CD34− Cells at the Apex of the Human Hematopoietic Stem Cell Hierarchy Have Distinctive Cellular and Molecular Signatures

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SUMMARY

In addition to well-characterized CD34+ hematopoietic stem and progenitor cells (HSPCs), the human hematopoietic stem cell (HSC) hierarchy contains a rare CD34− population with severe combined immunodeficiency-repopulating capacity. However, little is known about the molecular characteristics of these CD34− cells or their relationship to the CD34+ populations. Here, we show that the self-renewing Lin−CD34−CD38−CD93hi population contains cells that not only function as HSCs, but can also be placed above the CD34+ populations in the hematopoietic hierarchy. These cells have an active Notch pathway, in which signaling through Delta4 is crucial for maintenance of the primitive state, and combined signals from Jagged1 and TGF-β are important in controlling its quiescence. They are also refractory to proliferative signals and show a repressed canonical Wnt pathway, in part regulated by Notch. Overall, therefore, CD34− cells represent an immature and quiescent human HSC population maintained through a distinctive network of cellular signaling interactions.

INTRODUCTION

Although most human stem cell and progenitor activity is associated with CD34 expression, the existence of primitive hematopoietic cells in humans and rhesus monkeys that are negative for CD34 expression has been described (Goodell et al., 1997). Furthermore, a rare population of human cells devoid of CD34 expression with severe combined immunodeficiency (SCID)-repopulating cell (SRC) activity has also been identified (also defined as Lin−CD34−CD38−, or simply denoted as −/−) (Bhatia et al., 1998). It has additionally been reported that CD34 expression can be induced on these cells in vitro by hematopoietic cytokines (Nakamura et al., 1999); however, such cells have a weaker SCID-repopulating activity compared to primary, unmanipulated CD34+ hematopoietic stem and progenitor cells (HSCs and HPCs, also called HSPCs) (Nakamura et al., 1999). This implies that the induction of CD34 expression from primary −/− cells does not produce true CD34+ SRCs, because CD34 expression on human HSPCs can be downregulated, both in vitro and in vivo, without compromising repopulating capacity (Dao et al., 2003; Kuci et al., 2003). Therefore, it has been thought that the acquisition of CD34 expression on primary −/− cells could be merely an upregulation of this antigen and not a developmental differentiation toward a CD34+ SRC type.

We have previously shown that only the CD34+ (mouse homolog of AA4.1 or C1qR) subfraction within the Lin−CD34−CD38− cell population has SCID-repopulating capacity (Danet et al., 2002). Our understanding of the biology of primary Lin−CD34−CD38−CD93hi cells and the contribution of this rare population to the maintenance of human hematopoiesis remains limited. The unique cellular and molecular features that distinguish these cells from CD34+ HSPCs, as well as the signaling pathways that regulate their properties, have yet to be elucidated. Here, we report that this rare population has the ability to self-renew and is more primitive than the Lin−CD34−CD38−(+/−) cell population, because it is able to generate self-renewing +/+ cells in vivo and is more competent in serial transplants than the +/+ cell population. We further demonstrate that the combination of Notch, transforming growth factor β (TGF-β), and (repressive) canonical Wnt pathways plays a central role in their unique biology.

RESULTS

Lin−CD34−CD38−CD93hi Population Resides at the Top of the Human HSPC Hierarchy

It has previously been reported that human CD34+ cells can be generated from cord blood (CB) Lin−CD34− in immunodeficient mice, and that these CD34+ cells are capable of producing secondary grafts (Wang et al., 2003). To further elucidate the in vivo relationship between the −/− and +/+ populations, we began by purifying (>99% purity) the two populations from CB and transplanted them into primary nonobese diabetic (NOD)/SCID-β2−/− recipient mice using an equivalent number of SRCs (~1.2 SRCs/mouse; Figure S1A available online). At 3 weeks
Figure 1. In Vivo Self-Renewal Capacity of Different CB Populations

(A) Approximately 1.2 Lin− CD34+ CD38− (−/−; 75,000 cells) and Lin− CD34+CD38− (+/−; 750 cells) SRCs were intravenously (i.v.) injected into separate primary NOD/SCID−/− recipients, and at different weeks after transplant mice were sacrificed and human graft was determined. Calculation of the SRC frequency for each population can be seen in Figure S1. w, weeks.

(B) Approximately 5,000 −/−/+ and 750 +/− cells were i.v. injected into primary NSG recipients, and human graft was determined at 12 and 24–26 weeks after transplant. BMs were sampled at 12 weeks for determining human chimerism in mice, eq., equivalent.

(C) Secondary transplant with Lin− CD34+ CD38− (×12,500, ○25,000, △40,000, □60,000, ○90,000) and Lin− CD34+CD38− (◆1,250, ○2,400, ▲4,800, ■10,000) cells that were FACS-sorted from primary recipients’ BM originally transplanted with CB −/− (left) or +/− (right) cells.

(D) Ex vivo replating potential of engrafted Lin− CD34+CD38− (+/−/lo) cells isolated from primary NSG mice transplanted with CB −/−/+ or +/− cells at 24–26 weeks posttransplant (n = 6). (E) Frequency of +/−/lo populations found in the primary mouse BM at different time points and in the different mouse strains shown in (A) and (B).

(F) qRT-PCR for the expression of p16INK4a and p21CIP1 in the +/−/lo populations used for secondary transplant (C) and ex vivo replating assays (D), respectively (n = 5–7). ND, not detected.

