HEMATOPOIESIS AND STEM CELLS

Notch signals are required for in vitro but not in vivo maintenance of human hematopoietic stem cells and delay the appearance of multipotent progenitors

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Key Points

- Notch signals expand human HSC (CD90low) cells in vitro and delay the expansion of CD45RAint and CD45RAhi cells in vitro.
- HSCs expanded in vitro are equal to ex vivo CD90low cells in immune reconstitution.

All blood cell lineages start from hematopoietic stem cells (HSCs), which were recently shown to represent a heterogeneous group of cells. In mice, Notch signaling promotes the maintenance of “stemness” as well as the expansion of self-renewing HSCs in vitro. Additionally, human CD34+ cells were shown to expand in vitro in response to Notch signals. However, it is unclear whether Notch directly affects all HSCs, and whether this role is relevant in vivo. Here, we developed culture conditions that support the maintenance of CD34+CD133+CD90lowCD38+CD7+CD10+CD45RA− (CD90low) cells, phenotypically defined HSCs, as well as 2 early progenitor cells (CD34+CD38−CD7−CD10−CD45RAint [RAint] and CD34+CD38−CD7−CD10−CD45RAhi [RAhi]) that were functionally equivalent to multipotent progenitor-2 and lymphoid-primed multipotent progenitor, respectively, found in cord blood. Using a genetic approach, we show that Notch signals were required for HSC preservation, with cultured HSCs being equal to ex vivo HSCs in their ability to reconstitute immunodeficient mice; however, dnMaml-transduced HSCs were not maintained in vitro. Interestingly, Notch signaling did not appear to be required for the self-renewal of human HSCs in vivo. Our findings support the notion that Notch signals maintain HSCs in vitro that have hematopoietic-reconstituting ability in vivo and delay the appearance of 2 newly described early progenitor cells. (Blood. 2014;123(8):1167-1177)

Introduction

The hematopoietic system has been conceptualized as operating within a hierarchical model, with the hematopoietic stem cell (HSC) at the apex.1 Human umbilical cord blood (CB) CD34+ cells would appear to be the most quiescent HSCs capable of giving rise to CD34−CD90lowCD49f+ HSCs with long-term reconstituting ability.2-4 Whether HSCs are affected by Notch signaling remains controversial. For instance, Notch signaling does not appear to be required for the maintenance of adult HSCs in mice.2 In contrast, Notch signaling was shown to play an important role in the expansion of self-renewing HSCs in vitro.6

In humans, although a prominent role for Notch signaling is well documented based on the expansion of CD34+ cells in vitro, which includes both HSCs and progenitor cells, no quantitative and functional direct comparison in the efficiency of reconstitution to fresh ex vivo HSCs has been reported.7-13 Nonetheless, Notch appears to affect human HSCs because CD34− cells were recently shown to have active Notch signaling. However, in contrast to the experimental approach used in mice, which shows that Notch is not required for HSC maintenance, no similar genetic manipulations have been reported for human HSCs.4,5

Several human progenitors able to differentiate into lymphomyeloid cells have been found in CB and/or bone marrow (BM).14-18 Nevertheless, it remains difficult to establish a clear hierarchical structure starting from an HSC. We sought to address this by starting cultures with purified CB-derived HSCs and functionally defined intermediates to yield a composite and hierarchical relationship. We modified the OP9-DL1 cell coculture system19,20 to support the expansion of HSCs and made use of OP9 cells expressing 2 different levels of Delta-like-4 (DII4),21 therefore enabling the titration of Notch signaling on HSCs. Additionally, using a genetic approach, we found that Notch signaling during the coculture period was required for the maintenance of HSCs while delaying the appearance and frequency of downstream progenitors. However, in vivo competition experiments revealed that Notch signaling was not required for the maintenance of human HSCs within the BM.

