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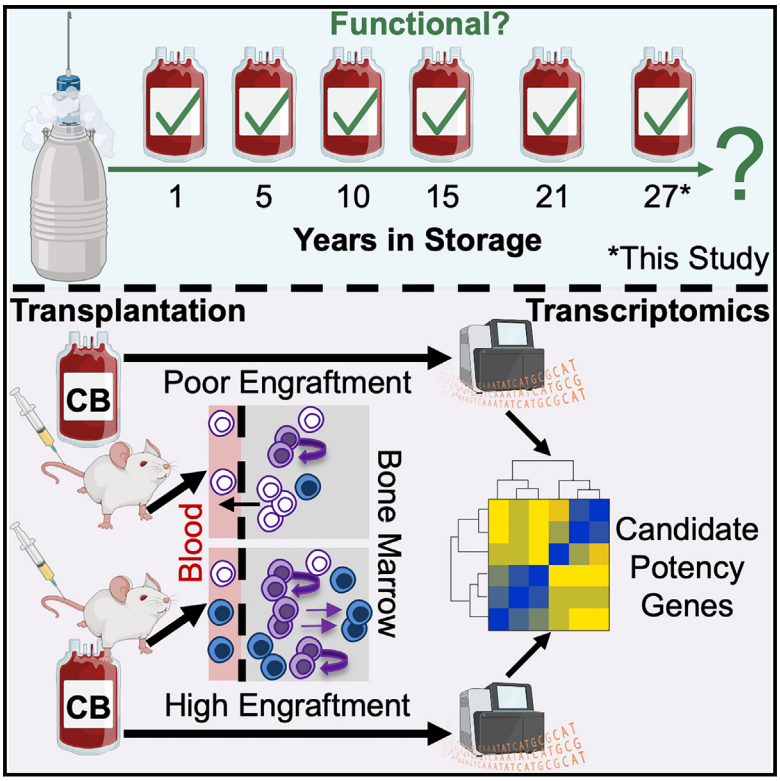
Insights into highly engraftable hematopoietic cells from 27-year cryopreserved umbilical cord blood

Graphical abstract



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In brief

Broxmeyer et al. show that long-term cryopreserved umbilical cord blood retains highly engraftable hematopoietic cells. Transcriptomic profiling reveals gene signatures associated with engraftment. Thus, older cord blood units should not be excluded from clinical selection, and omics approaches are a promising strategy to identify molecules associated with cord blood potency.

Highlights

- Umbilical cord blood transplantation is a life-saving treatment
- Long-term cryopreserved umbilical cord blood is often excluded from clinical use
- In mouse models of transplantation, 27-year-old cord blood is highly functional
- Transcriptomics reveal gene signatures associated with cord blood engraftment

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Report

Insights into highly engraftable hematopoietic cells from 27-year cryopreserved umbilical cord blood

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SUMMARY

Umbilical cord blood transplantation is a life-saving treatment for malignant and non-malignant hematologic disorders. It remains unclear how long cryopreserved units remain functional, and the length of cryopreservation is often used as a criterion to exclude older units. We demonstrate that long-term cryopreserved cord blood retains similar numbers of hematopoietic stem and progenitor cells compared with fresh and recently cryopreserved cord blood units. Long-term cryopreserved units contain highly functional cells, yielding robust engraftment in mouse transplantation models. We also leverage differences between units to examine gene programs associated with better engraftment. Transcriptomic analyses reveal that gene programs associated with lineage determination and oxidative stress are enriched in high engrafting cord blood, revealing potential molecular markers to be used as potency markers for cord blood unit selection regardless of length of cryopreservation. In summary, cord blood units cryopreserved for extended periods retain engrafting potential and can potentially be used for patient treatment.

INTRODUCTION

Since the first lifesaving umbilical cord blood (CB) transplantation almost 35 years ago,[1] more than 40,000 transplantations have been performed using CB as a source of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs).[2] CB is promising for use in transplantation because there are no risks to donors, CB transplantations result in lower incidences of graft-versus-host disease compared with bone marrow (BM) following partially human leukocyte antigen (HLA)-matched transplantation, and it is readily available because of the growth of CB banking.[3–5] The reduced requirement for strict HLA matching and its availability increases access for those lacking a match in donor registries, including under-represented racial minorities.[6] Additionally, CB may give enhanced graft-versus-leukemia effects in patients with acute leukemia[5] and banked CB may provide a more favorable donor source during high transmission periods of pandemics/endemics, when fresh donor sources are at risk of infection. The primary limitation of CB transplantation is slower neutrophil and platelet recovery following transplantation compared with BM or mobilized peripheral blood (PB), attributed to lower total numbers of HSCs/HPCs in single CB units (CBUs).[7] Slower recovery leads to complications, including increased risk of infection, graft failure, and prolonged hospitalization after transplantation.[7] Thus, it is critical to define criteria for CBU selection to maximize the potential for transplantation with reduced complications.

