biotec, Auburn, CA). Signals were visualized by incubation with anti-mouse or anti-rabbit secondary Abs followed by ECL (Amersham Biosciences, Piscataway, NJ).

Statistical analyses

Paired or unpaired Student t test was used for two-group comparison, and one-way ANOVA for more than two-group comparison; p < 0.05 was considered statistically significant. Results are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Enhanced Notch signaling in FA MPPs

To examine whether FA murine HSCs can balance self-renewal with differentiation, we crossed two FA mouse models (Fanca+/− and Fanca−/−) with a reporter strain in which Notch-driven eGFP expression acts as a sensor for HSC differentiation (21). We first used ImageStream to analyze Notch signaling in Notch-eGFP expression in Lin−Sca1+c-kit+ (LSK) cells, a population containing HSCs and multipotential progenitors. We observed higher Notch-eGFP expression in LSK cells from FA mice compared with wild type (WT) littermates (Fig. 1A). Consistent with this observation, flow cytometric analysis showed that, compared with WT counterparts, FA LSK cells expressed higher eGFP (Fig. 1B, 1D). It appeared that Notch-eGFP was also expressed in Lin−Sca1+c-kit− cells (Fig. 1C), a population consisting mostly of myeloid progenitors from mice of either genotype, albeit at lower levels than more primitive LSK cells. However, no difference in Notch-eGFP expression was observed between WT and FA Lin−Sca− c-kit− cells (Fig. 1C, 1D).

Because Notch signaling favors lymphoid while interfering with myeloid differentiation (31–34), we next determined whether increased Notch expression in FA LSK cells was associated with alteration in lineage differentiation. Surprisingly, we did not see reduced myeloid differentiation in FA mice. Specifically, when we examined the subsets in the stem–progenitor compartment further (i.e., granulocyte-macrophage progenitors, common myeloid

![FIGURE 1](http://www.jimmunol.org/Downloadedfrom/)

**FIGURE 1.** Increased Notch-eGFP expression in FA HSPCs. (A) eGFP+ LSK cells from Fanca−/− mice are brighter than those from WT. Whole BM cells from Notch-eGFP-Fanca+/− or Notch-eGFP-Fanca−/− mice were isolated followed by Lin− cell depletion. Lin−Sca1+c-kit+ (LSK) cells were labeled and subjected to ImageStream analysis at original magnification ×40. Representative images were shown (n = 5–7). (B) Enhanced Notch signaling in FA LSK cells. Whole BM cells from Notch-eGFP-Fanca−/−, Notch-eGFP-Fanca+/−, or WT littermates were isolated for cell surface marker staining. LSK cells were gated for eGFP expression analysis with flow cytometry. (C) No difference in Notch expression between FA and WT Lin−Sca1+c-kit+ cells. Cells described in (A) were gated for Lin−Sca1+c-kit− (Lin−S−K+) population followed by flow cytometry analysis for eGFP expression. (D) Quantification of eGFP expression in LSK and Lin−S−K+ cells. Results are means ± SD of three independent experiments (n = 12 per group). **p < 0.05, ***p < 0.01. (E and F) FA deficiency does not alter myeloid differentiation (E) or lymphoid differentiation (F). Cells described in (B) were subjected to flow cytometric analysis for myeloid or lymphoid progenitors. (G) FA deficiency does not affect T cell differentiation in vitro. Four thousand LSK cells isolated from either Fanca+/− or Fanca−/− mice were seeded to 24-well plates precoated with OP9-DL1 cells. Suspension cells were collected on days 14 and 21, followed by flow cytometric analysis for CD3, CD4, CD8, CD19, and γδTCR. Representative image (upper panels) and quantification (lower panels) are shown. Results are means ± SD of three independent experiments (n = 5–7 per group).
FIGURE 2. Enhanced Notch signaling in Fanca<sup>-/-</sup> MPPs. (A) Percentage of GFP<sup>+</sup> cells in LT-HSC and MPP populations. Whole BM cells from Notch-eGFP-Fanca<sup>+</sup>/+ or Notch-eGFP-Fanca<sup>-/-</sup> mice were isolated and stained for SLAM markers followed by flow cytometric analysis. Representative GFP expression (upper panels) from three cell populations—LT-HSC (Lin<sup>-</sup>Sca1<sup>-</sup>c-kit<sup>+</sup>CD150<sup>-</sup>CD48<sup>+</sup>), MPP1 (Lin<sup>-</sup>Sca1<sup>-</sup>c-kit<sup>-</sup>CD150<sup>-</sup>CD48<sup>-</sup>), or MPP2 (Lin<sup>-</sup>Sca1<sup>-</sup>c-kit<sup>-</sup>CD150<sup>-</sup>CD48<sup>-</sup>) and quantification (lower panels) were shown. Results are means ± SD of three independent experiments (n = 9 per group). (B) Intracellular expression levels of GFP in LT-HSC and MPP subsets. The levels of GFP expression from cell populations described in (A) were plotted for histogram display. (C) eGFP expression is positively correlated with active Notch1 in HSPCs. Whole BM cells from