Together, our findings support the notion that Notch signals can extend the period of time in which HSC function can be maintained in vitro by delaying the emergence of at least 2 early progenitors, which we have characterized here providing a more complete view of the early stages of human hematopoiesis.
Methods

Umbilical CB samples

Human CB samples were obtained by syringe extraction and collected in a blood-pack unit containing citrate phosphate dextrose anticoagulant (Baxter Healthcare) from consenting mothers following delivery in accordance with approved guidelines established by the research ethics board of Sunnybrook Health Sciences Centre. Within 24 hours of collection, CB mononuclear cells were isolated as previously described.22

Mice

NOD.Cg-PrkdcscidIl2rgtmWjlt/Sz (NOD/SCID/e−/− γc−/−) and Rag2−/− γc−/− BALB/c mice were purchased from The Jackson Laboratory and housed and bred in a pathogen-free facility. All animal procedures were approved by the Sunnybrook Health Science Centre Animal Care Committee.

Generation of OP9-DL4

OP9-Ctrl, OP9-DL4hi, and OP9-DL4low cells were generated and maintained as previously described.22

CB purification

CB cells were lineage depleted using the Stem-Sep human progenitor cell enrichment kit (StemCell Technologies) according to the manufacturer’s protocol, and further purified as described in detail in the supplemental Methods (available on the Blood Web site).

Cocultures

OP9-Ctrl, OP9-DL4hi, and OP9-DL4low cells were plated and maintained as previously described,22-24 and used in cocultures as described in detail in the supplemental Methods.

For clonal analysis, sort-deposited cells were cultured for an additional 14 to 21 days under T or myeloid conditions as described in detail in the supplemental Methods.

In vivo reconstitutions

Donor cells were sorted for the indicated markers and then injected intrahepatically into neonatal immune-deficient mice irradiated with 2 Gy. After 10 weeks, mice were sacrificed and thymus, BM, and spleen were analyzed by flow cytometry. Details of the cells used and analysis are described in the supplemental Methods.

dnMaml construct and retroviral transductions

The sequence encoding for amino acids 8 to 74 from the mouse Mastermind like 1 (Maml1) gene was fused, in frame, to a 5′-enhanced green fluorescent protein (eGFP) sequence using described cloning methods. This dominant-negative form of MAML1 (dnMaml) was then cloned into retroviral or lentiviral vectors to be used for transduction experiments of CB cells as described in detail in the supplemental Methods.

Precursor-product relationships

In vitro-derived CD45RAlow cells were sorted on day 18 from cultures, on OP9-DL4hi-DL4low or -Ctrl. Sorted cells (10 000 cells) were then cultured again on the OP9 cells they were derived from, in Stem. After 2 and 3 days, analysis of CD45RA expression on gated CD34−CD38−CD7−CD10− cells was performed.

Quantitative polymerase chain reaction analysis

Total RNA was isolated in TRIzol reagent and reverse transcribed using Superscript-III and Oligo(dT) primers (Invitrogen). Diluted complementary DNA samples from cultures were used as described in detail in the supplemental Methods.

Statistical analysis

Student t unpaired and paired 2-tailed tests were performed using Excel and Prism software.

Results

Characterization of early hematopoietic subsets in CB

We have used CB to identify early hematopoietic subsets using previously described markers,22-23 with the addition of Rhodamine-123 (Rho) and CD133 to further characterize human HSC and multipotent progenitor (MPP) subsets. Analysis of CD45RA expression from Lin−CD135+ cells showed the presence of 3 populations, with the most abundant fraction being CD45RA−. As shown in Figure 1A, the CD45RA− fraction (CD45RA− cells) was further analyzed for CD133 expression and Rho uptake, and we identified the HSC fraction as having a CD90low phenotype, which was found only in the CD45RA−CD133−CD135+ Rho− fraction. Not surprisingly, CD90− cells made up the vast majority of the Rho− subset, likely corresponding to an MPP-enriched subset. CD135+ CD45RA− CD133+ Rho− CD90low cells, which were also CD38lowCD7+CD10− (not shown), hereafter referred to as HSCs, were used in subsequent experiments.