Median bars are shown; *p ≤ 0.03, **p ≤ 0.003. See also Figure S1.

posttransplant, the +/− HSPCs were able to give rise to myeloid engraftment, whereas such engraftment from −/− cells was not seen until 6 to 9 weeks (Figure 1A). Although this myeloid engraftment was undetectable at 3 weeks in the −/− population, the cells did localize in the mouse bone marrow (BM), remaining undifferentiated and quiescent (Figure S1B) at early time points, suggesting that they had successfully homed. Notably, most of these homed −/− cells expressed CD93 (Figure S1B), confirming our previous finding that only the subfraction of −/− cells expressing the complement receptor CD93 show SRC activity (Danet et al., 2002). In CB, the Lin− CD34− CD38− population was almost all CD7+ (and also CD45RA+; Figure S2A), and therefore probably represents natural killer progenitors (Storms et al., 2000). As such, for achieving a more purified population, the CD34− CD93hi/lo(CD7/CD45RA)− (−/−/+ ) population was used in subsequent experiments.

We repeated the primary transplantation experiments using −/−/+ and −/−/lo populations and transplanted them into separate IL2Rγnull NOD/SCID (NSG) mice. Although we observed that −/−/+ cells yielded a >50-fold-lower engraftment than +/− cells at 12 weeks, the engraftment level produced by −/−/+ cells eventually matched or surpassed the levels produced by +/− cells at 24–26 weeks (Figure 1B).

For addressing the self-renewal capacity of primary −/− cells, BM from each group of NOD/SCID−/− mice was recovered at 12 weeks posttransplant and pooled, and human cells were again sorted via fluorescence-activated cell sorting (FACS) for populations phenotypically similar to the initial populations, CD45+Lin− CD34− CD38− (−/−/lo) and CD45+ Lin− CD34−CD38− (+/−/lo), then transplanted into secondary recipients.

Both −/−/lo and +/−/lo populations derived from primary −/−-grafts were able to engraft (Figure 1C); however, cells derived from primary +/− transplants only showed engraftment in the +/−/lo compartment (Figure 1C). Furthermore, +/−/lo cells derived from the primary −/− population were more efficient at engrafting secondary recipient mice as compared to those derived from primary +/−-grafts, in that at least an ~2-fold lower number of +/−/lo cells were required for engraftment (1,250 cells derived from −/− versus 2,400 or more cells derived from +/−; Figure 1C). Also, the −/−/lo population derived from the
primary -/- retained a CD93+CD117+HLA-DR- subpopulation, a phenotype corresponding to the original CB fraction previously shown to have SRC activity, whereas a similar subpopulation derived from +/- HSPCs was not present (Figure S1C) (Danet et al., 2002). As a result, -/-+ cells derived from CB +/- HSPCs, despite containing CD93+ cells, failed to engraft even at doses ~7-fold higher as compared to those derived from CB -/- cells (Figure 1C). Further phenotypic analysis demonstrated that these cells represent macrophages (CD117+ HLA-DR+CD41b-; Figure S1C).

Furthermore, +/-+ cells recovered from NSG mice transplanted with CB -/-+ cells have better ex vivo replating capacity as compared to the similar population derived from CB +/- cells (Figure 1D). A higher frequency of +/-+ cells was generated from the CB -/-+ and -/-+ cells compared to the +/- population in both animal models used (Figure 1E). Additionally, higher expression of CDKN2A (p16INK4a) and CDKN1A (p21CIP1) transcripts were found in +/-+ cells derived from CB +/- HSPCs than CB -/- or +/-+ cells, suggesting cell-cycle arrest and signs of exhaustion (Figure 1F).

**Lin-CD34-CD38-CD93hi HSCs Are Quiescent and Immature**

Given their ability to generate +/- cells, self-renew, and produce long-term engraftment with fewer signs of exhaustion, we were interested in whether the -/-+ cells are a more quiescent and primitive population as compared to the +/- population, with respect in particular to the four +/- subfractions that have been recently described based on the expression of CD45RA, CD90, and CD49f (CD45RA-CD90+CD49f+ or CD45RA+CD90-CD49f-), referred to herein as 90+/49f+/- (Notta et al., 2011). To delineate the developmental relationship between the -/-+ and +/- compartments, we performed a comparison with the aim of finding a +/-+ subpopulation closely related to the -/-+ cells. We also attempted to further dissect the -/-+ fraction using the same antigens. However, we found that -/-+ cells are CD45RA+; CD90- (Danet et al., 2002), and CD49f+ (Figure S2A). These cells are also CD133+ Tie2 (Figure S2B), and as such, we could not further divide the -/-+ population using recently published surface antigens important for subfraction +/- cells.

To assess their relative states of quiescence, we performed cell-cycle analysis and found that the -/-+ cells are largely Ki67 negative compared to the +/- population and all of its subpopulations that are Ki67 low (Figure 2A), indicating that the -/-+ population is highly quiescent. We confirmed that the -/-+ population expresses higher levels of cell-cycle regulators responsible for maintaining the cells in G0 state than any of the +/- subfractions, such as E2F4, RBL2 (p130), CDKN1B (p27KIP1), and p21CIP1 (Figure 2B). We further confirmed the increase of Rbl2 at the protein level (Figure 2C).

In addition to cell-cycle regulators, there are transcription factors that are known to be important for the development, activity, and proliferation of HSCs, which could be differentially expressed, as compared to +/- HSPCs (referred to here as “HSC fingerprint” transcription factors). Given that the +/- population has little to no clonogenic activity in vitro (Danet et al., 2002) and displays a delayed engraftment kinetic in vivo, we compared the expression of these HSC fingerprint transcription factors between the -/-+ and the +/- subpopulations. We observed that -/-+ cells express lower levels of GATA2, LMO2, TAL1, RUNX1A, and MYB (Figure 2D) compared to all the +/- fractions described here. The lower expression of these factors was again confirmed at the protein level for Lmo2 and Scl/Tal1 (Figure 2E). Taken together, Lin-CD34+CD38-CD93hi is the most quiescent population of cells with HSC activity and probably represents a very immature population.