(Figure legend continues)
progenitors, and megakaryocyte-erythrocyte progenitors), we saw no difference between WT and FA mice (Fig. 1E) although a slight increased portion of common lymphoid progenitors was observed in FA mice (Fig. 1F). Consistent with this finding, the frequencies of CD4+ single-positive T cells, CD8+ single-positive T cells, CD4+CD8+ double-positive T cells, and T cells bearing αβTCR+ or γδTCR+ cells derived from hematopoietic stem progenitor cells (HSPCs) at different stages of coculture with OP9-DL1 feeder cells in vitro (35) were similar between the genotypes (Fig. 1G). HSCs differentiate through intermediate stages, generating at least two subsets of multipotential progenitors (MPP1 and MPP2 (36). Only LT-HSCs have the ability to self-renew. Therefore, we subdivided the LSK fraction using signaling lymphocytic-activation molecule markers (SLAM; i.e., CD150 and CD48) (37). We observed lower LT-HSC (LSKCD150+CD48−) number, but higher MPP1 (LSKCD150+CD48+) cells in both FA−/− and Fanca+/+ mice than in WT mice (Fig. 1H). Therefore, a reduction in LT-HSCs is associated with an increase in the number of MPP1 cells in the BM of FA mice.

Because we observed higher Notch-eGFP expression in FA LSK cells than in WT LSK cells, we next analyzed Notch signaling in more defined HSPC populations. Surprisingly, WT and FA mice showed strikingly different patterns of Notch-eGFP expression in LT-HSCs versus MPPs. Specifically, the percentage of WT LT-HSCs expressing Notch-eGFP was significantly higher than that of Fanca−/− LT-HSCs (Fig. 2A). In contrast, FA MPPs expressed much higher Notch-eGFP than WT MPPs (Fig. 2A). This finding was confirmed by analyzing the intracellular level of Notch-eGFP expression in single cells from these subsets (Fig. 2B). Furthermore, intracellular staining using an Ab specific for the active Notch1 (NICD) (29, 38, 39) showed a positive correlation between eGFP expression level and active Notch signal in vivo (Fig. 2C). Thus, these results link Notch signaling to specific stages of HSPC frequencies.

Because loss of cellular quiescence contributes to HSC exhaustion (40), we next determined the cell cycle status of FA HSCs by PY/Hoechst staining. We noticed that both total and eGFP-positive LSK cells from FA mice showed significantly decreased G0 quiescent cells compared with those from WT mice (Fig. 2D).

To examine further the potential linkage between Notch signaling and HSC quiescence, we analyzed cell-cycle status of eGFP-positive LT-HSCs. Similar to the result obtained with the heterogeneous LSK cells, we observed significantly reduced quiescent Notch-eGFP+ LT-HSCs in FA mice compared with WT Notch-eGFP+ LT-HSCs (Fig. 2E). Therefore, the data indicate that Notch signaling fails to rescue loss of quiescence in FA HSCs.

Inverse correlation between Notch signaling and self-renewal capacity in FA HSCs

The observations that Notch-eGFP expression was low in FA LT-HSCs but high in FA MPP cells suggested that alteration in Notch signaling in FA LT-HSCs and MPP cells could influence their capacity to self-renew and differentiate. To test this hypothesis, we used FACS to divide the Notch-eGFP+ LSK population into Notch-eGFPhi (mean fluorescence intensity values: 49.41 ± 3.29 for Fanca+/+ and 68.59 ± 3.07 for Fanca−/−) and Notch-eGFPlo (mean fluorescence intensity values: 20.25 ± 2.68 for Fanca+/+ and 26.4 ± 4.72 for Fanca−/−) fractions (Fig. 3A) and determined the self-renewal capacity of these two populations. CFU assay showed that, although the first plating showed that both WT and the FA Notch-eGFPlo fractions had similar capacity to generate colonies, replying of the FA Notch-eGFPlo LSK cells yielded much fewer colonies (Fig. 3B), suggesting a possible loss of in vitro self-renewing capacity. To complement these in vitro studies, we performed competitive BM transplantation with 500 LSK cells purified from both fractions (expressing the marker CD45.2) along with 300,000 CD45.1+ competitor cells. Interestingly, we found that FA Notch-eGFPlo LSK cells still displayed defects in hematopoietic repopulation compared with the same number of WT Notch-eGFPhi LSK cells (Fig. 3C).

