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The Notch2–Jagged1 interaction mediates stem cell factor signaling in erythropoiesis

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Stem cell factor (SCF), the ligand for the c-kit receptor, is essential for the production of red blood cells during development and stress erythropoiesis. SCF promotes erythroblast proliferation and survival, while delaying erythroid differentiation through mechanisms that are largely unknown. In cultures of primary human differentiating erythroblasts, we found that SCF induces an increase in the expression of Notch2, a member of the Notch family implicated in the control of cell growth and differentiation. Functional inhibition of either Notch or its ligand Jagged1 inhibited the effects of SCF on erythroid cell expansion. SCF also induced the expression of Hes-1 and GATA-2, which may contribute to transduce Notch2 signals in response to SCF. Transduction of primary erythroid precursors with a dominant-negative Notch2 mutant inhibited both basal and SCF-mediated erythroblast expansion, and counteracted the effects of SCF on erythroblast differentiation. These findings provide a clue to understand the effects of increased proliferation and delayed differentiation elicited by SCF on the erythroid compartment and indicate Notch2 as a new player in the regulation of red cell differentiation.

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Stem cell factor (SCF) is a cytokine produced by bone marrow stromal cells that stimulates the expansion of hematopoietic stem and progenitor cells.¹ SCF is specifically essential for erythroid cell development, as mice defective for SCF or its receptor c-kit die before or shortly after birth due to severe macrocytic anemia.^{1–4} We and others have shown that SCF promotes erythroblast expansion, retards differentiation and protects erythroid precursors from apoptosis.^{5–7} Among the effectors activated by SCF in differentiating erythroid cells, p38, ERK and Bcl-2/Bcl-XL have been implicated in stimulating erythroblast survival and proliferation.^{7,8} However, the mechanisms through which SCF modulates erythroid differentiation remain mostly unclear.

Notch proteins are a family of highly conserved transmembrane receptors that regulate cell-fate decisions, proliferation, differentiation and survival. Notch receptors are synthesized as 300-kDa precursors that are cleaved by furin-like convertases in the *trans*-Golgi compartment. The resulting N-terminal and C-terminal fragments are assembled into mature heterodimers and exported to the cell surface, where Notch signal transduction is initiated by ligand binding and endocytosis. Subsequent cleavage by ADAM10/TACE/Kuz/SUP-17 and by the γ -secretase complex leads to the release of the Notch intracellular signal-transducing fragment named Notch intracellular domain (NICD). NICD then translocates to the nucleus, where it displaces co-repressors associated with the DNA-binding transcription factor CSL (CBF-1/SuH/Lag-1)

and forms a transcriptionally active complex with CSL, Mastermind and co-activators leading to activation of Notch target genes.9 In the hematopoietic system, Notch family members are expressed on both human and murine hematopoietic stem cells (HSCs), where they promote cell expansion, self-renewal and immortalization.¹⁰ Although canonical Notch signaling has been shown to be dispensable for HSC maintenance,¹¹ the Notch family still has a complex role in the hematopoietic system by way of modulating cell proliferation, survival and differentiation into multiple lineages. The clearest effects of the Notch system can be observed during lymphopoiesis, wherein Notch activation favors the CD8 and $\alpha\beta$ cell-fate decisions.¹² In the myeloid system, Notch signaling has been reported to modulate differentiation in vitro and in vivo, with cell context-dependent effects that vary between different experimental models and conditions.¹³ Several in vitro studies have associated Notch signaling with an inhibition of erythroid, granulocytic or megakaryocytic differentiation.^{14–16} However, Notch has also been reported to increase erythroid maturation or proliferation, to induce monocyte death, or to promote megakaryocytic maturation.17-20 In vivo studies on Notch function in the hematopoietic system have been precluded for a long time because of the embryonic lethal phenotype of mice being homozygously deficient in Notch1 or Notch2.21,22 Later, an inducible Notch1 knockout mouse model revealed an essential role for Notch1 in T-cell lineage induction while leaving all the other

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Abbreviations: SCF, stem cell factor; NICD, Notch intracellular domain; HSC, hematopoietic stem cells; GpA, Glycophorin A; Notch2 Intra, intracellular Notch2 mutant; Notch2 Extra, extracellular Notch2 mutant; Notch2 FL, full-length Notch2; GFP, green fluorescent protein; 7-AAD, 7-amino-actinomycin D; HPC, hematopoietic progenitor cell; RV, retroviral vector; C/G, copies per genome; BASO, basophilic erythroblasts; POLY, polychromatophilic erythroblasts; ORTHO, orthochromatic erythroblasts; MFI, mean fluorescence intensity; JAG1, Jagged1

hematopoietic lineages unaltered.¹² In apparent contradiction, mice deficient for the Notch mediator RBP-Jk were found to generate excessive numbers of erythroid cells in the yolk sac, suggesting that the Notch pathway regulates erythroid homeostasis through elimination of developing erythroblasts.²³ More recently, multilineage effects of Notch signaling on myeloid homeostasis were observed in fucosylationdeficient mice, which display a Notch-dependent increase in granulocytic cells but a decrease in bone marrow TER119⁺ cells and circulating erythrocytes.²⁴