**Active Notch Signaling in Lin-CD34-CD38-CD93hi HSCs**

Given the differences we observed between the populations with respect to cell cycle and the expression of HSC fingerprint transcription factors, we wanted to determine which signaling pathways regulate the quiescent state and immaturity of the -/-+ population. We first looked at the Notch pathway, because it has been shown to regulate differentiation and cell-cycle progression in human HSPCs (Delaney et al., 2005; Lauret et al., 2004).

We found that Notch3 expression was absent, but -/-+ cells express Notch1 and Notch2, as well as high levels of Notch4, compared to the +/- fractions (data not shown and Figure 3A). Additionally, -/-+ cells express the components of the Notch pathway, in particular DTX1 (DELTEx1, a potential negative regulator when high Notch activity is present), and the downstream target genes at a higher level compared to the other cell populations (Figure 3B). Hes1 protein was also found to be highly expressed in the -/-+ population (Figure 3C). Additionally, we looked at the cleaved forms of Notch, which are indicative of Notch activity. The -/-+ cells have very low levels of cleaved Notch1 but express the cleaved Notch2 (Figure 3D) and, perhaps most importantly, show nuclear expression of the intracellular form of Notch4 (N4-IC; ~30% nuclear localization; Figure 3D). Considering the fast turnover of this pathway (Fryer et al., 2004; Ilgman et al., 2011), this indicates that -/-+ cells have significant Notch4 receptor processing. Moreover, this expression pattern appears to be unique to the -/-+ population; we found that +/- HSPCs have a different pattern of Notch receptor cleavage (Figure 3D).

In order to see whether CB HSPCs are likely to receive Notch signals from their natural environment, we performed immunohistochemistry on tissue from the placental villi and umbilical cords (Figure 3E), where CB HSPCs would normally reside, especially the placenta villi, which represents a large surface area. We observed that the placenta villi and umbilical cord arteries and vein express Jagged1 (Jag1) and high levels of Delta4 (Dll4) (Figure 3E) but Delta1 could not be detected (Dll1; Figure S3A).

**Notch Signaling Is Important in the Maintenance of the Distinctive Features of Lin-CD34-CD38-CD93hi HSCs**

Because the -/-+ population has an active Notch pathway, we were interested in whether it played a significant role in the maintenance of these cells. As we are not yet able to maintain -/-+ cells in vitro without a supportive feeder layer for more than a few days before they become apoptotic, we were not able to silence the components of the pathway by using a small hairpin RNA-lentivirus approach to investigate its role. Instead, we cultured the cells on the stromal cell line, S17, which maintains the -/-+ cells and promotes their differentiation. Importantly,
S17 has no significant expression of Notch ligands, which allows it to be a vehicle for the overexpression of ligands of interest. We sorted /C0/+ cells and cultured them for 5 days on S17 alone or S17 expressing Dll1, Dll4, or Jag1. There was an increase in HES1 expression after coculture with each of the Notch ligands, and markedly so for Dll4 (Figure 4A), demonstrating that /C0/+ cells respond to these ligands. We then analyzed apoptosis, cell-cycle progression, expression of CD34, and ability to form colony-forming units (cfu) following coculture. No differences in apoptosis by AnnexinV (Figure S3 D) or upregulation of proapoptotic genes (Figure S3 E) were observed between the different conditions. We observed that Dll1 had no effect on the regulation of CD34 expression, cfu formation, and cell-cycle progression of /C0/+ cells (Figures S3B and S3C). Because we did not observe any effect of Dll1 on this immature population, at least on the parameters studied, or detect Dll1 expression in the placenta (Figure S3 A), we did not pursue further analysis of this ligand. Instead, we performed a more in-depth study of the effects of the other two ligands, given that in preliminarily experiments we observed that Dll4 and Jag1 appeared to specifically regulate differentiation and cell-cycle progression, respectively, of /C0/+ cells (Figure S3F). Indeed, in addition to decreasing CD34 expression (Figure 4B), coculture of /C0/+ cells on S17-Dll4 decreased primary cfus >2-fold without significantly altering the type of colonies generated (Figure 4C). However, in secondary cfu assays, cells that were cultured on S17-Dll4 increased cfus by ~10-fold (Figure 4C), suggesting a similar latency in differentiation as seen in the in vivo experiments.

In the presence of Dll4, many transcription factors in the cultured cells were maintained at the same level as uncultured /C0/+ cells compared to those cultured on S17 alone, including GATA2, LMO2, and TAL1 (Figure 4D). With respect to Jag1, a greater proportion of cells cocultured on S17-Jag1 were maintained in G0 compared to those cultured on S17 alone (Figure 4E). Expression analysis indicated that many G0-G1 transition regulators were maintained in S17-Jag1 cocultured cells at a similar level as uncultured cells (Figure 4F).

Genes commonly regulated through contact with either Jag1 or Dll4, such as SPI1 (PU.1), MPO, and p21CIP1 (presumably acting through the common RBPJ-k-Hes1 axis) could have
been triggered by the cleavage of different Notch receptors. However, we observed that many of the changes in gene expression after coculture on S17-Jag1 or S17-Dll4 were mutually exclusive (Figures 4D and 4F), without changes in HES5 and HEY1 expression (data not shown). Also, DTX1 expression was maintained in /C0+/ cells when interacting only with Dll4 (Figure S3G). Notably, the addition of the g-secretase inhibitor DAPT to the S17 cocultures resulted in the abrogation of the effects of Jag1 and Dll4, confirming that the effects seen acted through the canonical Notch pathway (Figure S3F).

As reported above, following /C0+/ cell transplantation, a period of latency is observed, and with this in mind we hypothesized that these immature HSCs could be receiving Notch signals from the mouse BM. We first confirmed that the same Notch receptors continue to be expressed on the /C0+/ cells when interacting only with Dll4 (Figure S3G). Notably, the addition of the g-secretase inhibitor DAPT to the S17 cocultures resulted in the abrogation of the effects of Jag1 and Dll4, confirming that the effects seen acted through the canonical Notch pathway (Figure S3F).