This result prompted us to reevaluate the LSK population used to identify engrafting HSCs by dividing the LT-HSCs and MPP1 population into Notch-eGFP− and Notch-eGFP+ cells (Fig. 4A). As shown in Fig. 4B, eGFP+ LT-HSCs from Fanca−/− mice showed reduced CFUs compared with WT eGFP+ LT-HSC in the second plating, indicating an impaired in vitro self-renewal capacity. In contrast, Fanca−/− eGFP+ LT-HSCs show a colony-forming ability comparable to their WT counterparts in both platings (Fig. 4B). On the other hand, both eGFP+ and eGFP− MPP1 cells from Fanca−/− mice showed defective colony-forming capacity in the second plating (Fig. 4B). These results suggest that Notch signaling has distinct roles in self-renewal of LT-HSCs and MPP cells in vitro.

To examine the in vivo role of Notch signaling in self-renewal of LT-HSCs and MPP cells, we transplanted 50 LT-HSCs or 1000 MPP1 cells from each of the two fractions into lethally irradiated recipients to investigate their repopulation potential. Fanca−/− Notch-eGFP+ LT-HSCs showed a typical repopulating deficit at each time point after transplantation (Fig. 4C, 4D). The results of a previous report (19), WT Notch-eGFP+ LT-HSCs exhibited enhanced long-term repopulating capacity. However, Fanca−/− Notch-eGFP+ LT-HSCs displayed quantitatively defective long-term repopulating activity with less than 20% donor-derived cells, compared with more than 50% repopulated by WT Notch-eGFP+ LT-HSCs at 16 and 24 wk after transplantation (Fig. 4C, 4D). All LT-HSCs, regardless of genotype status or Notch-eGFP expression, generate HSPCs and all mature lineages 24 wk after transplantation (Supplemental Fig. 1). Neither WT nor FA MPP1 cells, regardless of Notch-eGFP expression, provided long-term reconstitution (Fig. 4C, 4D). In fact, Notch-eGFP+ FA MPP1 progenies were almost undetectable at 4 wk after transplantation (Fig. 4C, 4D). These results indicate that Notch signaling fails to rescue repopulating defect of FA LT-HSCs and impairs the short-term repopulating ability of FA MPPs.

FA deficiency in MPPs deregulates the gene network of Notch and NF-κB pathways

To understand further the effect of enhanced Notch signaling mechanistically in FA MPPs, we purified MPP1 subsets and used pathway-specific gene array analysis to define the gene-expression signatures of each population (Supplemental Fig. 2A). Because FA cells have defects in DNA repair, Cell-cycle control, antioxidant defense, inflammatory response, and apoptotic signaling, we focused on these pathways as well as the Notch signaling pathway.
FIGURE 3. Self-renewal defect in Fanca−/− Notch-eGFPlo HSPCs. (A) Flow-based purification of GFPlo and GFPhi subsets. The upper 30% of Notch-eGFP LSK cells (GFPhi) or lower 30% of Notch-eGFP LSK cells (GFPlo) were sorted from mice with the indicated genotypes. Representative flow images (left panel) and histogram overlay (right panel) are shown. (B) Replating defects of Notch-eGFPlo LSK cells. One hundred GFPlo or GFPhi LSK cells were seeded in duplicate and cultured in cytokine-supplemented methylcellulose medium for 7 d. Colonies were then counted, isolated, replated, and cultured for an additional 7 d. Error bars indicate SD of three experiments (n = 9). (C) Hematopoietic reconstitution defects of Fanca−/− Notch-eGFPlo LSKs. Five hundred GFPlo or GFPhi LSK (CD45.2+) cells plus 300,000 cells (CD45.1+) were injected via the tail vein into lethally irradiated recipient (BoyJ) mice. Donor-derived chimera was assessed by flow cytometric analysis 16 wk after BM transplantation. Representative dot (Figure legend continues)
A substantial increase in the expression of genes involved in the NF-κB and Notch signaling pathways was observed in the Fanca<sup>−/−</sup> or Fancc<sup>−/−</sup> MPP1 cells (Table II; Supplemental Fig. 2B, 2C). These genes include NF-κB target genes \textit{Gadd45b}, \textit{Icam1}, \textit{Irf1}, \textit{Rela} (p65), \textit{Sod2}, \textit{Stat1}, \textit{Tgfrsf1b} (TNF receptor II) and \textit{Xiap}, as well as Notch signature genes \textit{Hes1}, \textit{Hey1}, \textit{Hoxb4}, \textit{Fzd1}, \textit{Fzd2}, \textit{Nfkb1}, \textit{Notch1}, \textit{Notch2}, and \textit{Numb} (Supplemental Fig. 2B, 2C).