Cocultures with a modified OP9 system for HSC expansion

To examine how Notch signals could influence the CB HSC fraction, these cells were cultured on OP9-Ctrl cells (green fluorescent protein [GFP] alone) or on OP9 cells expressing different levels of DI4 (OP9-DL4low and OP9-DL4hi21) (supplemental Figure 1C), together with cytokines3,26 to support survival and expansion of HSCs and early progenitors. Cocultures were analyzed on a weekly basis for the expression of markers that define HSCs. By day 21, we noted that cocultures grown on OP9-DL4hi and -DL4low cells displayed a greater frequency of CD34+ cells than those on OP9-Ctrl cells (Figure 1B). An additional and expected effect of Notch signaling was the generation of CD34+CD7+ cells, early T-lineage cells,19 present in DL4hi and DL4low cocultures starting with either HSCs or MPPs, but not in DL4low cultures (not shown).

Based on these observations, we looked specifically at CD34−CD7− cells at different time points, and found that CD45RA and CD38 were expressed at different levels and in different proportions on CD34−CD7− gated cells (Figure 1C). In particular, the CD45RA− and CD45RAint subsets were clearly present in cultures grown on DL4hi but not in Ctrl, while the CD45RAhi subset was abundantly present in cocultures lacking Notch signaling (Ctrl).

Because cocultures initiated with HSCs under Notch signaling conditions contained a substantial fraction of CD45RA− cells, we analyzed whether CD90low cells were present after 3 weeks. Supplemental Figure 1E shows CD90low cells only in DL4hi and DL4low cocultures starting with either HSCs or MPPs, but not in OP9-Ctrl cultures, nor in cultures initiated with downstream CD90− cells (supplemental Figure 1F).

We calculated the expansion of CD45RA− (CD133+CD34+CD38−CD7−CD45RA−) cells, and phenotypically defined HSCs (pHSCs) as (CD133+CD34+CD38+CD7−CD45RA−CD90low) based on the absolute cellularity recovered from the starting HSCs (Figure 2A). CD45RA− and pHSCs cultured on Ctrl expanded minimally (Figure 2A-B). However, when cultured with DL4hi, CD45RA−, and pHSCs cultured sixfold to 12-fold and twofold to fourfold after 2 to 3 weeks, respectively (Figure 2A-B). A smaller, but significant, expansion was also seen with DL4low. Similar data were obtained when OP9-DL1hi cells were used (data not shown). These results suggest that a high or low level of Notch signaling...
retards differentiation of pHSCs, allowing for some cellular expansion. We conclude that the modified OP9-DL4 coculture system appears to maintain cells that are phenotypically identical to the input HSCs.

In vivo potential of HSCs generated in vitro

Although phenotypically defined HSCs were maintained and expanded in the presence of Notch signals in vitro, the functional potential of these cells remained to be verified. We therefore compared the capacity of isolated DL4^hi-derived day 14 (2 × 10^3) pHSCs with that of 25-fold more phenotypically defined MPP (CD90^-pMPP) cells, isolated in parallel DL4^hi-derived day 14 (5 × 10^4), to repopulate sublethally irradiated NOD/SCID/γc null (NSG) mice. After 10 weeks, BM samples from pHSC- and pMPP-reconstituted mice contained macrophages (CD33+CD14^+), granulocytes (CD33^CD15^+), and lymphoid cells (CD19^+ B or CD7^+ T cells) (Figure 2C-D). In all, 8 mice were analyzed in 2 groups of 4 (Figure 2E), and absolute numbers of myeloid or lymphoid cells were similar between the 2 groups. Therefore, both cultured pHSC and pMPP cells have lymphomyeloid reconstitution potential.

We next wanted to compare day 14 in vitro–grown pHSCs with freshly isolated HSCs for their ability to repopulate immune-deficient mice. NSG mice were injected with fresh HSCs ranging from 0.5 to 3 × 10^3 cells/mouse, and the resulting expansion of CD45RA^-cells after 10 weeks within the BM of host mice was used to determine the expected cellular yield from injected HSCs (Figure 2F). A linear relationship was observed with the absolute number of donor CD45RA^- cells in the BM correlating directly with the number of injected HSCs. We then extrapolated the number of CD45RA^-cells recovered from mice injected with 2 × 10^3 day 14 in vitro–grown pHSCs and found that this corresponded to the equivalent of 0.5 × 10^3 freshly isolated HSCs, or a ~0.25 times equivalency in cellular outcome (Figure 2F). In contrast, very few CD45RA^- were found in mice injected with pMPP cells, even though 25-fold more cells were injected. We conclude that pHSCs cultured in the presence of Notch signaling act as SCID-mouse repopulating cells (SRCs)^27 similar to freshly isolated HSCs.