To examine its role, we temporarily blocked the Notch pathway in vivo before the cells began differentiating (at 5 weeks posttransplant) by administering DAPT, either 4 times (4 3 DAPT) or 8 times (8 3 DAPT), to mice transplanted with /C0+/ cells. We observed an /C24 5-fold increase in engraftment with 4 3 DAPT compared to controls, and 8 3 DAPT resulted in an additional increase in engraftment (14-fold total; Figure 4I). However, this increase in engraftment appears to be at the expense of the CD34+ compartment (Figure 4J). This observation is further supported by the limited cfu capacity of the human cells isolated from mice treated 8 3 (Figure 4K). Without the known toxicity of DAPT treatment, we could expect an abrogation of the engraftment if a continuous DAPT treatment was performed.

To better understand any differential role of the Notch pathway between the /C0+/ and +/- cells, we performed similar experiments with the four +/- subfractions. We first observed that the 90*49f* population, despite having a lower primary cfu capacity, had better replating capacity compared to other subfractions.
Figure 4. Primitive and Quiescent Features of Lin−CD34−CD38−CD93hi HSCs Maintained by Notch Signaling

(A) HES1 expression in −/−/+ cells that have been cultured for 5 days on S17 cells expressing the indicated ligand. Cells cultured on S17 were used as a reference (n = 3).

(B) CD34 expression on noncultured (NC)−/−/+ and 5-day-cultured cells on S17 cells expressing the indicated ligand (n = 5). Representative FCM plot can be seen in Figure S3 F.

(C) Primary and secondary cfu assays evaluated with −/−/+ and 5-day-cultured cells on S17 or S17-Dll4 stromal cell lines (n = 3, each in triplicate).

(D) qRT-PCR for the expression of different differentiation and “HSC fingerprint” genes as shown in (A). NC−/−/+ cells were used as a reference population (n = 3–5).

(E) Cell-cycle analysis of NC−/−/+ and 5-day-cultured cells on S17 cells expressing the indicated ligand (n = 3–5).

(F) Expression of different cell-cycle regulator genes as in (A). NC−/−/+ cells were used as a reference population (n = 3–5).

(G) Representative FCM histograms for Notch receptor expression on human−/−/+ cells in primary NSG recipients’ BMs that were transplanted with ~5,000 CB−/−/+ cells 4–6 wks earlier. MFI ratio values are shown (n = 2, each with pool of 2–3 BMs).

(legend continued on next page)
TGF-β Signaling Also Participates in the Maintenance of the Quiescent State of Lin⁺CD34⁻CD38⁻CD93⁺ HSCs

During our analyses, we observed that the expression of p57<sup>KIP2</sup> (CDKN1C) is the most abundant cyclin-dependent-kinase (CDK) inhibitor in −/−/+ cells, and its expression was reduced following coculture of −/−/+ cells with S17 but remained higher than that of p21<sup>CIP1</sup> in uncultured primary −/−/+ cells (data not shown). Given that p57<sup>KIP2</sup> is a known target of TGF-β signaling and that TGF-β1 is highly expressed in the placenta villi (but only slightly in the mesenchyme surrounding the umbilical cord arteries and vein) (Figure 5A), we wanted to evaluate whether this pathway might also contribute to the cell-cycle status of −/−/+ cells.

First, we confirmed that −/−/+ cells express high levels of the TGF-β receptors (TGF-βRs), downstream effector genes, TGFβ1, and some of its activators via quantitative RT-PCR (qRT-PCR) (Figure 5B). We further confirmed the expression of TGRβ1R (Figure S5A), the nuclear localization of Foxo3a (Figure S5B), and the phosphorylation of Smad-3 (p-Smad3) (Figure S5C). The expression of these components of TGF-β signaling suggests that these cells are capable of receiving TGF-β signals efficiently from the placenta villi. We therefore first tested the response of these cells with TGF-β in vitro by culturing −/−/+ cells with TGF-β1 and observed an upregulation of p-Smad3 (Figure 5C). Using the S17 coculture system we pulsed the −/−/+ cells with low doses of TGF-β1 (2.5 ng/ml) and/or a function-blocking antibody twice over the course of 5 days. We observed that TGF-β1 was able to block cell-cycle progression (Figure 5C) and found that expression of p57<sup>KIP2</sup> and E2F1 was brought back to levels similar to those seen in uncultured −/−/+ cells (Figure SD), an effect not observed with Jag1.

Second, we wished to evaluate the importance of the TGF-β pathway in −/−/+ cells in vivo. Following primary transplantation, the levels of p-Smad3 remain unregulated in the engrafted −/−/+ population (Figure 5E), and we hypothesized that temporarily blocking the TGF-β pathway would cause the −/−/+ cells to exit G0 and result in better engraftment levels. To test this, we injected mice with 4× or 8× SB431542, a TGF-β inhibitor, 5 weeks after they were transplanted with −/−/+ cells. We observed an ~7-fold increase in the engraftment levels of mice

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(Figure S4A). Additionally, this 90*49f<sup>+</sup> population gave a higher level of engraftment when transplanted with a limited cell dose as compared to others (Figure S4B). Altogether, this supports the report that this population is highly enriched with HSCs (Notta et al., 2011).

Upon culture with different Notch ligands, each subfraction responded differently to the different ligands (data not shown) despite a comparable upregulation of HES1 in the different subpopulations (Figure 4L). We focused on the impact of Dll4 given that it had the strongest effect in repressing primary cfu formation while enhancing the replating potential of the cells as compared to cultures on S17 alone, specifically on the two CD90<sup>-</sup> populations (data not shown, Figure 4M). Indeed, coculture on S17-Dll4 repressed CEBPA, MPO, and GATA1 expressions in these subpopulations (Figure S3H) as compared to the control condition. In contrast, interaction with Dll4 induced a substantial upregulation of GATA1 expression in the two CD90<sup>-</sup> subpopulations that resulted in an enhanced burst-forming unit erythroid (BFU-E) formation compared to cultures with S17 alone (Figure 4M). In analyzing cell-cycle regulation, we observed an increase in p21<sup>CIP1</sup> expression that resulted in an increase in G1 in all subpopulations cultured on S17-Jag1 (Figures 4N and S3).