As Notch family members are known to modulate the differentiation of hematopoietic progenitor cells, we investigated whether SCF could modulate the expression of Notch in erythroid progenitors and precursors. We found that SCF induced the expression of the Notch family member Notch2 in differentiating erythroblasts. Interfering with Notch function by γ -secretase inhibitor treatment or expression of a dominant-negative Notch2 mutant in primary erythroid precursors resulted in decreased erythropoiesis, indicating a necessary role of Notch2 in mediating SCF effects on erythroblast proliferation and differentiation. Altogether, these results show a new mechanism that involves SCF and Notch to control erythropoiesis, which may contribute to maintain postnatal erythroid homeostasis.

Results

SCF modulates the proliferation and differentiation of primary human erythroblasts. To investigate the mechanisms responsible for SCF's effects on erythroid proliferation and differentiation, we took advantage of a serum-free liquid culture system that allows the production of virtually pure populations of differentiating erythroblasts starting from CD34⁺ hematopoietic progenitors (Figure 1a).^{7,25,26} Erythroblasts grown in these culture conditions gradually



Figure 1 SCF stimulates the proliferation and delays the differentiation of primary erythroblasts in unilineage culture. CD34⁺ cells derived from the peripheral blood of healthy individuals were cultured in standard erythroid medium to obtain a pure population of differentiating erythroid cells. (a) May–Grünwald–Giemsa staining of erythroblast populations at different days of culture. (b) Expression of c-kit and Glycophorin A in erythroblasts at different days of culture. (c) Effect of SCF on erythroblast proliferation. Cells were cultured in standard erythroid medium with (SCF) or without (Untreated) 100 ng/ml SCF. Statistically significant differences between untreated and treated samples are indicated as **P < 0.01 and ***P < 0.001. (d) Effect of SCF on erythroblast differentiation. Erythroblasts were cultured in the presence (SCF) or in the absence (Untreated) of 100 ng/ml SCF statistically significant differentiation stage was evaluated by morphological analysis (left panel), where the differences between untreated and treated samples are indicated as **P < 0.001. Cells were stained with May–Grünwald–Giemsa (central panels) and the differentiation stage was evaluated by morphological analysis (left panel), where the differences between untreated and treated samples are indicated as **P < 0.01 and ***P < 0.001. Cells were also stained with anti-Glycophorin A (right panels) are representative of at least four experiments performed with cells from different donors, whereas the results shown in **c** and **d** (left panels) are means ± S.D. of four independent experiments. Abbreviations: BASO, basophilic erythroblasts; MFI, mean fluorescence intensity; POLY, polychromatophilic erythroblasts; ORTHO, orthochromatic erythroblasts

become >90% positive for c-kit expression, whereas this expression tends to decrease during the later stages of maturation (Figure 1b). Conversely, Glycophorin A (GpA) begins to be expressed around day 3 of differentiation. reaching $98 \pm 2\%$ positivity at day 14 of culture (Figure 1b). The addition of SCF to erythroblast cultures results in a large increase in cell proliferation (Figure 1c) with a concomitant delay of erythroid differentiation that manifests with the prevalence of immature basophilic erythroblasts at advanced stages of culture and expression of lower levels of GpA (Figure 1d).

SCF induces Notch2 expression ervthroid in precursors. The mechanisms used by SCF to increase the proliferation and retard the differentiation of hematopoietic precursors are scarcely understood. As Notch family members have an important role in maintaining the undifferentiated state of hematopoietic stem cells, we investigated whether SCF could modulate Notch proteins to exert its effects on the immature erythroid compartment. Therefore, we investigated the expression of Notch1 and Notch2, the main members of the Notch family expressed in the hematopoietic system, on cultures of primary erythroblasts untreated or treated with SCF. A short (days 6-8) treatment with SCF was chosen to investigate the ability of this cvtokine to modify Notch expression in the absence of major alterations of erythroid differentiation. Erythroblast treatment with SCF decreased Notch1 RNA expression while increasing Notch2 RNA (Figure 2a). Notch1 protein levels remained unaltered upon SCF treatment, whereas both the mature and immature forms of Notch2 increased significantly (Figure 2b). To verify that the increase in Notch2 expression induced by SCF stimulation in ervthroid precursors was not due to the presence of a less mature ervthroblast population. we stained erythroid precursors with anti-Notch2/anti-GpA antibodies at day 8 of culture, untreated or treated for 2 days with SCF. Such staining confirmed Notch2 induction by SCF, with the increase in Notch2 expression detected by FACS staining (1 log fluorescence scale) being coherent with that detected by western blot (~ 10 times fold). Importantly, however, simultaneous GpA staining did not reveal major differences in GpA expression following the short-term treatment with SCF, indicating that the SCF-induced increase in Notch2 was due to protein induction and not