Effect of Notch inhibition on the maintenance/expansion of HSCs and/or CD45RA^-subsets derived in vitro

To test whether the in vitro expansion of pHSCs and/or CD45RA^-cells is regulated by canonical Notch signaling, we transduced ex vivo HSCs with the dnMaml-GFP construct^5 to prevent cells from responding to Notch signals. HSC cells expressing dnMaml-GFP and/or control
yellow fluorescent protein (YFP) were generated and cultured on Ctrl, DL4hi, and DL4low cells for an additional 2 to 3 weeks. Analysis of CD34+CD90low cells (HSCs) placed on OP9-Ctrl, -DL4hi, and -DL4low cells and analyzed by flow cytometry at days 7, 14, and 21 of culture. The cellular fold expansion from the input number (5×10^3) is shown for (A) CD90low and (B) CD45RA cells. Bar graphs show mean and SE of 6 to 8 independent experiments. Statistical significance is indicated as (*) for P < 0.05, and (**) for P < 0.01. Cocultures were initiated with HSCs placed on OP9-DL4hi cells for 14 days, (C) phenotypically defined pHSC/CD90low, and (D) phenotypically defined pMPP/CD90high cells were isolated by flow cytometry, and 2×10^5 pHSC/CD90low and 5×10^5 pMPP/CD90high cells were injected into sublethally irradiated NSG neonatal mice. Ten weeks after injection, BM was analyzed by flow cytometry for the expression of the indicated marker. Numbers within each plot indicate the percentage of cells in the indicated gates or quadrants. (E) Absolute numbers of CD14+, CD15+, and CD19+ cells recovered. (F) A linear correlation between the absolute numbers of CD45RA cells recovered from the BM and the number of freshly sorted HSCs injected into mice is shown (R^2 = 0.87). Absolute numbers of CD45RA cells recovered from (C) and (D) were plotted on the linear graph and the corresponding number of HSCs injected was extrapolated. A total of 8 mice were analyzed.

**Is Notch signaling required for the maintenance/expansion of HSCs in vivo?**

We next tested whether this effect was also the case in vivo using competitive transplantation experiments. Equivalent numbers of sorted HSCs expressing either dnMaml-Ctrl–transduced cells (Figure 2B).
and YFP\(^1\) cells. In both donor types, myeloid and B-lymphoid cells were similarly observed. Analysis of the BM from 10 independently reconstituted mice is shown in supplemental Figure 2D-G. Similar results were found in the spleen. Analysis of the thymus showed CD45\(^1\) donor cells, but composed nearly entirely of YFP\(^1\) cells, with very few GFP\(^1\) cells (supplemental Figure 2B). The YFP\(^1\) cells included mainly T cells that expressed high levels of CD3. These findings clearly demonstrate effective abrogation of Notch signaling in GFP\(^1\) cells. To test the critical question of whether Notch signaling plays any role in the maintenance and/or self-renewal of human HSCs (SRC) in vivo, absolute numbers of donor-derived CD45RA\(^{-}\) and pHSCs were compared in the BM, and were found similar in dnMaml-GFP and YFP-injected mice (Figure 3C-E). These results strongly suggest that Notch inhibition did not have an effect on the maintenance and/or self-renewal of SRCs within the BM microenvironment.