We then went on to investigate whether this pathway is important for the maintenance of +/− HSPCs in vivo as we observed for the −/−/+ population. We confirmed that the Notch receptor expressions were upregulated in the −/+ subfractions in the BM recovered from primary recipients (Figure 4O). We also observed that Hes1 is similarly expressed in the four fractions, albeit at a lower level than in CB cells (Figures 3C and 4P). However, when we temporarily blocked the Notch pathway by administering DAPT 4× or 8× to mice transplanted with +/− HSPCs, we observed an ~50% reduction in engraftment only when mice were treated 8× as compared to control (Figure 4Q). Furthermore, the 8× treatment reduced the −/+ compartment (Figure 4R) by affecting all the −/+ subfractions equally (Figure S3J). Altogether, these results suggest that although both −/−/+ and −/+ populations can respond efficiently to Notch signals in vitro, albeit differently; the Notch pathway plays a larger role in controlling the differentiation of −/−/+ cells to +/− HSPCs than the latter to subsequent progenitors (e.g., CD34⁺CD38<sup>-</sup>) in vivo.
treated 4 × with SB431542; however, when mice were treated 8 ×, engraftment was almost abrogated (Figure 5F), suggesting that the TGF-β pathway is strongly involved in the maintenance of −/−/+/ cells in a quiescent state in vivo. Unlike the DAPT treatment, the frequency of +/− cells generated from −/−/+/ cells after SB431542 treatment was the same as for controls (data not shown), suggesting that the cell-cycle progression of −/−/+/ cells is not necessarily coupled with differentiation.

Again, to understand the relationship between the −/−/+/ population and the +/− HSPCs, we looked at TGF-β signaling in the four +/− subfractions. First, we observed that p-Smad3 was present (Figure 5G) and all the fractions were able to respond equally to TGF-β1 in the S17 coculture system, demonstrated through a decrease in cycling cells (Figure 5H). However, when we temporarily blocked the TGF-β pathway in vivo by administering SB431542, we observed no effect on the level of engraftment, even after 8 × (Figure 5I). Additionally, we observed a low level of p-Smad3 expression, which is indicative of low TGF-β signaling, in all the engrafted +/− subfractions (Figure 5J). These observations are in contrast to those seen in the −/−/+/ cells and

**Figure 5. Quiescent Nature of Lin−CD34−CD38−CD93hi HSCs through the TGF-β/p57kip2 Axis**

(A) Double-fluorescent immunostaining for TGF-β1 and αSMA or CD31 on paraffin sections of human placenta bed and umbilical cords (scale bars represent 100 μm).

(B) Expression of different components of the TGF-β pathway in the different CB HSPC populations by qRT-PCR. −/−/+ cells were used as the reference (n = 3–5).

(C) Cell-cycle analysis of NC −/−/+ cells and 5-day-cultured cells on S17 stromal alone or with two pulses of TGF-β1 (2.5 ng/ml) or TGF-β1+TGF-β1-blocking antibody (n = 4).

(D) qRT-PCR for different cell-cycle regulator genes as in (C). NC −/−/+ cells were used as the reference population (n = 3–5).

(E) Representative FCM histogram of p-Smad3 expression in engrafted −/−/+ cells in primary recipients’ BM transplanted with ~5,000 CB −/−/+ cells 4–6 weeks earlier. MFI ratio is shown (n = 2, each with a pool of 2–3 BMs).

(F) Approximately 5,000 CB −/−/+ cells were transplanted into each NSG mouse. Five weeks later, mice were treated either with vehicle or 4 × or 8 × with SB431542. Engraftment was analyzed at 12 weeks. Median bars are shown.

(G) p-Smad3 expression in CB +/− HSPCs and different Lin−CD34−CD38−CD45RA−CD49f− fractions (n = 4).

(H) Cell-cycle analysis of +/− subfractions cultured for 5 days on S17 stroma alone or with two pulses of TGF-β1 (2.5 ng/ml) or TGF-β1+TGF-β1-blocking antibody (n = 4).

(I) 750 CB +/− cells were transplanted into NSG mice, and 5 weeks later mice were treated with vehicle or 4 × or 8 × with SB431542. Engraftment was analyzed at 12 weeks. Median bars are shown.

(J) p-Smad3 expression in different human +/− subpopulations in primary NSG recipients’ BMs at 4–6 weeks posttransplantation with CB 750 +/− HSPCs. MFI ratio values are shown (n = 5).

± and error bars shown represent SD; *p ≤ 0.03, **p ≤ 0.003. See also Figure S5.
are indicative that TGF-β signaling plays a larger role in the maintenance of the quiescent state of /C0/+ cells as compared to +/- HSPCs in vivo, at least in the context of xenograft environments.

**Lin**CD34**−**CD38**−**CD93** hi** HSCs Have a Repressed Canonical Wnt Pathway

Our observations indicated that /−/−/+ cells are refractory to signals that induce proliferation. We therefore decided to investigate the canonical Wnt pathway, given that it has been shown to drive HSC proliferation both in vitro and in vivo (Kirstetter et al., 2006; Scheller et al., 2006).

Using a T cell factor/lymphoid-enhancing factor (TCF/LEF) reporter assay, we detected an absence of basal canonical Wnt activity in the /C0/+ population as compared to the +/-HSPCs (Figure S6A). Additionally, we observed a weak stabilization and accumulation of β-catenin when stimulated with canonical Wnt3A compared to all other +/- fractions (Figure 6A). Further activation of the pathway through inhibition of GSK3β by LiCl (Figure 6A) did not alter the outcome, indicating that the /−/−/+ population did not have a higher level of β-catenin proteasomal degradation compared to the other populations. These results suggest that these immature cells have minimal Wnt pathway activity and are resistant to canonical Wnt ligands. Looking at the expression of the different receptors, we could not find any substantial differences that could account for the different Wnt activity seen in the different hematopoietic populations (Figure S6B).

