

# CD34 expression does not correlate with immunophenotypic stem cell or progenitor content in human cord blood products

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## Key Points

- The CD34<sup>+</sup> compartment of human cord blood contains a range of HSPC immunophenotypes, among which the Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>CD127<sup>+</sup>CLP is rare.
- There is no correlation between the frequencies of CD34<sup>+</sup> cells and immunophenotypic HSC in umbilical cord blood products.

## Introduction

Since the seminal studies by Till and McCulloch that first characterized the self-renewing, multipotent hematopoietic stem cell (HSC) in a murine model,<sup>1-5</sup> the understanding of human hematopoiesis has accelerated through the delineation of human HSC and progenitor subsets by the expression of cell surface antigens.<sup>6,7</sup> Although the differentiation capacities of hematopoietic stem and progenitor cells (HSPCs) and their corresponding immunophenotypes have been extensively studied, little is known about the relative distribution of HSPC subsets between individuals. Here, we characterize the heterogeneity of the CD34<sup>+</sup> compartment within human umbilical cord blood (UCB) and note a lack of correlation between the frequencies of CD34<sup>+</sup> cells and progenitor subtypes. Additionally, we identified common lymphoid progenitors (CLPs) as a particularly rare population.

## Methods

### Study design

Immunophenotyping of CD34-enriched cord blood products was performed on 50 samples. As this is a pilot study, the lack of comparable analyses in the existing literature precludes calculation of ideal sample size and predicted power a priori. Resource availability (antibodies, time, and laboratory space) was the primary determinant of sample size.

### Isolation of CD34<sup>+</sup> HSPCs

HSPCs were isolated from the UCB of term deliveries ( $\geq 34$  weeks of gestation) at Lucile Packard Children's Hospital-Stanford. UCB collections were performed through the Binns Program for Cord Blood Research with donor consent and institutional review board approval. Within 24 to 48 hours of collection, mononuclear cells obtained by density gradient separation of whole blood (Ficoll Paque Plus, GE Healthcare; 400g, room temperature, 30 minutes, deceleration off) were labeled with the human CD34 Microbead Kit Ultrapure for positive selection of CD34<sup>+</sup> HSPCs according to the manufacturer's protocol (Miltenyi Biotec, San Diego, CA).

### Immunophenotyping of CD34<sup>+</sup> HSPCs

Following positive selection, 100 000 CD34<sup>+</sup> cells per sample were stained with antibodies against markers of HSPC differentiation at optimal concentrations (supplemental Table 1). At least 30 000 live events (propidium iodide negative) were acquired on a BD FACS Aria II flow cytometer and analyzed using FlowJo software (version 9).

### Statistics

Data are reported in the text as median (interquartile range [IQR]). The relationship between CD34<sup>+</sup> expression and progenitor subtypes was assessed using nonparametric Spearman correlation and simple linear regression (supplemental Table 2).

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The authors agree to make renewable materials, data sets, and protocols available to other investigators without unreasonable restrictions. Requests can be directed to the corresponding author by e-mail at sruthim@stanford.edu.

The full-text version of this article contains a data supplement.

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**Table 1. Distribution of HSPC subpopulations by immunophenotype, percentage, and absolute cell count**

Cell type	Immunophenotype	Percentage	Absolute no.
HSC	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> CD45RA <sup>-</sup> CD90 <sup>+</sup>	6.7 (0.1-17.3)	5.8 × 10 <sup>4</sup> (3.9 × 10 <sup>2</sup> -9.4 × 10 <sup>5</sup> )
MPP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> CD45RA <sup>-</sup> CD90 <sup>-</sup>	10.6 (1.1-37.5)	7.9 × 10 <sup>4</sup> (1.4 × 10 <sup>3</sup> -2.5 × 10 <sup>6</sup> )
CMP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>+</sup> CD45RA <sup>-</sup> CD123 <sup>+</sup>	33.6 (3.2-45.8)	3 × 10 <sup>5</sup> (4.1 × 10 <sup>3</sup> -1.9 × 10 <sup>6</sup> )
GMP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>+</sup> CD45RA <sup>+</sup> CD123 <sup>+</sup>	20.8 (7.1-51.9)	1.9 × 10 <sup>5</sup> (1.8 × 10 <sup>3</sup> -2 × 10 <sup>6</sup> )
MEP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>+</sup> CD45RA <sup>-</sup> CD123 <sup>-</sup>	11.4 (3.8-22.9)	8.9 × 10 <sup>4</sup> (1.4 × 10 <sup>3</sup> -6.8 × 10 <sup>5</sup> )
CLP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>+</sup> CD127 <sup>+</sup>	0.0071 (0-0.064)	5.6 × 10 <sup>1</sup> (0-7.8 × 10 <sup>2</sup> )
Pro-B/NK	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>+</sup> CD45RA <sup>+</sup> CD10 <sup>+</sup>	1.2 (0.2-4.4)	1.1 × 10 <sup>4</sup> (1.5 × 10 <sup>2</sup> -2 × 10 <sup>5</sup> )
MLP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> CD45RA <sup>+</sup> CD10 <sup>+</sup>	1.1 (0.3-4.6)	9 × 10 <sup>3</sup> (4.4 × 10 <sup>2</sup> -1.1 × 10 <sup>5</sup> )
LMPP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> CD45RA <sup>+</sup> CD90 <sup>v</sup>	3.3 (0.9-16.5)	3.1 × 10 <sup>4</sup> (3.9 × 10 <sup>2</sup> -1.1 × 10 <sup>6</sup> )