**Effect of Notch engagement on the expansion of CD45RA subsets derived in vitro**

We next examined the consequences of the Notch-driven expansion of HSCs on early progenitors, namely the CD45RA\(^{int}\) and CD45RA\(^{hi}\) fraction (as shown in Figure 1C). We calculated the expansion of each progenitor fraction at weekly intervals based on the absolute number of cells recovered. Figure 4A shows that both CD45RA\(^{int}\) and CD45RA\(^{hi}\) fractions expanded the most in the absence of Notch signals (Ctrl). In the presence of Notch signaling, the generation of
CD45RA<sup>int</sup> and CD45RA<sup>hi</sup> cells was not favored in particular with DL4<sup>hi</sup> (Figure 4 middle panels). These data suggest that a high or low level of Notch signaling expands CD45RA<sup>2</sup> and pHSCs, maintains them in an undifferentiated state, and restrains their differentiation into both CD45RA<sup>int</sup> and CD45RA<sup>hi</sup> progenitors.

**Precursor-product relationship of CD45RA fractions derived in vitro**

We next determined precursor-product relationships between CD45RA<sup>int</sup> and CD45RA<sup>hi</sup> fractions generated in vitro. CD45RA<sup>int</sup> were sorted from day 21 Ctrl, DL4<sup>hi</sup>, and DL4<sup>low</sup> cocultures and then recultured (new day 0) under the same conditions from which they were originally derived. As shown in Figure 4B, >80% of CD45RA<sup>int</sup> became CD45RA<sup>hi</sup> after only 2 days of further culture on Ctrl or DL4<sup>low</sup>, but cells progressed more slowly to CD45RA<sup>hi</sup> when re-cultured in the presence of a high Notch signal. This indicated that CD45RA<sup>int</sup> were precursors of CD45RA<sup>hi</sup> cells. We concluded that CD45RA<sup>2</sup> give rise to CD45RA<sup>int</sup> and then to CD45RA<sup>hi</sup>, with Notch signaling restraining this developmental progression.

**Functional characterization of progenitor cells: bulk and clonal assays**

We next addressed the differentiation potential of CD45RA<sup>int</sup> and CD45RA<sup>hi</sup> subsets and examined their ability to generate colony-forming units (CFUs) in methylcellulose cultures. Figure 5A (top left panels) shows that CD45RA<sup>int</sup> cells derived from either Ctrl or DL4<sup>low</sup> cultures gave rise to BFU-E, GM, G, and M colonies. In striking contrast, very few if any BFU-E colonies were found in the CD45RA<sup>hi</sup> fraction from OP9-DL4<sup>low</sup> or -Ctrl cultures. Nevertheless, CD45RA<sup>hi</sup> cells have clear myeloid potential in that they gave rise to GM, G, and M colonies at higher frequencies than ex vivo MPP cells (Figure 5A). Therefore, CD45RA<sup>int</sup> and CD45RA<sup>hi</sup> cells both possess myeloid potential but CD45RA<sup>hi</sup> cells appear to have lost erythroid potential.

As shown in supplemental Figure 1A, a similar CD45RA<sup>int</sup> subset is present in CB cells, which are CD45RA<sup>int</sup><sup>CD135</sup><sup>1</sup><sup>CD133</sup><sup>1</sup><sup>CD7</sup><sup>2</sup><sup>CD10</sup><sup>2</sup><sup>Rholow/int</sup> (ex vivo CD45RA<sup>int</sup>). By comparing the myeloid potential of in vitro–derived CD45RA<sup>int</sup> cells to ex vivo CD45RA<sup>int</sup> cells, we observed that both CD45RA<sup>int</sup> cells display erythromyeloid potential (Figure 5A third top panel). To further characterize the BFU-E potential within this population, we fractionated ex vivo CD45RA<sup>int</sup> cells into Rhoint and Rholow fractions as shown in supplemental Figure 1A and tested each fraction separately. Only the Rhoint fraction gave rise to BFU-E although both fractions generated GM, G, and M CFU colonies (Figure 5B).