An alternative explanation could lie downstream of the receptors. Indeed, we observed higher levels of expression of AXIN1 and CSNK1A1 and lower levels of TCF7L2 expression (Figure 6B) (at the protein level also, Figure S6C), confirming low levels of canonical Wnt signaling in /C0/+ cells compared to the other CD34**+** populations. Interestingly, we found high levels of LEF1, but this was limited to the short splicing variant form, which lacks the β-catenin binding domain (SF-LEF1, Figure 6B). This Lef1 isoform has been demonstrated to function as a repressor for the same genes that the long-form Lef1/β-catenin complex activates (Hovanes, et al., 2001). As such, we wondered whether this short form of Lef1 had any functional significance. Again, given that it is difficult to manipulate /C0/+ cells for any significant period of time in vitro, we were not able to overexpress the long form of Lef1 (LF-Lef1) to compete with the endogenous SF-Lef1 that is more abundant in this population. As an alternative approach, we transiently overexpressed ΔLef1 (a form with a

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**Figure 6. Repressed Canonical Wnt Pathway in Lin**CD34**−**CD38**−**CD93** hi** HSCs**

(A) FCM staining for total β-catenin expression in the stated CB HSPC subpopulations. Noncultured, NC; stimulated with Wnt3A alone, +; or in combination with LiCl, ++; for 8 hr (n = 6).

(B) qRT-PCR for the expression of components of the canonical Wnt pathway in the different CB HSPC populations. +/- cells were used as the reference (n = 4).

(C) Primary cfu colonies generated from noncultured (NC); cultured (C); or nucleofected then cultured without any plasmid (Nu+C), with the construct over-expressing ΔLEF1, or with the control vector (pcDNA3) +/- cells.

(D) Secondary colonies derived from +/- HSPCs that were subjected to different conditions as described in (C).

(E) Expression of a selected group of genes in +/- HSPCs that have been treated as in (C). Cultured cells were used as the reference (n = 3).

(F) Expression of different LEF1 isoforms in NC −−/−/−/+ cells and after culturing in different S17 stromal cell lines for 5 days. Cells cultured on S17 were used as the reference (n = 3).

Error bars shown represent SD; *p ≤ 0.03. See also Figure S6.
Figure 7. Cellular and Molecular Features of BM Lin−CD34−CD38−CD93hi Cells

(A) Frequency of −/−/+ cells and different +/- subpopulations found in CB (left), GMPB (middle), and BM (right), n = 5–7. Representative FCM plots of this analysis are shown in Figure S7.

(B) Cell-cycle profile of human adult BM −/−/+ and +/- populations determined by Ki67 and DAPI staining (n = 3).

(C) qRT-PCR for different cell-cycle regulator genes. The −/−/+ population was used as the reference population (n = 3).

(legend continued on next page)
mutated β-catenin binding domain that mimics the functions of SF-Left1) in +/− HSPCs to see whether an abundance of this variant would compete with the long form and repress canonical Wnt or other non-Wnt target genes. We nucleofected +/− HSPCs with the ΔLEF1 expression vector and subsequently cultured them for 3 days with cytokines known to promote differentiation. When overexpressing ΔLEF1, the capacity of the cells to form primary colonies was reduced compared to the controls to numbers similar to the cultured, nonnucleofected condition (Figure 6C). However, in replating assays, the numbers of secondary colonies were increased compared to controls (Figure 6D). From this it appears that the ΔLEF1 variant was able to suppress their differentiation and increase replating capacity. Additionally, we analyzed these cells by qRT-PCR for genes affected by the nucleofection with ΔLEF1. Of the panel of genes analyzed, CEBPA, TCF7L2, and CTNNB1 (β-catenin) were downregulated in the ΔLEF1 nucleofected cells as compared to the control vector (Figure 6E). CEBPA is not a canonical Wnt target gene, given that expression was not increased following stimulation with Wnt3A in vitro, in contrast to target genes such as TCF7L2, CTNNB1, and CCND1 (Figure 6D).

To investigate whether this reduction in CEBPA was linked to that observed following coculturing of −/+ cells with DiI4 (Figure 4D), we analyzed SF-LEF1 expression following exposure to DiI4. Interestingly, we observed that −/+ cells maintained their SF-LEF1 levels upon treatment with DiI4 (Figure 6F), suggesting that DiI4 acts on −/+ cells by blocking differentiation partly through the regulation of SF-LEF1 expression.

The Lin−/CD34−/CD38−/CD93hi Population Exists in Human BM and Has Similar Features to Its CB Counterpart

It has been demonstrated that human BM Lin−/CD34− cells can engraft a preimmune mouse model (Zanjani et al., 1998), BM Lin−/CD34−/CD38− cells can engraft NOD/SCID mice (Bhatia et al., 1998), and human granulocyte colony-stimulating factor-mobilized peripheral blood (GMPB) Lin−/CD34−/CD38− cells can repopulate NOD/SCID mice (Lemoli et al., 2003). With this in mind, we were interested to see whether a cell population phenotypically similar to the CB −/−/+ cell population existed in human adult hematopoietic tissues. Phenotypically, these cells can be found in GMPB and BM samples, albeit at a 10-fold-lower frequency (relative to CD34+ cells) compared to CB (Figure 7A). We were able to identify a nonendothelial hematopoietic population comprising ~0.174% and ~0.194% of BM and GMPB CD34+ cells, respectively (Figures 7A, S7A, and S7B). Interestingly, in BM, we showed that −/+ cells were more quiescent than +/− HSPCs (Figures 7B and 7C). We also observed that these BM −/+ cells had substantial cell-surface expression of different Notch receptors (Figure 7D) and higher expression of the components of the Notch pathway and Hes1 expression compared to +/− HSPCs (Figures 7E and 7F). Furthermore, similar to their CB counterparts, these quiescent BM cells had a higher level of basal TGF-β signaling but low or absent basal canonical Wnt activity (Figures 7G–7J). Collectively, these data support the existence of a −/+ cell population in adult BM, which shares some of the cellular and molecular features of its CB counterpart.