**FIGURE 4.** Inverse correlation between Notch signaling and self-renewal capacity of Fanca<sup>−/−</sup> HSPCs. (A) Flow-based purification of GFP<sup>−</sup> and GFP<sup>+</sup> subsets. GFP<sup>−</sup> and GFP<sup>+</sup> cells from LT-HSC or MPP1 compartments of Notch-eGFP-Fanca<sup>−/−</sup> or Notch-eGFP-WT mice were sorted. Representative images (left panels) and histogram overlay (right panels) were shown. GFP<sup>−</sup> and GFP<sup>+</sup> subsets were then subjected to CFU assay (B) and BM transplantation (C). (B) Increased Notch signaling results in replating defect in Fanca<sup>−/−</sup> MPPs. Twenty LT-HSCs or 200 MPP1 cells were seeded in duplicated and were cultured in cytokine-supplemented methylcellulose medium (MethoCult 3434). Subsequently, colonies were counted on day 7 and then isolated, replated, and cultured for another 7 d. Error bars indicate SD of three experiments (n = 9). (C) Increased Notch signaling leads to self-renewal defects in Fanca<sup>−/−</sup> HSCs. Fifty LT-HSCs or 1000 MPP1 (CD45.2<sup>+</sup>) cells plus 300,000 cells (CD45.1<sup>+</sup>) were injected via the tail vein into lethally irradiated recipient (BoyJ) mice. Donor-derived chimera was assessed using flow cytometric analysis at 4, 8, 16, and 24 wk after transplantation. (D) Quantification of self-renewal capacity of Fanca<sup>−/−</sup> HSCs. Experiments described in (C) were quantified. Results are means ± SD of three independent experiments (n = 9 per group). *p < 0.05, **p < 0.01, ***p < 0.001.
Inflammatory stress–activated Notch signaling compromises HSC self-renewal

Because our gene expression analysis suggests a potential NF-κB-Notch crosstalk in FA HSPC cells and because FA HSPC cells are hypersensitive to inflammatory stress (41), we next determined whether TNF-α, a NF-κB activator and a major inflammation mediator in FA (42), would enhance Notch signaling in FA HSPCs. To this end, we treated Notch-eGFP-Fanca<sup>−/−</sup> mice and Notch-eGFP-WT littermates with TNF-α, and we examined Notch-eGFP expression in BM LSK cells. Interestingly, we found that TNF-α affected eGFP-positive HSPCs in both WT and FA mice. However, the effect of TNF-α was exacerbated significantly in FA mice compared with WT mice. Specifically, the frequency of GFP<sup>+</sup> LSK cells was increased further in TNF-α–treated Notch-eGFP-Fanca<sup>−/−</sup> mice (Fig. 5A). This increase was associated with a further decrease in HSC quiescence (Fig. 5B). To substantiate these phenotypic analysis, we performed competitive BM transplantation by injecting 500 GFP<sup>+</sup> LSK or GFP<sup>+</sup> LSK cells isolated from the mice treated with TNF-α along with 300,000 congenic WT BM cells into lethally irradiated recipients and determined donor-derived chimera 24 wk after transplantation. We observed dramatically increased impairment in hematopoietic repopulation of GFP<sup>+</sup> LSK cells from TNF-α–treated Notch-eGFP-Fanca<sup>−/−</sup> mice (Fig. 5C). In fact, donor-derived cells from recipients transplanted with TNF-α–treated FA eGFP<sup>+</sup> LSK cells were almost undetectable at 24 wk after transplantation (Fig. 5C). It is also noteworthy that Notch-eGFP<sup>+</sup> HSPCs, regardless of genotypes, were more vulnerable to TNF-α treatment than Notch-eGFP<sup>+</sup> HSPCs in the context of long-term hematopoietic repopulation (Fig. 5C). To assess whether the enhanced expression of Notch-eGFP in TNF-α–treated Fanca<sup>−/−</sup> mice required canonical NF-κB signaling, we crossed Notch-eGFP-Fanca<sup>−/−</sup> mice with mice carrying a conditional gene encoding the p65 subunit of NF-κB (p65<sup>fl/fl</sup>) (22). Inducible deletion of p65 was accomplished by Cre-mediated deletion of floxed p65 alleles using a Cre-ERT2 strain (23) and tamoxifen (Supplemental Fig. 3). We found that inactivation of p65 resulted in attenuation of Hes1 and Hey1 expression in LSK cells from TNF-α–treated Notch-eGFP–positive WT and Fanca<sup>−/−</sup> mice (Fig. 5D). Furthermore, p65 deletion caused a significant reduction in Notch target gene expression in untreated Notch-eGFP-positive Fanca<sup>−/−</sup> LSK cells (Fig. 5D).