The lymphoid potential of CD45RA<sup>int</sup> and CD45RA<sup>hi</sup> cells generated in vitro was also assessed. The ability to generate T and B cells was observed in both CD45RA<sup>int</sup> (Figure 5C) and CD45RA<sup>hi</sup> cells (Figure 5D). The comparison of in vitro CD45RA<sup>int</sup> to ex vivo CD45RA<sup>int</sup> revealed that ex vivo CD45RA<sup>int</sup> also had T- and B-lineage potential (Figure 5C). However, a lower frequency of B cells was consistently found as compared with in vitro–derived CD45RA<sup>int</sup> cells. When ex vivo–derived CD45RA<sup>int</sup>Rholow cells were further fractionated into Rhoint and Rholow cells, almost no B cells were generated in the Rhoint fraction whereas the Rholow fraction did give rise to B cells (Figure 5E).
To define the potential of these fractions at the clonal level, we repeated these analyses under limiting dilution conditions. We found that every single clone of CD45RAint and CD45RAhi was able to generate T cells regardless of the presence or absence of Notch signaling during the first 3 weeks of culture. Interestingly, T cells were also generated from ex vivo CD45RAint clones at a similar frequency (Figure 5B; supplemental Figure 3A, supplemental Tables 1 and 2). Additionally, B cells, as well as macrophages and granulocytes (supplemental Figure 3, supplemental Tables 1 and 2), were also present at comparable frequencies from day 21 cocultures of clonally sorted CD45RAint and CD45RAhi cells.

Because the majority of CD45RAint or CD45RAhi clonal outcomes contained both CD19+ cells and CD33+ cells (supplemental Table 3) and given the T-cell frequencies observed (supplemental Table 2), our clonal analysis indicates that both CD45RAint and CD45RAhi cells were able to give rise to T, B, and myeloid cells. Therefore, a substantial fraction of both CD45RAint and CD45RAhi cells are lymphomyeloid precursors, the former compatible with an MPP.2
type of cell and the latter with a lymphoid-primed MPP (LMPP) type of cell.28

Ex vivo CD45RAintRholow cells have a similar potential to in vitro–generated CD45RAhi cells (supplemental Figure 5). Therefore, ex vivo–derived CD45RAintRhoint cells are LMPP-like cells. However, ex vivo CD45RAintRhoint cells have the potential to generate at the clonal level GM, G, M, and T cells (supplemental Tables 1 and 2). Only a fraction of them also have in vitro erythroid potential. So, at least 2 subsets are present in ex vivo CD45RAint cells, a minority subset with erythromyeloid and lymphoid potential and a majority subset with myeloid and T-cell potential, but devoid of B and erythroid potential in vitro.

Molecular characterization of CD45RA subsets

To further characterize the in vitro–generated CD45RAint and CD45RAhi cells, we performed a quantitative polymerase chain reaction (qPCR) analysis. Figure 6 shows that DTX1 (Deltex1) was expressed only on CD45RAint and CD45RAhi cells grown from DL4low cultures (Figure 6 top left panel) consistent with Notch signaling in these cultures. GATA1 and GATA2 transcripts were expressed in CD45RAint cells but were present at low levels in CD45RAhi cells. GATA1 expression was high in CD45RAint cells from DL4low cultures, which possessed a higher BFU-E frequency. On the other hand, IKZF1 (Ikaros) transcripts were expressed at higher levels in CD45RAhi cells from either Ctrl or DL4low cultures. This, together with the low levels of GATA1, confirmed a similar pattern of expression to that of mouse cells with similar differentiation potential. Additionally, SPI1 (PU.1), HOXB2, MLL3, and TAL1 (SCL) (Figure 6) were expressed at higher levels in CD45RAhi than in CD45RAint cells from either Ctrl or DL4low cultures, correlating with their earlier stage of differentiation. Taken together, the gene expression patterns found in both lymphomyeloid progenitors generated in vitro correspond to the pattern of gene expression predicted from their differentiation potential.