DISCUSSION

The CD34− SRC was first described in 1998 by Dick and colleagues (Bhatia et al., 1998) and has subsequently been confirmed in other studies (Danet et al., 2002; Wang et al., 2003). Contrary to the report by Dao et al. (2003), our results are consistent with previous observations that +/− HSPCs are not able to give rise to a functionally equivalent −/+ population capable of producing secondary grafts in vivo when employing the same primary and secondary recipient mouse model (Wang et al., 2003), in that −/+ cells derived from +/− HSPCs are differentiated cells, such as macrophages, and not −/+ cells that have downregulated CD34 expression (Dao et al., 2003). Building on this, we demonstrate the self-renewal capacity of the CB CD34− SRC in the mouse model, which Wang et al. (2003) failed to show, perhaps because of the lower number of cells injected as compared to this study.

We show that −/+ cells, when uncultured, have almost no plating capacity and produce a delayed and low level of primary engraftment at 12 weeks posttransplant. A close inspection of published data shows that all of the −/+ subfractions that have been recently described can give rise to myelo-lymphoid repopulation at 5 weeks (Notta et al., 2011). Throughout our study of the −/+ cells, we did not observe an enhanced level of engraftment at early time points by injecting more cells or by changing mouse models. We obtained quite similar levels of engraftment in NOD/SCID-J2−/+ and NSG mice as in our previous report in NOD/SCID mice, reflecting comparable SRC frequencies in different mouse models when using intravenous delivery: 1 in ~75,000 –/+ cells in NOD/SCID (D.B., unpublished data), 1 in 62,500 –/+ cells in NOD/SCID-J2−/−, and 1 in ~7,500 –/+ cells in NOD/SCID (Danet et al., 2002), compared to 1 in ~6,100 –/+ cells in NSG (F.A.-A., unpublished data). This differs from the well-documented repopulating behavior of +/− HSPCs, wherein SRC frequency changes with the mouse strain. Also, unlike +/− cells, where the SRC frequency can be increased 15-fold when injected intrabone (Yahata et al., 2003), we observed only a ~2-fold increase with −/+ cells using...
the same route (F.A.A., unpublished data). However, the engraftment level produced by $-/-/+\) cells matched or surpassed the level generated by $+/-$ HSPCs at 6 months post-transplant. We suggest that interpretations should be made with caution when comparing SRC frequencies between $-/-/+\) and $+/-$ cells (or with any of the subfractions) at a given time point, because these populations have distinct engraftment kinetics. More importantly, the newly generated $+/-$ cells from CB CD34$^-$ cells have better secondary repopulation and replating capacities compared to those from CB $+/-\) HSPCs.

Having identified the potential of this population, we wished to characterize the cellular and molecular mechanisms that might dictate its behavior. First, we demonstrate that $-/-/+\) cells are highly quiescent, a hallmark of stem cells. Furthermore, the expression of important HSC developmental regulators, denoted here as an HSC fingerprint, suggests that the $-/-/+\) population represents an immature cell type. Indeed, the molecular features indicative of a quiescent and primitive cell state are reflected by the in vitro and in vivo behaviors observed.

We were therefore interested in dissecting the signaling pathways responsible for controlling the unique features of the $-/-/+\) population. We found that the Notch pathway is not only active in freshly isolated CB $-/-/+\) cells but also plays an important role in the maintenance of the immature and quiescent features of these cells. We show that $-/-/+\) cells demonstrate a unique Notch4 expression and cleavage pattern. Moreover, when these cells interact with DI4, but not with DI1 or Jag1, the upregulation of CD34 expression and induction of clonogenic activity are halted, which is accompanied by the repression of HSC fingerprint transcription factors. This, in combination with the high DI4 expression found in their native environment, supports the idea that the immature features of $-/-/+\) cells are dictated, at least in part, through this pathway.

Despite each of the $+/-$ subfractions responding distinctively in vitro to each of the ligands, our data suggest that $+/-$ cells are less dependent on Notch signals in vivo compared to $-/-/+\) cells. When we temporarily blocked this pathway, $-/-/+\) HSCs gave rise to more $+/-$ HSPCs, resulting in a higher level of engraftment in vivo; however, a prolonged inhibition led to exhaustion of the system. This is in contrast to the effect on $+/-$ HSPCS, wherein the effects of Notch inhibition are only seen after extended inhibition of this pathway. One of the most interesting aspects of this study is that many of the changes in gene expression in $-/-/+\) cells and in different $+/-$ subpopulations after coculture with different Notch ligands appear to be exclusive. This suggests that the effects of Notch signaling are context dependent and cell specific and that attention is therefore required when different cell populations are compared.

With respect to cell-cycle regulation, it appears that the quiescent state of $-/-/+\) cells is regulated, at least in part, by a combination of Jag1 and TGF-β signaling. Our results suggest that Notch signaling driven by Jag1 binding can sustain cells in G0 by blocking the G0-G1 transition, whereas TGF-β1 stimulation can sustain p57$^{kip2}$ expression. Given that we saw considerable TGF-β signaling in $-/-/+\) cells, we believe that p57$^{kip2}$ might be one of the key factors that prevent $-/-/+\) cells from entering and progressing through G1 phase. Furthermore, we have shown that different $+/-$ subfractions can respond efficiently to TGF-β signals in vitro as previously shown for the bulk $+/-\) population (Scandura et al., 2004). Interestingly, only $-/-/+\) cells seem to have a more active TGF-β signaling pathway in vivo as compared to any of the $+/-$ subpopulations. As a result, engrafted $-/-/+\) cells respond when this pathway is inhibited, whereas $+/-$ HSPCs do not.