Table II. Increased expression of genes involved in the NF-κB pathway and Notch signaling pathway

<table>
<thead>
<tr>
<th>Pathway-Specific Array</th>
<th>Symbol</th>
<th>Accession No.</th>
<th>Fold Upregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NF-κB signaling pathway</td>
<td>Ccr5</td>
<td>NM_009917</td>
<td>3.04</td>
</tr>
<tr>
<td></td>
<td>Gadd45b</td>
<td>NM_008655</td>
<td>8.79</td>
</tr>
<tr>
<td></td>
<td>Icam1</td>
<td>NM_010493</td>
<td>6.26</td>
</tr>
<tr>
<td></td>
<td>Irf1</td>
<td>NM_008390</td>
<td>4.84</td>
</tr>
<tr>
<td></td>
<td>Rela</td>
<td>NM_009045</td>
<td>6.17</td>
</tr>
<tr>
<td></td>
<td>Sod2</td>
<td>NM_013671</td>
<td>9.10</td>
</tr>
<tr>
<td></td>
<td>Stat1</td>
<td>NM_009283</td>
<td>9.96</td>
</tr>
<tr>
<td></td>
<td>Tnfrsf1b</td>
<td>NM_001610</td>
<td>5.37</td>
</tr>
<tr>
<td></td>
<td>Xiap</td>
<td>NM_009688</td>
<td>8.91</td>
</tr>
<tr>
<td>Notch signaling pathway</td>
<td>Fzd1</td>
<td>NM_021457</td>
<td>5.71</td>
</tr>
<tr>
<td></td>
<td>Fzd2</td>
<td>NM_020510</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td>Hes1</td>
<td>NM_008235</td>
<td>5.79</td>
</tr>
<tr>
<td></td>
<td>Hey1</td>
<td>NM_010423</td>
<td>5.79</td>
</tr>
<tr>
<td></td>
<td>Hoxb4</td>
<td>NM_010459</td>
<td>3.79</td>
</tr>
<tr>
<td></td>
<td>Nfkbi</td>
<td>NM_008689</td>
<td>3.59</td>
</tr>
<tr>
<td></td>
<td>Notch1</td>
<td>NM_008714</td>
<td>6.52</td>
</tr>
<tr>
<td></td>
<td>Notch2</td>
<td>NM_010928</td>
<td>9.81</td>
</tr>
<tr>
<td></td>
<td>Numb</td>
<td>NM_010949</td>
<td>3.28</td>
</tr>
<tr>
<td></td>
<td>Pdpk1</td>
<td>NM_011062</td>
<td>3.52</td>
</tr>
</tbody>
</table>

These results suggest that inflammatory stress activates Notch signaling and compromises hematopoietic repopulating capacity of HSPCs.