Figure 2 SCF induces Notch2 expression in erythroid precursors. CD34⁺ cells were cultivated for 6 days in standard erythroid medium to generate differentiating erythroblasts, which were treated for 2 days (until day 8 of culture) with SCF 100 ng/ml and then processed for (a) reverse-transcriptase PCR analysis, (b) western blotting and (c) flow cytometry analysis. Lower panels in a and b represent the quantification (mean ± S.D.) of bands obtained in three independent experiments. (d) Flow cytometry analysis of Notch2 expression at different days of unilineage envthroid culture. The panel on the lower right (293T N2FL) shows a positive control of Notch2 staining represented by 293T embryonic kidney cells transfected with pCDNA3 Notch2 full length. Flow cytometry profiles shown in c and d are representative of three experiments performed with cells from different donors. (e) Erythroblasts at day 4 of differentiation were cultivated for 4 days in standard erythroid medium in the presence or absence of 5 µM γ-secretase inhibitor L685,458 and/or 100 ng/ml SCF as indicated. Bars represent the mean ± S.D. of the number of cells counted at day 8 and expressed as fold increase versus the untreated sample. The difference between samples treated with SCF alone or with SCF + L685,458 was statistically significant with *P<0.05, calculated over three independent experiments

due to a differentiative shift (Figure 2c). In line with a prevalent role of Notch2, but not of Notch1, in the modulation of erythroid differentiation, we found that Notch2 expression progressively increased, peaking at around days 5-7 of erythroid unilineage culture (Figure 2d), whereas Notch1 protein expression progressively decreased during erythroid maturation as compared with the levels found in CD34⁺ hematopoietic progenitors (Supplementary Figure 1a). Erythroblast response to inhibition of the Notch system was subsequently investigated at days 4-8 of unilineage culture, when high Notch2 expression reflects the pro-ervthroblast/ basophilic ervthroblast stage of the vast majority of cells, which possess a high proliferation potential and the homeostasis of which is therefore particularly susceptible by modulation through external stimuli. To investigate whether Notch modulation interfered with the functional effects of SCF stimulation, we treated erythroid precursors with SCF in the presence or absence of L-685,458, an

374

inhibitor of γ -secretase that is known to inhibit the production of functional Notch proteins. While a short-term (days 4–8) treatment with L-685,458 alone did not significantly interfere with basal erythroid proliferation, γ -secretase inhibition interfered with SCF-induced cell expansion (Figure 2e). Longer treatments with L-685,458 resulted in cell toxicity (data not shown).

The Notch ligand Jagged1 is expressed during erythropoiesis and contributes to SCF-mediated erythroblast expansion. To identify the Notch ligand potentially responsible for Notch2 binding and activation, we investigated the expression of four Notch ligands, Jagged1, Jagged2, Delta-like1 and Delta-like3, in differentiating erythroid cells. We found that only Jagged1 RNA was expressed at detectable levels throughout erythroid maturation (Figure 3a), whereas the other ligands showed very low or absent RNA expression (Supplementary



Figure 3 Jagged1 is expressed during erythropoiesis and is involved in SCF-mediated cell expansion. (a) Real-time PCR analysis of Jagged1 (JAG1) expression at different days of unilineage erythroid culture. Bars represent the mean \pm S.D. of three independent experiments. Peripheral blood lymphocytes (PBLs) were used as positive control. (b) Western blot analysis of Jagged1 (JAG1) expression at different days of unilineage erythroid culture, untreated or previously treated for 2 days with 100 ng/ml SCF. (d) Western blot analysis of Jagged1 expression in erythroblasts treated as in **c**. The upper panel represents the quantification of three independent experiments. (e) Erythroblasts at day 4 of differentiation were cultivated for 4 days in standard erythroid medium in the presence or absence of 15 μ g/ml anti-Jagged1 neutralizing antibody and/or 100 ng/ml SCF as indicated. Bars represent the mean \pm S.D. of the number of cells counted at day 8 and expressed as fold increase *versus* the untreated sample. The difference between samples treated with SCF alone or SCF + anti-JAG1 was statistically significant with **P*<0.05, calculated over three independent experiments



Figure 4 Hes-1 and GATA-2 levels increase upon SCF stimulation of differentiating erythroblasts. CD34 + cells were cultivated for 6 days in standard erythroid medium to generate erythroblast populations, which were treated for 2 days (until day 8 of culture) with SCF 100 ng/ml and then processed for real-time PCR analysis (a) and western blotting (b). Bars represent the mean ± S.D. of three experiments performed with cells from different donors