One of the most intriguing characteristics of $-/-/+\) cells is their unresponsive nature to external stimuli, something that has been a major obstacle to their study both in vitro and in vivo. With respect to this, we decided to focus on the canonical Wnt pathway, one that might act to block differentiation. We demonstrate that $-/-/+\) cells have an absent or low basal canonical Wnt pathway and are refractory to canonical Wnt signals. Unexpectedly, we observed an exclusive expression of the short isoform of the transcription factor LEFT1 in this quiescent immature cell population. We were able to confirm the importance of this variant of LEF1 by showing that it not only represses the expression of TCF4 and CTNNB1, but also of CEBPA, which is normally induced by hematopoietic cytokines. This suggests that SF-Left1 in $-/-/+\) cells represses the expression of some of the components of the canonical Wnt pathway, rendering them unable to convey Wnt signals, alongside transcription factors induced by other stimuli. Remarkably, we also found that the expression of SF-LEFT1 seems to be regulated by Notch signaling through DI4. This finding provides additional support for the importance of DI4 interactions in $-/-/+\) cells and suggests an additional connection between the Notch pathway and (repressive) canonical Wnt signaling in this immature and quiescent population.

We attempted to further subfractionate this $-/-/+\) population with antigens recently used to subfractionate the $+/-$ HSPC population, hoping to find a potential developmental link with the $+/-$ compartment. Although phenotypically these immature $-/-/+\) HSCs differ from the 90$^{th}$ subfraction, both populations appear to share some biological features. For example, in vitro replicating potential, response to DI4 signals, and in vivo self-renewal capacity are similar between these two populations as compared with the remaining $+/-$ subfractions (Notta et al., 2011). However, none of the $+/-$ subpopulations, including the 90$^{th}$ HSCs, have the degree of dormancy and immaturity that the $-/-/+\) HSCs have, as seen through their different engraftment kinetics, SRC frequencies, and response to proliferative signals. These and other results strongly suggest that the CD34$^-$ SRC represents a distinctive class of human HSCs (Bhatia et al., 1998; Danet et al., 2002; Wang et al., 2003). We would also like to emphasize that antigens that are currently used to subfractionate the $+/-$ population are not of any use in further subdividing the $-/-/+\) cells, and interpretation of comparative studies between such different cell types should be performed with caution.

Finally, we uncovered only small differences between the four $+/-$ subfractions in terms of their molecular signatures and signaling pathways at the basal level. These findings were not totally surprising, given that published microarray data did not reveal differences between the subfractions for the signaling pathways illustrated here (Notta et al., 2011).

Taking these results together, we provide an extensive cellular and molecular characterization of $-/-/+\) cells (Figure 7L). The data strongly supports the idea that the $-/-/+\) population
Characterization of Human CD34⁻ HSCs

contains bona fide HSCs and can be placed in the human hematopoietic hierarchy above +/- HSBCs. Given the existence of these CD34⁺ cells in human adult hematopoietic tissues (Bhatia et al., 1998; Lemoil et al., 2003; Zanjani et al., 1998), we propose that these +/-/+ HSCs could represent the human equivalent of the mouse +/-CD45⁻lin Rho SP and/or the CD34⁺KLSCD150⁺ HSCs (Dykstra et al., 2007; Morita et al., 2010) based on their similar in vivo engraftment behavior of delayed primary and efficient secondary engraftment.

This work provides the foundation for future research into the hierarchy of human HSCs and the developmental relationship of these cells with +/- long-term HSCs. Importantly, the characterization of the CD34⁻ compartment provides the implication for both scientific and clinical communities that these cells might be of importance for human cell therapy.

EXPERIMENTAL PROCEDURES

Primary Cells
CB was obtained after informed consent at St Bartholomew’s and the Royal London Hospitals. The protocol was approved by the East London Ethical Committee. Two or more CB samples were pooled for each experiment, and mononuclear cells (MNCs) were obtained by density centrifugation using Ficoll-Paque (GE Healthcare Life Sciences). Mature cells were removed via immunomagnetic depletion using StemSep Human Hematopoietic Progenitor Cell Enrichment Cocktail (STEMCELL Technologies) according to the manufacturer’s protocol. In some experiments, MNCs depleted of CD34⁺ cells with the EasySep Human CD34 Positive Selection Kit were used to isolate lineage-negative CD34⁻ cells. Cells lacking lineage markers (Lin⁻) were used for all intracellular immunostaining for determining the activity of the signaling pathways and were obtained as fast as possible, with all the procedures performed cold (including staining, cell washing, and cell collection during depletion) and carried out with PBS containing 2% CB serum in order to preserve as much as possible the natural activity of the pathways. Human BM MNCs were purchased from Lonza Biologics, and Lin⁻ cells were obtained as described for CB MNCs.

Immunophenotyping
Cells were incubated with antibodies (see Table S1) for 30 min at 4°C in PBS/2% fetal bovine serum, washed, and resuspended in DAPI-containing solution before analysis on an LSR II flow cytometer (BD Biosciences). When unconjugated or biotin-conjugated primary antibodies were used, the appropriate secondary antibody or streptavidin binding step was used, respectively, with gated or biotin-conjugated primary antibodies were used, the appropriate secondary antibody or streptavidin binding step was used, respectively, with washes performed between incubations. For all multicolor analysis, Fluorescence Minus One controls—wherein all other specific staining is in the same threshold. Gates were set up to exclude nonviable cells and debris during analysis.

Please refer to Supplemental Experimental Procedures for the remaining methodologies, materials, and reagents used for the preparation of this manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.05.025.

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