CB is cryopreserved upon collection, facilitating accumulation of over 5 million CBUs in public and private banks.[8] The number of banked CBUs continues to grow faster than units are depleted for use in transplantation. Currently, the duration of cryopreservation is considered when selecting CBUs for transplantation, with preference given to units that have been more recently preserved (excluding those cryopreserved for >15 years).[9] Despite lower incidences of graft-versus-host disease associated with CB transplantation, several recent studies have demonstrated that HLA matching of CB correlates with overall patient outcome.[5] Excluding older units decreases the pool of possible donor units, which may in turn decrease the diversity of HLA types available for a given patient. It is then essential to assess

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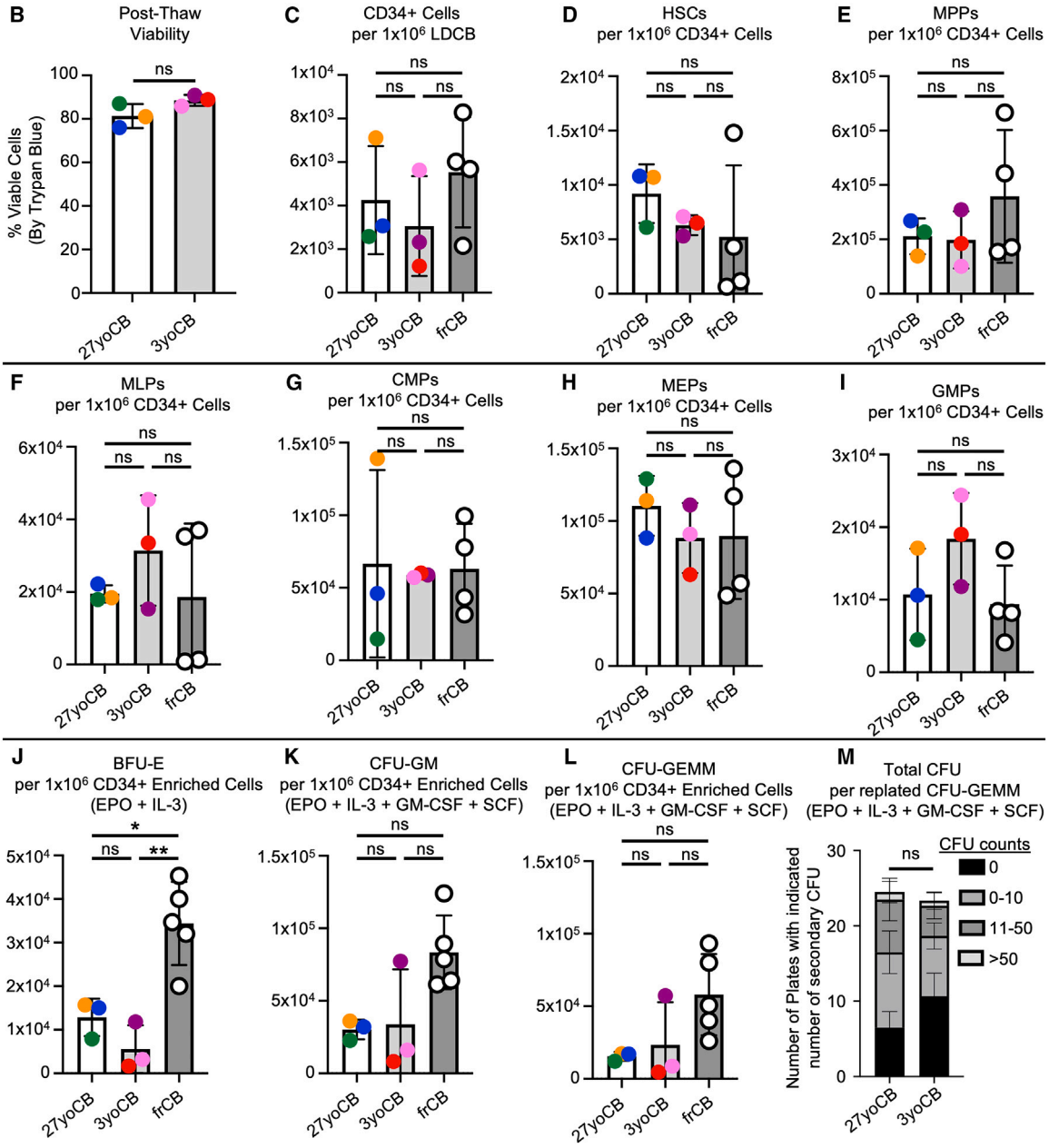
Group	Cord Blood Unit	Age of Cord at Thaw	TNC (pre-freeze)	Post-Thaw Viability	TNC (post-thaw)	LDCB
27yoCB	103239P (239)	27 years, 2 months, 4 days	1.368×10^9	81%	1.108×10^9	1.82×10^8
	103397P (397)	27 years, 1 month, 11 days	1.306×10^9	87%	1.136×10^9	5.99×10^8
	103410P (410)	27 years, 1 month, 7 days	1.561×10^9	76%	1.186×10^9	2.94×10^8
3yoCB	415963P (415)	3 years, 4 months, 30 days	1.197×10^9	90%	1.03×10^9	5.70×10^8
	609534P (609)	2 years, 9 months, 1 day	1.178×10^9	88%	1.07×10^9	7.60×10^8
	320005P (320)	2 years, 9 months, 21 days	1.220×10^9	85%	1.08×10^9	5.70×10^8