Inhibition NF-κB or Notch partially restores FA HSPC function

To determine the functional relevance of Notch–NF-κB crosstalk in FA HSPCs, we sought to test whether inhibition of NF-κB or Notch signaling could restore FA HSPC function. We took two approaches on NF-κB inhibition: genetic ablation of p65, a subunit of NF-κB transcription complex (22), and pharmacologic inhibition of NF-κB. We found that p65 deletion in MPP1 cells of Notch-eGFP-Fanca<sup>−/−</sup> mice reduced Notch expression to nearly WT levels (Fig. 6A). To substantiate this finding further, we used BAY11-7082, an inhibitor of cytokine-induced IκB phosphorylation (28). Consistent with p65 knockout, BAY11-7082 treatment also significantly reduced Notch-eGFP expression in FA MPP1 cells (Fig. 6A). Phenotypic analysis of the HSPC subsets in the LSK compartment revealed that inhibition of NF-κB by either p65 deletion or BAY11-7082 treatment increased LT-HSCs and decreased MPP1 cells in Fanca<sup>−/−</sup> mice to near WT levels (Fig. 6B). Thus, these data provide molecular evidence that links Notch–NF-κB crosstalk to regulation of FA HSC differentiation.

To determine the functional consequence of inhibiting NF-κB and Notch signaling in FA HSPCs, we blocked either NF-κB by conditional deletion of p65 in Fanca<sup>−/−</sup> mice or Notch signaling by treating the mice with the γ-secretase inhibitor DAPT, which inhibits Notch signaling (24, 25), and we assessed cell cycle status and repopulating capacity of HSPCs. Deletion of p65 partially rescued the loss of quiescence of Fanca<sup>−/−</sup> but not WT HSPCs (Fig. 6C). For DAPT treatment, we found a dose-dependent (up to 100 mg/kg body weight) inhibition of Notch signaling evidenced by a decreased generation of the active form of Notch 1 (NICD) without affecting non-Notch signaling protein Stat1, which paralleled a decreased level of eGFP-positive MPP cells in Fanca<sup>−/−</sup> mice (Supplemental Fig. 4A, 4B). Consistent with this finding, mice treated with DAPT at the dose of 100 mg/kg body weight showed effective inhibition of the expression of Notch target genes without affecting NF-κB transactivation in MPP cells (Supplemental Fig. 4C). Consequently, cell-cycle analysis demonstrated that DAPT treatment restored quiescence of LSK cells from Fanca<sup>−/−</sup> mice (Fig. 6C). Furthermore, LSK cells from Fanca<sup>−/−</sup> mice subjected to p65 deletion exhibited a significant increase in long-term hematopoietic repopulation when transplanted to lethally irradiated recipients compared with untreated controls (Fig. 6D). A notable increase, albeit not statistically significant, in hematopoietic repopulation was also observed in DAPT-treated Fanca<sup>−/−</sup> LSK cells. These results indicate that blocking the NF-κB or Notch signal can partially correct the functional defect of FA HSPCs.

Discussion

The current study identifies a potential interaction between the FA pathway and Notch signaling in HSC differentiation and establishes a role of FA proteins in the control of balance between renewal and lineage commitment. There are several findings that highlight the significance of our study: 1) loss of murine FA proteins results in enhanced Notch signaling in MPPs, which is correlated with decreased phenotypic and functional LT-HSCs and increased formation of MPP1 progenitors; 2) deletion of the Fanca or Fancc gene deregulates genes in the Notch signaling and the NF-κB pathway; 3) TNF-α stimulation enhances Notch signaling in Fanca<sup>−/−</sup> and Fancc<sup>−/−</sup> LSK cells, leading to decreased HSC quiescence and compromised HSC self-renewal; 4) inflammation-
Notch signaling is activated in Fanca<sup>−/−</sup> and Fancc<sup>−/−</sup> MPP cells requires NF-κB; genetic ablation or pharmacologic inhibition of NF-κB reduces Notch signaling in FA MPPs to near WT levels and significantly increases LT-HSCs and decreases MPP1 cells in FA mice; and blocking either NF-κB or Notch signaling partially restored FA HSC quiescence and self-renewal capacity.