In vivo potential of in vitro–generated CD45RAint and CD45RAhi and ex vivo CD45RAint cells

We next assessed the in vivo developmental potential of in vitro CD45RAint and CD45RAhi cells and ex vivo CD45RAint cells to reconstitute BM and thymi of BALB/c Rag2/2 gc null mice. A relatively low number (105) of CD45RAint and CD45RAhi cells from DL4low or Ctrl cultures (2-4 weeks) was injected into immune-deficient neonatal mice. CD45RAint and CD45RAhi cells isolated from DL4low cultures engrafted mice with donor CD451 cells in the BM and thymus, including B and myeloid cells in the BM and T cells in the thymus, respectively, when analyzed 4 to 7 weeks postinjection (Figure 7A, B,D). We noted that different frequencies of CD4+CD8− cells, which are mainly immature single-positive cells, reflect the different temporal kinetics of reconstitution from the CD45RAint and CD45RAhi cells.
In contrast, mice injected with similar numbers of CD45RAint and CD45RAhi cells from Ctrl cultures were unable to effectively reconstitute the thymus, although they did reconstitute BM, with B and myeloid cells (Figure 7A-B). Notably, high levels of CD45+ engraftment were seen in the thymus of 15 mice injected with CD45RAint from DL4low cultures as compared with 7 mice injected with CD45RAint from Ctrl cultures (Figure 7D). Nonetheless, injection of higher numbers of these populations enabled CD45RAint and CD45RAhi cells from Ctrl cultures to reconstitute the thymus (data not shown). Thus, it appears that Notch signaling facilitated but was not required for thymic reconstitution. Lastly, ex vivo CD45RAint cells engrafted both the BM (B and myeloid cells) and thymus (Figure 7C). Results from 7 independent mice are presented in Figure 7D.

Discussion

We have defined culture conditions suitable for the maintenance of human HSCs, thus allowing us to directly test the effects of Notch signaling on prospectively defined HSCs. Here, we have found that HSCs are specifically affected by Notch signals in vitro, but do not rely on Notch signals for their expansion in vivo. In addition, our culture system enabled us to identify and characterize 2 new early progenitors, functionally akin to MPP-2 (CD45RAint) and LMPP (CD45RAhi) cells that were also found in CB. The role of Notch on HSCs remains controversial. In aggregate, our current understanding points to a dichotomy in the role of Notch in HSC expansion: in vitro systems support a role for both mouse HSCs and human CD34+ cells while in vivo approaches discounted a role for mouse HSCs. Mechanisms of how Notch signaling influence the maintenance and expansion of human HSCs in vitro vs in vivo are complex and difficult to dissect because they require discrete fractionation of the different HSC subsets.

As described by Anjos-Afonso, "both the most primitive quiescent HSC (CD34- ) and its progeny CD34+CD90+CD49f+ express DTX1 and HES1, display different kinetics of reconstitution, and appear to be affected differentially upon Notch inhibition in vivo. Although a pharmacological inhibitor of Notch was used in this report, which is known to affect other nonhematopoietic tissues, maintenance and self-renewal of the CD34+ subset was affected in vivo. However, here we show that competitive transplantation experiments using dnMaml for Notch inhibition in HSCs possessed equal reconstitution abilities in vivo, consistent with the results in mice. Nevertheless, whether loss of Notch signaling affected the temporal kinetics of reconstitution, as seen in mice, was not addressed.
Our results not only serve to extend the findings from Bernstein and colleagues, but more importantly, we now show that, in culture, HSCs are an important target of Notch signals. Additionally, it appears that cells at an early stage (CD34 + CD38 - CD7 - CD10 + CD45RA - cells [CD45RA -]) are maintained and expanded in the presence of Notch signals. Thus, by clarifying the target population exposed to Notch signals, a clearer picture of which cells are affected by this pathway has emerged.

A mechanism for how Notch signaling influences the maintenance and expansion of human HSCs in vitro is likely to involve several pathways regulated by Notch targets, such as CMyc, Cdk1, and PTEN, all of which have been shown to affect HSC expansion/maintenance. However, the dichotomy between the requirements for Notch in vitro vs its dispensable role in vivo is likely due to the complex nature of the BM niche, in which Notch signals may be only one of many factors, while in vitro it becomes critically important. By removing HSCs from their normal BM niche, these cells would require a strong Notch signal in vitro to compensate for the absence of the other signaling queues (supplemental Figure 4A).