Data are shown as median (range). Percentages are among live CD34<sup>+</sup> cells.

CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; LMPP, lymphoid-primed multipotent progenitor; MEP, megakaryocyte/erythrocyte progenitor; MLP, multipotent lymphoid progenitor; MPP, multipotent progenitor; pro-B/NK, B/natural killer cell progenitor.

## Results and discussion

We analyzed the distribution of immunophenotypes within UCB-derived CD34<sup>+</sup> HSPCs harvested 24 to 48 hours after delivery (*n* = 50). All populations were identified as previously described (supplemental Figures 1 and 2).<sup>8-13</sup>

### Multipotent cell types: HSCs and MPPs

Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>CD90<sup>+</sup> HSCs comprised 6.7% (median, IQR 3.4%) of all CD34<sup>+</sup> cells (Table 1). However, their frequency was highly variable between donors, spanning a range of 0.1% to 17.3% of CD34<sup>+</sup> cells (a >2-log variability) (Figure 1B and Table 1). We also observed high interdonor variability in phenotypic MPPs (>1-log variability), which accounted for 10.6% (IQR 3.9%) of CD34<sup>+</sup> cells. We quantified the relationship between the frequencies of CD34<sup>+</sup> cells and both multipotent lineages and found no evidence of predictability between CD34<sup>+</sup> cells and either HSCs or MPPs by immunophenotype (Spearman *r* -0.009 and 0.069, respectively) (Figure 1A,B,F; supplemental Table 2). Total number of CD34<sup>+</sup> cells and both multipotent subpopulations exhibited a >1-log variability (Figure 1E; supplemental Figure 4). The Spearman coefficients correlating CD34<sup>+</sup> cell number with number of HSCs and MPPs are high (0.94 and 0.943, respectively), suggesting a strong predictability between these populations on first glance (Figure 1F; supplemental Figure 4; supplemental Table 2). However, interpretation of correlative analysis using absolute cell counts is prone to error and bias for the following reasons: (1) the manipulation of CD34-enriched samples prior to analysis by flow cytometry, including sample dilution, centrifugation, and overall handling, results in cell loss and changes in volume that affect the calculation of cell number; (2) correlation and linear regression are not suitable methods of analysis in circumstances when one variable is used to define the other (here, the number of CD34<sup>+</sup> cells is required to calculate the number of progenitor cells); and most importantly, (3) unlike HSPC subpopulation frequency, subpopulation cell counts are directly affected by the total number of cells in a given sample (ie, a sample with a low frequency of HSCs may contain a greater number of HSCs than a sample with a high frequency of HSCs by virtue of a greater number of cells overall). Consequently, the interpretation of any correlation between absolute number of cells in the context of this study is inconclusive.