Figure 1b). Jagged1 expression was confirmed at the protein level and appeared to be present during the central phases of erythroid differentiation (Figure 3b). Then, we determined whether SCF was able to increase Jagged1 expression. Erythroid precursors at day 6 of unilineage culture were stimulated for 2 days with SCF and analyzed for Jacqed1 RNA and protein expression. Both Jagged1 RNA and protein remained unvaried upon SCF treatment, suggesting that SCF acts rather by reinforcing Notch2 expression (Figure 3c and d). To rule out a potential role of other Notch ligands in mediating SCF effects, we assessed whether SCF was able to modify the expression of Jagged2, Delta-like1 and Delta-like3, but RNA levels of such factors remained unchanged upon SCF treatment (Supplementary Figure 1c). To understand whether Jagged1 had a role in SCF-mediated modulation of erythropoiesis, we cultivated erythroid precursors for 4 days (days 4-8) in the presence or absence of SCF and anti-Jagged1 neutralizing antibodies. We found that blocking Jagged1 receptor-ligand interactions reduced SCF-mediated erythroid cell expansion, suggesting the presence of an autocrine signaling mechanism involving Notch2 and Jagged1 expression on erythroid precursors (Figure 3e). Even in the absence of increased protein expression, the ability of anti-Jagged1 neutralizing antibodies to inhibit SCF-induced proliferation indicates that basal levels of Jagged1 provide a sufficient stimulus to activate Notch2 and support SCF-mediated erythroid expansion.

SCF modulates the expression of Notch mediators in erythroid precursor cells. In order to depict a possible mechanism of action downstream of Notch2 and responsible for SCF modulation of erythropoiesis, we assessed RNA and protein expression of two major transducers of Notch signals, Hes-1 and Hey-1. As Notch has previously been shown to modulate GATA-2 expression in hematopoietic cells to inhibit myeloid differentiation, we also analyzed the expression of GATA-2 and its relative GATA-1 in erythroid precursors at day 8 of differentiation, untreated or previously treated for 2 days with SCF. We found that Hes-1 RNA and protein levels, but not Hey-1 levels, strongly increased upon SCF stimulation (Figure 4a and b). Likewise, SCF increased RNA and protein levels of the antidifferentiative factor GATA-2, whereas the pro-erythroid factor GATA-1 remained unvaried (Figure 4a and b). Upregulation of Notch2, Hes-1 and GATA-2 by SCF suggests that this cytokine activates signaling pathways downstream of Notch2 that are responsible for the modulation of erythropoiesis.

Interfering with Notch2 function inhibits the effects of SCF on erythroblast proliferation and differentiation. In order to confirm Notch2's involvement in SCF signaling, we searched for a method to stably interfere with Notch2 activity throughout the erythroid cell maturation. To do so, we developed Notch2 mutant molecules based on pioneer studies demonstrating that specific Notch truncations resulted in constitutively active and dominant-negative forms of the receptor.²⁷ The constitutively active Notch2 mutant (Notch2 Intra) was constructed by truncating all the extracellular part of the molecule, whereas a dominantnegative Notch2 (Notch2 Extra) was produced by removing the intracellular part of the receptor (Figure 5a). Specifically, the Notch2 Extra mutant was constructed in order to maintain all the extracellular and transmembrane region of Notch2 but excluding the region that interacts with CBF-1, which was demonstrated to encompass a conserved region adjacent to the cdc10/ankyrin repeats.²⁸ The activity of the two mutants was confirmed by evaluating their ability to modulate the activation of a multimerized CBF-1 binding sequence upstream of the SV40 promoter cloned upstream of the luciferase sequence (Figure 5b). The constitutively active and dominant-negative Notch2 mutants were cloned in a bicistronic retroviral vector carrying the GFP reporter gene. A full-length Notch2 gene could not be used in this expression system as its large size (~7400 bp) exceeded the packaging threshold of the virus. Retroviral constructs containing Notch2 mutants were used to transduce cycling CD34⁺ hematopoietic progenitors, which were subsequently sorted for GFP expression and induced to undergo erythroid differentiation through culture in standard erythroid medium. The expression of the truncated Notch2 proteins was detected in packaging cells and in Notch2 Extra-transduced

375

npg 376

erythroblasts, whereas sufficient numbers of erythroid precursors for immunoblot analysis could not be collected for the Notch2 Intra sample (Figure 5c). In fact, we observed

that on Annexin V/7-AAD staining, the Notch2 Intratransduced sample revealed a higher rate of apoptotic erythroblasts as compared with the vector-transduced and