Several previous studies also suggest a crosstalk between the FA proteins and Notch targets. Tremblay et al. (18, 43) identified the interaction between components of the FA core complex and HES1, a Notch target that participates in many of the Notch-assigned functions including proliferation, differentiation, apoptosis, self-renewal, and asymmetric cell division regulation. Another study demonstrated that FA core complex interacts with HES1 and an-
FIGURE 6. Inhibition of NF-κB partially rescues FA HSC phenotype. (A) Inhibition of NF-κB reduces Notch-eGFP expression in Fanca<sup>−/−</sup> MPP1 to nearly WT levels. Whole BM cells from genetic ablation (3 d vehicle- or tamoxifen-injected Notch-eGFP-Fanca<sup>+/+</sup>p65<sup>f/f</sup>-CreER or Notch-eGFP-Fanca<sup>−/−</sup>p65<sup>f/f</sup>CreER mice) or pharmacologic inhibition (BAY 11-7082-treated Notch-eGFP-Fanca<sup>+/+</sup> or Notch-eGFP-Fanca<sup>−/−</sup>mice) of NF-κB were isolated for flow cytometric analysis. MPP1 cells were gated for GFP expression. Representative flow graphs (left panel) and quantification (right panel) are shown. Results are means ± SD of three independent experiments (n = 9 per group). (B) Inhibition of NF-κB increases LT-HSCs and decreases MPP1 in Fanca<sup>−/−</sup> mice. Cells described in (A) were subjected to SLAM analysis using flow cytometry. Representative flow graphs (left panel) and quantification (right panel) are shown. (Figure legend continues)
Inhibition of NF-κB

300,000 cogenic WT BM cells (CD45.1+) were transplanted to lethally irradiated recipients. Donor-derived chimerism was determined 24 wk after BM transplantation. Representative flow graphs (left panel) and quantification (right panel) were shown. Results are means ± SD of three independent experiments (n = 9 per group). (C) Inhibition of NF-κB or Notch partially restores HSPC quiescence. BMCs from genetic ablation (3 d vehicle- or tamoxifen-injected Notch-eGFP-Fanca−/− or DAPT-treated Notch-eGFP-Fanca−/− mice) or DAPT-treated Notch-eGFP-Fanca−/− mice were isolated for cell cycle analysis. Representative flow graphs (left panel) and quantification (right panel) were shown. Results are means ± SD of three independent experiments (n = 9 per group). (D) Inhibition of NF-κB or Notch partially restores HSPC self-renewal capacity. Five hundred LSK cells (CD45.2+) from mice described in (C) along with 300,000 cogenic WT BM cells (CD45.1+) were transplanted to lethally irradiated recipients. Donor-derived chimerism was determined 24 wk after BM transplantation. Representative flow graphs (left panel) and quantification (right panel) were shown. Results are means ± SD of three independent experiments (n = 9 per group). *p < 0.05.

Recent evidence of interaction between Notch and NF-κB suggests that the crosstalk between these two important signaling pathways could influence tissue homeostasis (7, 8, 51). On the one hand, it has been shown that transcriptional repressor CBF1, the essential transcription factor downstream in Notch signaling, interacts with a dual NF-κB/CBF1-binding site (κB2) in the IκBα promoter and de-represses IκBα gene transcription, leading to NF-κB activation in hematopoietic progenitor cells (8, 52). On the other hand, it was found that activation of the canonical NF-κB pathway by TNF-α stimulation induced optimal expression of Notch targets, such as Hes1 and Hey1, through a mechanism involving epigenetic activation of the Notch gene promoters by TNF-α stimulation (7). In this study, we provide several lines of evidence demonstrating the crosstalk between NF-κB and Notch pathways in the regulation of HSC maintenance. First, we found that TNF-α stimulation enhanced Notch signaling in Fanca−/− and Fancc−/− MPP cells to near WT levels, and it significantly increases the proportion of quiescent HSCs toward a less quiescent state, leading to impaired stem cell function.

Recent evidence of interaction between Notch and NF-κB suggests that the crosstalk between these two important signaling pathways could influence tissue homeostasis (7, 8, 51). On the one hand, it has been shown that transcriptional repressor CBF1, the essential transcription factor downstream in Notch signaling, interacts with a dual NF-κB/CBF1-binding site (κB2) in the IκBα promoter and de-represses IκBα gene transcription, leading to NF-κB activation in hematopoietic progenitor cells (8, 52). On the other hand, it was found that activation of the canonical NF-κB pathway by TNF-α stimulation induced optimal expression of Notch targets, such as Hes1 and Hey1, through a mechanism involving epigenetic activation of the Notch gene promoters by TNF-α stimulation (7). In this study, we provide several lines of evidence demonstrating the crosstalk between NF-κB and Notch pathways in the regulation of HSC maintenance. First, we found that TNF-α stimulation enhanced Notch signaling in Fanca−/− and Fancc−/− MPP cells to near WT levels, and it significantly increases the proportion of quiescent HSCs toward a less quiescent state, leading to impaired stem cell function.
References