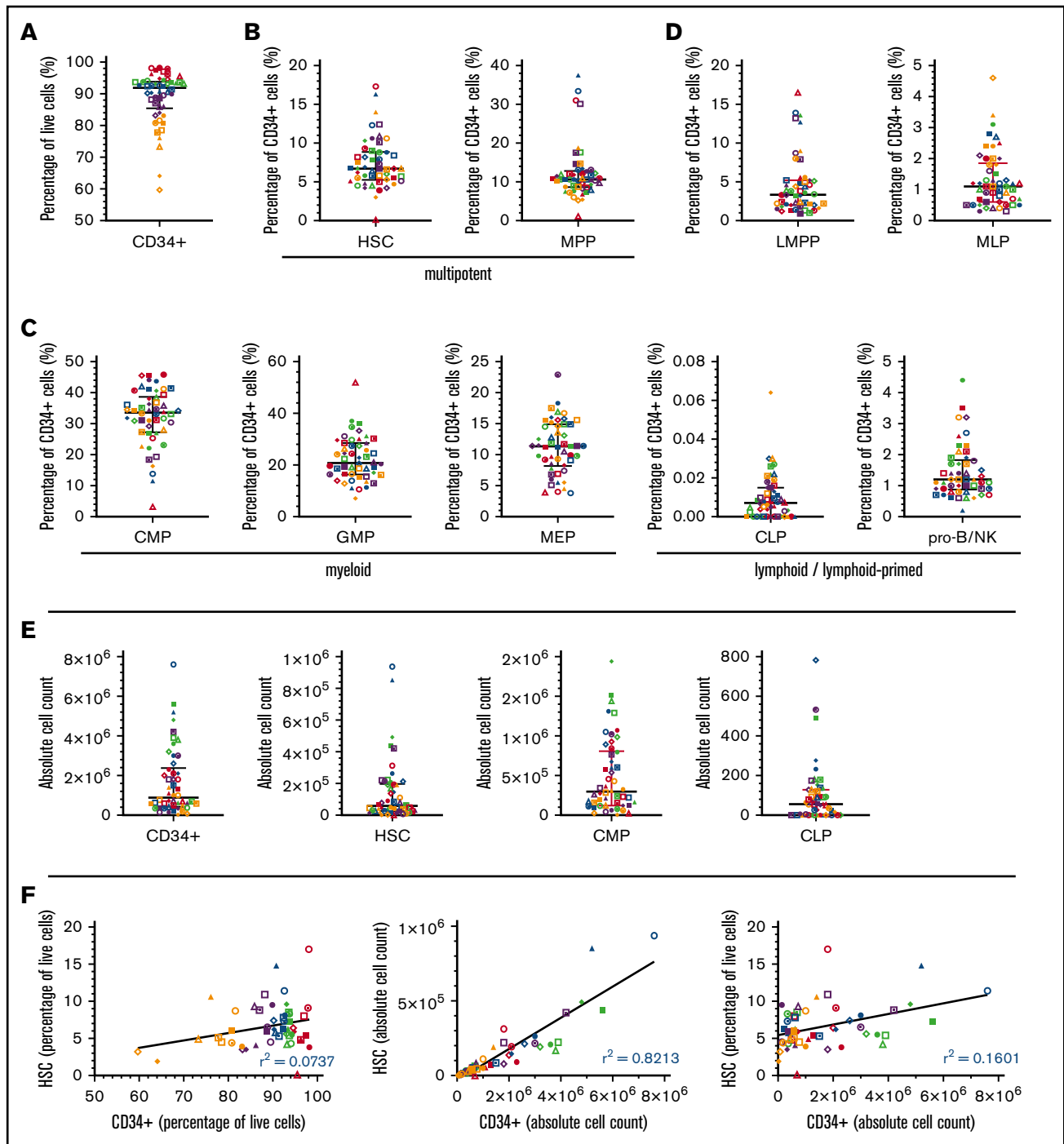
### Myeloid progenitors: CMPs, GMPs, and MEPs

Myeloid precursors within the Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup> compartment comprised the overwhelming majority of HSPCs (supplemental Figure 3). We observed that 33.6% (IQR 10.8%) of the CD34<sup>+</sup> cells isolated from cord blood express the CMP immunophenotype (Figure 1C, Table 1), suggesting a bias toward the myeloid lineage. Although the heterogeneity and differentiation capacity of HSCs are well studied,<sup>14-16</sup> the challenge of classifying populations within a fluid hematopoietic model solely by immunophenotype and the resulting paucity of comparable data makes it difficult to compare our data with existing literature. The Spearman correlation between the frequencies of total CD34<sup>+</sup> cells and phenotypic myeloid cells is weak, ranging from -0.196 to 0.331, indicating low predictability between these populations (supplemental Table 2).

### Lymphoid and lymphoid-primed progenitors: LMPPs, MLPs, CLPs, and pro-B/NKs

Phenotypic lymphoid cells in these UCB samples were notably less prevalent than their myeloid counterparts (Figure 1D; supplemental Figure 3). MLPs accounted for only 1.1% (IQR 1.2%) of CD34<sup>+</sup> cells. Similarly, B/natural killer (NK)-cell progenitors comprised 1.2% (IQR 1%) of CD34<sup>+</sup> cells. Our analysis revealed no relationship between the frequencies of CD34<sup>+</sup> cells and lymphoid progenitors, only a weakly negative correlation (supplemental Table 2).