npg 377

Notch2 Extra-transduced samples (Figure 5d). To explain this observation, we hypothesized that an elevated Notch2 activity may be incompatible with the first stages of erythroid differentiation, resulting in the death of cells that incorporated a high number of transgene copies, while only cells with a low copy number of constitutively active Notch2 gene could escape apoptosis and mimic SCF signaling. This hypothesis was confirmed by a transgene dosage experiment revealing a decrease in the number of integrated viral copies at late differentiation stages (day 12) in the Notch2 Intra-transduced population but not in the vector-transduced population (Supplementary Figure 1d). Then, we evaluated the proliferative capacity of erythroblasts transduced with the mutant Notch2 genes. The proliferation of cells expressing Notch2 Extra was evaluated both in the presence and in the absence of SCF, whereas the growth of cells expressing Notch2 Intra was evaluated only in standard erythroid medium, as the mutant gene itself was expected to mimic the effects of SCF. Erythroblasts transduced with Notch2 Extra showed a severely impaired rate of proliferation both in the presence and in the absence of SCF, indicating that dominant-negative Notch2 interfered with both basal and SCF-induced erythroid expansion (Figure 5e). Erythroblasts expressing the constitutively active Notch2 mutant also showed an impaired proliferation, according to the increased percentage of dving cells in the early maturation phases (Figure 5d and e). When we evaluated the differentiation of erythroblast carrying the Notch2 Extra or the Notch2 Intra genes, we found that the Notch2 Intra sample contained increased numbers of immature cells as compared with the vector-transduced sample (Figure 5f, left panel). Conversely, Notch2 Extra-expressing erythroblasts showed an increased presence of mature cells as compared to the control (Figure 5f, left panel). Accelerated erythroid differentiation of Notch2 Extra-expressing erythroblasts was observed also when cells were cultivated in the presence of SCF, indicating that Notch2 contributes to SCF-mediated delay of erythroid differentiation (Figure 5f, right panel). The presence of mature erythroblasts in culture transduced with the dominant-negative Notch2 Extra gene was confirmed by GpA staining, which revealed increased numbers of GpA^{high} cells in the Notch2 Extra sample than in the control (vector-transduced) sample, both in basal conditions and in the presence of SCF (Figure 5g).

Discussion

The Notch signaling system has a complex role in regulating cell proliferation, survival, differentiation and fate specification. The context-dependent effects of Notch activation are particularly apparent in the regulation of hematopoiesis, where in different systems the Notch pathway has been reported to promote or suppress apoptosis, proliferation and differentiation or even to be dispensable for hematopoiesis.^{11,29,30} Redundancy between Notch receptors, complexity of intracellular networks and threshold effects may all contribute to explain the different effects of Notch on hematopoiesis.¹³ In this context, studies on differentiating primary hematopoietic cells or on simple vertebrates may provide an important contribution to clarify the role of Notch in the control of hematopoietic cell production.

The process of erythroid maturation involves sequential waves of proliferation and differentiation that are mainly controlled by erythropoietin. SCF has a key role in the regulation of ervthropoiesis as demonstrated by the impaired development of late erythroid progenitors displayed by mice deficient for SCF or its receptor c-kit.¹⁻³ Interestingly, c-kit mutation also results in a severe impairment of stress erythropoiesis, underlying the importance of SCF both in the basal erythropoiesis and in the recovery from acute anemia.³¹ The molecular pathways responsible for SCF-mediated erythroid proliferative and antiapoptotic effects have been reported to involve multiple effectors including p38, MAP kinase and Bcl-2/Bcl-XL.^{7,8} In contrast, pathways activated by SCF that affect erythroid differentiation have been poorly characterized, except for the finding that SCF modulates the activity of cyclin/cyclin-dependent kinases to inhibit erythroid cell maturation.32

As both Notch and SCF are able to delay the differentiation of hematopoietic progenitors, we investigated a possible link

Figure 5 Dominant-negative Notch2 inhibits the effects of SCF on erythroblast proliferation and differentiation. Constitutively active and dominant-negative Notch2 mutants (indicated as Notch2 Intra and Notch2 Extra, respectively) were constructed as described in Materials and methods and used to transduce cycling CD34 + cells, which were subsequently sorted for GFP expression and induced to undergo erythroid maturation by culture in standard erythroid medium. (a) Schematic representation of Notch2 mutants. (b) Effect of Notch2 mutants and full-length Notch2 (Notch2 FL) on the activation of a luciferase reporter gene. Bars represent the mean ± S.D. of three independent experiments. (c) Expression of Notch2 mutant proteins detected by western blotting in the packaging cell line Phoenix (Φ NX) and in erythroid progenitors (HPCs). (d) AnnexinV/7-AAD staining of erythroblasts at day 4 of culture transduced with the empty vector (Vector), with Notch2 Intra or Notch2 Extra. Numbers refer to the cumulative percentage of Annexin V⁺, 7-AAD⁺ and Annexin V⁺/7-AAD⁺ cells. (e) Proliferation curve of erythroblasts transduced with the empty vector (Vector), with Notch2 Intra or Notch2 Extra grown in the presence or absence of 30 ng/ml SCF from day 0. The proliferation of retrovirally transduced erythroblasts was followed only until day 12, as CD34 cells used for these experiments were maintained in cycling conditions before being committed to erythroid differentiation (as described in Materials and methods) and such treatment accelerated the subsequent maturation process. Statistical analysis performed by means of two-way ANOVA with Bonferroni post-tests showed the following statistical significance: Vector versus Notch2 Extra: *P<0.05 at day 9 and ***P<0.001 at day 12. Vector versus Notch2 Intra: **P<0.01 at day 9 and ***P<0.001 at day 12. Vector + SCF versus Notch2 Extra + SCF: ***P<0.001 at day 12. The experiment was repeated five times with cells derived from different donors. (f) Differentiation of erythroblasts transduced with the empty vector (Vector), with Notch2 Intra or with Notch2 Extra grown in standard erythroid medium (left panel) or in the presence of 30 ng/ml SCF (right panel). Bars represent the mean ± S.D. of three experiments performed with cells from different donors, showing a statistical significance of **P<0.01 for Vector versus Notch2 Intra and *P<0.05 for Vector versus Notch2 Extra (left panel) and *P<0.05 for Vector + SCF versus Notch2 Extra + SCF (right panel). (g) May-Grünwald-Giemsa staining (upper panels) or Glycophorin A staining (lower panels) of erythroblasts at day 10 of culture transduced with the empty vector (Vector) or with Notch2 Extra, grown in standard erythroid medium in the absence or presence of 30 ng/ml SCF as indicated. Numbers in the lower guadrants indicate the percentage of Glycophorin A^{bright} terminally differentiated erythroblasts. The panel on the lower right represents the mean ± S.D. of Glycophorin A stainings performed with cells transduced in four independent experiments. Abbreviations: BASO, basophilic erythroblasts; ORTHO: orthochromatic erythroblasts; POLY: polychromatophilic erythroblasts