We also showed that both in vitro–derived phenotypically defined HSC (CD45RA - CD90low) and phenotypically defined MPP (CD45RA - CD90 + ) populations were multipotent. However, upon transfer in to mice, in vitro–grown pHSCs were about 200 times more effective in generating CD45RA - cells than pMPPs, probably due to the fact that only CD90low cells were able to self-renew in vivo. In this regard, we found that in vitro–derived pHSCs approximated the SRC activity of freshly isolated HSCs when directly compared. Given the variability associated with in vivo manipulations, the use of a xenogeneic model system, and the relatively low numbers of cells injected, we can estimate that the 2 populations are likely similar in function.

The immediate stages following the differentiation of human CB HSCs are not fully appreciated, nor are the precursor-product relationships between these early progenitors clearly established. We now show that Notch signaling appears to delay the differentiation of CD45RA - cells in vitro, as the appearance of downstream CD45RA int and CD45RA hi cells is inhibited by Notch signals. We next characterized and showed that CD45RA - cells gave rise to CD45RA int, then to CD45RA int and then to CD45RA hi and CD10 + cells (P.B., M.M., J.C.Z.-P., manuscript in preparation) in vitro, following a progression in which each fraction of lymphomyeloid progenitors corresponds to a more differentiated stage of development.

CD45RA int cells obtained in vitro were shown at both population and clonal levels, and at the transcript level, to be functionally equivalent to phenotypically defined multipotent cells (pMPP-2), whereas the CD45RA hi cells qualify as LMP-like cells. Both subsets were able to reconstitute the same lymphomyeloid lineages when injected into immune-deficient mice. Interestingly, both subsets could reconstitute the thymus of immune-deficient mice, but when these in vitro cells had received Notch signals thymic reconstitution was greatly facilitated. In the absence of Notch signaling, thymic reconstitution occurs but only when higher numbers of cells are injected. So, Notch signals facilitate but are not required for thymic reconstitution. Similar functional subsets were characterized in CB (ex vivo CD45RA int). The presence of a weak erythroid differentiation potential in ex vivo Rhoint CD45RA int suggests that this phenotypically defined subset of CB cells could include both MPP-2 and GMP precursor cells. Interestingly, ex vivo CD45RA int Rhoint cells lacked erythroid potential, but had myeloid and lymphoid potential in vitro. Therefore, both subsets of ex vivo CD45RA int cells, Rhoint and Rholow, would appear to include cells that qualify as MPP-2 and LMPP, respectively.

Hao et al reported, at the clonal level, a Lin - CD34 + CD7 - early thymic lymphomyeloid precursor that has erythropoietic/myeloid potential, a finding unique to humans. The presence of thymic progenitors with such a potential correlates well with our in vitro–generated CD45RA int, as well as with ex vivo CD45RA int cells. Ex vivo CD45RA int Rhoint cells, described here as LMP-like cells, are at a more immature stage of differentiation than the cells recently described by Kohn et al, in that they possess GM and G myeloid potential and express CD45RA at only intermediate levels. Haddad et al characterized in CB an ex vivo CD34 + CD45RA hi cell, described as progeny of the in vitro–derived CD45RA int subset that shares characteristics with our in vitro–generated CD45RA int subset.

The model presented in supplemental Figure 4B summarizes our findings. The human hematopoietic hierarchy appears similar to the murine system except for the fact that unlike the murine system, human pMPP-like cells with erythroid potential are able to reconstitute the thymus. We showed that the Notch-dependent maintenance of human pHSCs leads to an arrest in the differentiation of each of the lymphomyeloid progenitors (MPP-2 and LMPP) derived from it.

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Authorship

Contribution: P.B. and P.S. performed and designed the research; M.M. and D.D. generated the OP9-DL4 lines; E.H. provided the umbilical CB samples; G.K. performed the flow cytometric cell sorting; and P.B. and J.C.Z.-P. wrote the paper.

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Notch signals are required for in vitro but not in vivo maintenance of human hematopoietic stem cells and delay the appearance of multipotent progenitors

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