While a consensus on the immunophenotypic definition of the CLP is yet to be reached,<sup>17-21</sup> this population was recently identified within the Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup> compartment of bone marrow with acquired expression of CD127.<sup>12</sup> By this definition, CLPs consistently formed less than 0.1% of CD34<sup>+</sup> cells (range, 0% to 0.06%) in all 50 UCB samples. A previous study by Hao et al demonstrated CD7<sup>+</sup> and CD10<sup>+</sup> progenitors with B-cell and NK-cell developmental potential in UCB, but not CD127<sup>+</sup> lymphoid progenitors.<sup>11</sup> This finding, consistent with our own observation of rare CD127<sup>+</sup> phenotypic CLPs, is paradoxical considering the known lymphoid potential of young HSCs. The low frequency of CLPs in the bulk UCB CD34<sup>+</sup> population may (1) suggest that lymphoid progenitors do not traffic in the fetal environment as HSCs and myeloid progenitors and (2) provide an additional mechanism for the delayed immune reconstitution seen following UCB transplants beyond the known naive phenotype of the T-cell compartment.<sup>22</sup>



**Figure 1. Heterogeneity of HSPC populations in CD34-selected cord blood products by percentage and absolute number.** Distribution of multipotent, myeloid-, and lymphoid-primed cell types as a percentage of CD34<sup>+</sup> HSPCs (n = 50). (A) CD34<sup>+</sup> cells. (B) HSCs and MPPs. (C) CMPs, GMPs, and MEPs. (D) LMPPs, MLPs, CLPs, and pro-B/NKs. (E) Distribution of total CD34<sup>+</sup> cells, HSCs, CMPs, and CLPs by absolute number. (F) Linear regression plots to assess the putative relationship between total CD34<sup>+</sup> cells and HSCs by percentage and absolute number (further addressed in the discussion). Immunophenotypes used to identify HSPC subtypes are defined in Table 1 and supplemental Figures 1 and 2. Each scatterplot contains 50 data points corresponding to 50 individual samples. Each data point is further indicated by a unique color and shape that remains consistent across all plots for sample tracing, with red points corresponding to 10 samples with the highest percentage of a given cell type (first quintile) and orange points corresponding to 10 samples with the lowest percentage of a given cell type (fifth quintile). Bars depict median and IQR.

Based on the unexpected high variability in UCB, a comparable analysis of the CD34<sup>+</sup> compartment of marrow products is indicated.

The interdonor variability of UCB HSPCs is demonstrated by 5 samples, selected to represent a broad range of HSCs (supplemental Figure 3). While the overall proportion of myeloid cells consistently

exceeded that of lymphoid lineages, there was a high degree of variation between the relative proportions of individual subpopulations. Interestingly, in 1 sample, HSCs accounted for only 0.1% of CD34<sup>+</sup> cells. These results support the conclusion that the CD34<sup>+</sup> compartment of human UCB, though often regarded as a single population for research and clinical applications, is a heterogeneous pool of stem and progenitor cells of varying immunophenotypic prevalence. In conjunction with the high variability of phenotypic HSCs, these data may have important implications in experimental and clinical contexts that rely on the functional properties of self-renewal and long-term engraftment.

In the clinical context, this study addresses the assumption that CD34<sup>+</sup> cell composition is an accurate and adequate measure of stem cell and progenitor content. Currently accepted metrics of UCB graft potency include total nucleated cell count, CD34<sup>+</sup> cell count, and colony-forming unit assays.<sup>23,24</sup> However, most graft composition data are obtained after cryopreservation or after transplantation, which may have direct implications in measurable clinical outcomes such as immune reconstitution and engraftment of stem cells, neutrophils, and platelets. To this end, we propose that grafts be subjected to quantification of progenitors in addition to total CD34<sup>+</sup> cells. Our antibody panel and staining protocol would address the need for real-time potency quantification at the time of transplant in 2 important ways. First, with a short time for execution, it would provide rapid turnaround. Secondly, the results would provide a baseline for assessing the aforementioned cell-dependent clinical outcomes. It may be that the time to neutrophil engraftment or immune reconstitution is explained by the frequency of committed progenitors (eg, CMPs and CLPs), which have no phenotypic correlation with the total CD34<sup>+</sup> cell content. Such immunophenotyping data would not supplant the utility of existing measures such as the functional colony-forming unit assay but would serve as a complement to better understand UCB biology and transplantation.