between the two systems. We observed that Notch2 was strongly induced upon SCF stimulation and that targeting Notch2 signaling neutralized the effects of SCF on erythroblast expansion and differentiation. The observation that dominant-negative Notch2 depresses erythroid proliferation is in agreement with previous reports showing that Notch inhibition results in reduced erythropoiesis. In particular, a 40% decrease of bone marrow erythroid cells was detected in fucosylation-deficient mice, which have a defective Notch signaling.²⁴ Interestingly, studies performed on primary human hematopoietic progenitors reported that the simultaneous presence of SCF and Jacged1 increased ervthroid colony formation,¹⁷ anticipating the link between SCF and the Notch pathway described in the present study. Our observation that Notch inhibition impairs erythropoiesis is apparently in contrast with the results obtained in other studies. Mice embryos deficient for the Notch mediator RBP-jk have been reported to display increased numbers of Ter119⁺ cells at the yolk-sac level, because of decreased apoptosis of developing erythroblasts.²³ In agreement with this observation, activation of Notch signaling in embryonic stem cells has been recently reported to inhibit primitive erythropoiesis.³³ This apparent discrepancy may be explained by hypothesizing distinct roles of Notch signaling in different phases of erythroid development. In early erythroid progenitors as well as during embryonic erythropoiesis. Notch signaling may create a conflict with the process of lineage commitment and result in cell death. Accordingly, we found that CD34+ hematopoietic progenitors transduced with constitutively active Notch2 undergo apoptosis when forced to undergo ervthroid differentiation by erythropoietin-containing medium. In contrast, in more mature erythroblasts, elevated Notch expression can result in increased proliferation and differentiative slowdown. Notably, mice with a conditional inactivation of Mind bomb-1, which is essential for endocytosis of Notch ligands and subsequent Notch signaling, exhibit expansion of the immature erythroid compartment, but reduction of circulating mature erythrocytes.³⁴ Similarly, mice with defective presenilin activity, which is necessary for γ -secretase cleavage and generation of intracellular Notch, have decreased numbers of mature erythrocytes.³⁵ According to these observations, common myeloid progenitors from mice expressing a dominant-negative form of Mastermind-like-1 that sequesters the intracellular domains of all mammalian Notch receptors produce only one-third of erythroid colonies compared with controls.¹⁹ Altogether, these evidences suggest that the Notch system has a complex role in regulating the size of the erythroid compartment, possibly by restraining the expansion of immature erythroblasts but at the same time by enhancing the production of more mature erythroid precursors and erythrocytes.

The present study adds a new player in SCF-mediated regulation of hematopoiesis, linking SCF-activated signaling pathways to Notch receptors and intracellular mediators. It may be hypothesized that the links between Notch and SCF are not limited to the hematopoietic system. In fact, a connection between SCF and Notch signaling pathways has been previously identified in neural stem cells, where SCF induces Notch expression, possibly contributing to stem cell proliferation.³⁶ Possible correlations between Notch and SCF

may also emerge from the melanocytic compartment, where mutations affecting either the Notch system or the c-kit/SCF system similarly lead to loss of melanocyte precursors and absence of hair pigmentation.³⁷ Future studies that link different systems regulating cell homeostasis are likely to provide new clues to understand hematopoietic regulation and indicate new potential applications for clinical intervention.