## References

1. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res*. 1961;14(2):213-222.
2. Becker AJ, McCulloch EA, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature*. 1963;197(4866):452-454.
3. Siminovitch L, McCulloch EA, Till JE. The distribution of colony-forming cells among spleen colonies. *J Cell Comp Physiol*. 1963;62(3):327-336.
4. Wu AM, Till JE, Siminovitch L, McCulloch EA. A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. *J Cell Physiol*. 1967;69(2):177-184.
5. Wu AM, Till JE, Siminovitch L, McCulloch EA. Cytological evidence for a relationship between normal hematopoietic colony-forming cells and cells of the lymphoid system. *J Exp Med*. 1968;127(3):455-464.
6. Doulatov S, Notta F, Laurenti E, Dick JE. Hematopoiesis: a human perspective. *Cell Stem Cell*. 2012;10(2):120-136.
7. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. *Science*. 1988;241(4861):58-62.
8. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell*. 2007;1(6):635-645.
9. Månsson R, Hultquist A, Luc S, et al. Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. *Immunity*. 2007;26(4):407-419.
10. Manz MG, Miyamoto T, Akashi K, Weissman IL. Prospective isolation of human clonogenic common myeloid progenitors. *Proc Natl Acad Sci USA*. 2002;99(18):11872-11877.
11. Hao Q-L, Zhu J, Price MA, Payne KJ, Barsky LW, Crooks GM. Identification of a novel, human multilymphoid progenitor in cord blood. *Blood*. 2001;97(12):3683-3690.
12. Pang WW, Price EA, Sahoo D, et al. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. *Proc Natl Acad Sci USA*. 2011;108(50):20012-20017.

In conclusion, our data provide a thorough map of the immunophenotypic heterogeneity of human cord blood and lay the foundation for clinical analyses of the cellular composition therein. We hope these results support future research endeavors and advance efforts to further optimize graft selection and prediction of hematologic recovery in HSC transplantation.

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## Authorship

Contribution: R.M. and A.R. provided the antibody panel and staining protocol; B.T.D. and K.I.W. provided instrumental guidance on immunophenotypic classification, especially of CLPs; B.T.D., R.A.-H., M.H.P., and K.I.W. contributed to data analysis; and all authors contributed to manuscript drafting and revision.

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13. Weissman IL, Shizuru JA. The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood*. 2008;112(9):3543-3553.
14. Mayani H. The regulation of hematopoietic stem cell populations. *F1000 Res*. 2016;5:1524.
15. Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I, Dick JE. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science*. 2011;333(6039):218-221.
16. Ema H, Morita Y, Suda T. Heterogeneity and hierarchy of hematopoietic stem cells. *Exp Hematol*. 2014;42(2):74-82.
17. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*. 1997;91(5):661-672.
18. Hoebeke I, De Smedt M, Stolz F, et al. T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34<sup>+</sup>CD38<sup>-</sup>CD7<sup>+</sup> common lymphoid progenitors expressing lymphoid-specific genes. *Leukemia*. 2007;21(2):311-319.
19. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med*. 2010;2(6):640-653.
20. Payne K, Baez I, Milford T, et al. IL-7 receptor expression defines a human common lymphoid progenitor in cord blood lymphopoiesis. *J Immunol*. 2011;186(1 suppl):153.4.
21. Ichii M, Oritani K, Yokota T, et al. The density of CD10 corresponds to commitment and progression in the human B lymphoid lineage. *PLoS One*. 2010;5(9):e12954.
22. Thomson BG, Robertson KA, Gowan D, et al. Analysis of engraftment, graft-versus-host disease, and immune recovery following unrelated donor cord blood transplantation. *Blood*. 2000;96(8):2703-2711.
23. Page KM, Zhang L, Mendizabal A, et al. Total colony-forming units are a strong, independent predictor of neutrophil and platelet engraftment after unrelated umbilical cord blood transplantation: a single-center analysis of 435 cord blood transplants. *Biol Blood Marrow Transplant*. 2008;17(9):1362-1374.
24. Page KM, Zhang L, Mendizabal AM, Gentry T, Balber A, Kurtzberg J. A novel scoring system, the cord blood Apgar, predicts engraftment after cord blood transplantation: optimization of selection of cord blood units (CBUs) for transplantation. *Blood*. 2010;116(21):351.