Materials and Methods

Antibodies and reagents. Human recombinant SCF, Epo, IL-3 and GM-SCF were purchased from Peprotech Inc. (Rocky Hill, NJ, USA). Rat monoclonal antibodies against Notch1 (bTAN20), Notch2 (C6516BDHN) and Jagged1 (TS115H) were the supernatants of hybridomas purchased from DSHB Hybridoma Bank (Iowa City, IA, USA). Alexa-647-conjugated anti-rat antibodies used for flow cytometry were purchased from Invitrogen-Molecular Probes (Carlsbad, CA, USA). Anti-Jagged1 blocking antibody and anti-c-kit were purchased from R&D Systems (Minneapolis, MN, USA). The -secretase inhibitor L-685,458 was from Sigma-Aldrich (St Louis, MO, USA). PE-conjugated anti-GpA was from Pharmingen (San Diego, CA, USA). Annexin V FITC and 7-amino-actinomycin D (7-AAD) were from Invitrogen-Molecular Probes.

Adult peripheral blood human progenitor cell (HPC) purification and culture. Peripheral blood was obtained from healthy donors after informed consent and approval by the institutional ethical committee (protocol N. CE-ISS 08/207). CD34⁺ hematopoietic progenitor cells (HPCs) were purified using the Midi-MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) and cultured in serum-free medium supplemented with 0.01 U/ml IL-3, 0.001 U/ml GM-CSF and 3 U/ml Epo (subsequently referred to as standard erythroid medium) as previously described.²⁵ These culture conditions routinely yield a progeny of $98 \pm 2\%$ GpA-positive cells. Alternatively, CD34⁺ cells were kept for 2 days in serum-free medium supplemented with cycling mixture (see below) for subsequent retroviral infection. In all the experiments, CD34⁺ cells were obtained from three different healthy donors and pooled. The differentiation stage of erythroblasts was routinely evaluated by May-Grünwald-Giemsa staining and cytological analysis. Images were taken with a Nikon Eclipse E1000 microscope equipped with a Nikon Plan Apo \times 60/1.4 NA oil immersion objective and a Nikon DXM 1200 digital camera with dedicated acquisition software (Nikon ACT-1 v. 2.1; all from Nikon Instruments, Tokyo, Japan).

Reverse-transcriptase PCR and real-time PCR. Total RNA was extracted by the standard guanidium thiocyanate-CsCl method and reverse-transcribed with oligo (dT) as a primer. To evaluate Notch1 and Notch2 expression, an aliquot of RT-RNA was amplified within the linear range (30 PCR cycles) with the primers indicated in Table 1. Samples were electrophoresed in 2% agarose, transferred to a nylon membrane and hybridized with the probes indicated in Table 1. Sample expression was normalized based on S26 expression. Real-time PCR was performed by TaqMan technology, using the ABI PRISM 7900 DNA Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to standard procedures. 18S RNA was selected as endogenous control. Commercial ready-to-use primers/probe mixes were used (Applied Biosystems): Hs00164982_m1 (Jagged1), Hs00171432_m1 (Jagged2), Hs00194509_m1 (Delta-like1), Hs00231112_m1 (GATA-1), Hs00231119_m1 (GATA-2) and 4319413E (Eukarvotic 18S rRNA).

To assess the number of vector copies integrated in the genome of retrovirally transduced erythroblasts, quantitative PCR was performed using primers complementary to the vector backbone sequence GFP: forward primer, 5'-CAGCT CGCCGACCACTA-3' at 600 nM final concentration; reverse primer, 5'-GGGCCGT CGCCGA-3' at 600 nM final concentration. As internal reference for normalization of human samples, we amplified a fragment of the human telomerase reverse transcriptase (*TERT*) gene, using the following set of primers: forward primer, 5'-GGGCACACGTGGCTTTTCG-3' at 200 nM final concentration; reverse primer, 5'-GGGCACACGTGGCTTTTCG-3' at 600 nM final concentration. A standard curve of genomic DNA carrying 11 retroviral vector (RV) copies, validated by Southern blot analysis, was constructed with DNA extracted from RV-transduced HeLa cells. Reactions were carried out in a total volume of 25 µl,

Table 1 Oligonucleotides used for the amplification of Notch genes and S26 control

Oligonucleotide name	Oligonucleotide sequence
Notch1 forward	5'-AACGCTTCAGACGGTGCC-3'
Notch1 reverse	5'-GCTGTTGCCCGTCTCCAG-3'
Notch1 probe	5′-CTCAGCAGCACCTGGATGCCGCTGACCTGC-3′
Notch2 forward	5'-AGCTCAGATTTGAGTGATGAA-3'
Notch2 reverse	5'-AGTTGCCTCCACATTATTGAC-3'
Notch2 probe	5'-ATGGCCCTGCACCTTGCAGCCCGCTACTCA-3'
S26 forward	5'-GCCTCCAAGATGACAAAG-3'
S26 reverse	5′-CCAGAGAATAGCCTGTCT-3′
S26 probe	5'-GAGCGTCTTCGATGCCTATGTGCTTCCCAA-3'
Notch2 extra forward	5'-CCCCCCGCGCTCGAGATGCCCGCCCTGCGCCCCGCTCTG-3'
Notch2 extra reverse	5'-CCCCCCGAATTCTCATTCTTCTGAGAGTAAGGCCTCATCTTCAG-3'
Notch2 intra forward	5'-GGGGGGCTCGAGATGGCAAGCAATCACAAGCGTCGTGAG-3'
Notch2 intra reverse	5'-GGGGGGGCTCGAGTCACGCATAAACCTGCATGTTGTTGTG-3'

in an ABI PRISM 7700 HT sequence detection system (Applied Biosystems). C/G (copies per genome) was calculated by using the equation: ((ng retroviral vector)/ (ng endogenous DNA)) \times (no. of RV integrations in the standard curve).

Reporter assays. The reporter construct pGL3-N8 was obtained by cloning a multimerized (8-mer) CBF1 binding sequence upstream of the SV40 promoter in the vector pGL3 Promoter (Promega, Madison, WI, USA). Transfections were performed in the 293T embryonic kidney cell line by lipofection (Lipofectamine, Invitrogen) following the manufacturer's instructions. Briefly, cells were seeded in 12-well plates on day 1. On day 2, cells were transfected with a mixture containing either pGL3 promoter or pGL3-N8, along with pRL-CMV (Promega), and the effector plasmids. On day 4 cells were lysed and the reporter activity was assayed using the Dual Luciferase Reporter Assay (Promega) in a TLX20 luminometer. Relative luciferase activity (RLA) was calculated as the ratio of firefly luciferase activity *versus* jellyfish activity and relative promoter activation (RPA) was calculated by dividing the RLA of the pGL3-N8 series by the RLA of the pGL3 series and then normalizing by the RPA of the empty vector.

Western blotting. Protein extracts were prepared by resuspending cell pellets in 1% NP40 lysis buffer (20 mM Tris/HCl, pH 7.2, 200 mM NaCl, 1% NP40) in the presence of protease inhibitors (Sigma). Concentration of lysates was determined by the Bradford assay (Bio-Rad Laboratories, Richmond, CA, USA) and equal amounts of proteins were used for SDS-PAGE. Samples were analyzed by standard immunoblot procedure and visualized by chemiluminescence (Super Signal West Pico Pierce, Rockford, IL, USA). The intensity of bands representing relevant proteins was quantified using Scion Image (Scion Corporation, http://www.scioncorp.com).

Construction of Notch2 mutants. The dominant-negative Notch2 mutant (Notch2 Extra) was obtained by amplifying the extracellular and transmembrane portions of full-length Notch2 (5340 nucleotides from the start codon). The constitutively active Notch2 mutant (Notch2 Intra) was obtained by amplifying the intracellular portion of full-length Notch2 starting from nucleotide 5095. Oligonucleotides used for amplification are reported in Table 1. The PCR products were verified by sequencing and cloned *Xhol/Eco*RI (Notch2 Extra) or *Xhol/Xhol* (Notch2 Intra) in a modified Pinco retroviral vector for subsequent transduction of CD34⁺ cells.³⁸

Retroviral infection of primary erythroblasts. Mutant forms of Notch2 (Notch2 intracellular and Notch2 extracellular) were cloned into a bicistronic retroviral vector obtained by modification of the Pinco vector under the control of Moloney long-terminal repeats together with the GFP reporter gene.³⁸ The amphotropic packaging cell line Phoenix was transfected by standard calcium-phosphate/chloroquine method, and culture supernatants containing retroviral particles were collected after 48 h. HPC infection was performed on CD34⁺ cells previously kept in serum-free medium supplemented with an SCF-free cycling mixture (100 U/ml IL-3, 100 ng/ml FLT3 ligand, 100 ng/ml thrombopoietin) for 48 h after purification. Cells were suspended at 5×10^4 /ml in the viral supernatant supplemented with cycling mixture and 8μ g/ml polybrene and centrifuged at 1800 r.p.m. for 45 min at 32°C, then placed back in the incubator for 1 h. Such infection cycle was repeated three times a day for two consecutive days. GFP-positive cells were separated by flow cytometry using a FACSAria

(Becton Dickinson, Omaha, CA, USA). Immediately after sorting, HPCs were placed in standard erythroid medium to induce erythroid differentiation.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA; http:// www.graphpad.com). Data are represented as the mean and standard deviation of independent experiments, with the statistical significance expressed by one asterisk (P < 0.05), two asterisks (P < 0.01) or three asterisks (P < 0.001). Results shown in Figures 1d, 2e, 3e, 5f and g were analyzed by Student's *T* test assuming equal variances. Results shown in Figures 1c and 5e were analyzed by means of two-way ANOVA with Bonferroni post-tests.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)