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if long-term storage of CB affects engraftment capacity to determine the importance of this criterion for CB selection. Our group has previously demonstrated efficient recovery of engraftable HSCs/HPCs from 10- to 21-year cryopreserved CB.[10–12] To examine whether older cryopreserved CBUs maintain functional viability, we performed comprehensive analyses on three CBUs cryopreserved for more than 27 years (Figure S1).

CB transplantation outcomes can be improved by elucidating potency markers to use as criteria for selection of units, by finding treatments that facilitate better engraftment or enhance numbers of HSCs/HPCs delivered in a single CBU by expansion or by better isolation procedures.[13] However, molecular markers that can be used to identify potent CBUs, to assess effects of cryopreservation, or that can be targeted for better engraftment, expansion, or isolation have not been elucidated on a global scale. Here we demonstrate recovery of highly engraftable HSCs/HPCs from 27-year-cryopreserved CB and examine the transcriptomes of units with variable engraftment capacities to gain insights into molecular programs that drive CB potency.

RESULTS

Long-term cryopreserved CB retains viable immunophenotypic HSCs/HPCs

CBUs cryopreserved for more than 27 years (27yoCB), approximately 3 years (3yoCB), and fresh, never cryopreserved CB (frCB) were assessed for viable immunophenotypic numbers of HSCs/HPCs. Post-thaw viability in total nucleated cells (TNCs) were statistically similar ($p > 0.05$) in cryopreserved units, with 81.3% ± 5.5% viability in 27yoCB and 88.5% ± 2.5% in 3yoCB (Figures 1A and 1B). Fluorescence-activated cell sorting by flow cytometry (FACS) to determine numbers of immunophenotypic HSCs/HPCs showed no significant differences ($p > 0.05$) in viable $CD34^+$ cells per 10^6 low-density CB (LDCB) cells recovered from 27yoCB (4,253 ± 2,486), 3yoCB (3,057 ± 2,295), or frCB (5,531 ± 2,532) (Figure 1C). Respectively, between 27yoCB, 3yoCB, and frCB we found statistically similar ($p > 0.05$) numbers per 10^6 $CD34^+$ cells of immunophenotypically viable HSCs (9,200 ± 2,685; 6,300 ± 916; 5,233 ± 6,581), multipotent progenitors (MPPs) ($2.1 \times 10^5 \pm 6.5 \times 10^4$; $1.9 \times 10^5 \pm 1.0 \times 10^5$; $3.5 \times 10^5 \pm 2.4 \times 10^5$), multilymphoid progenitors (MLPs) ($1.9 \times 10^4 \pm 2.3 \times 10^3$; $3.1 \times 10^4 \pm 1.5 \times 10^4$; $1.8 \times 10^4 \pm 2.0 \times 10^4$), common myeloid progenitors (CMPs) ($6.6 \times 10^4 \pm 6.4 \times 10^4$; $5.8 \times 10^4 \pm 1.5 \times 10^3$; $6.3 \times 10^4 \pm 3.1 \times 10^4$), megakaryocyte-erythroid progenitors (MEPs) ($1.1 \times 10^5 \pm 2.0 \times 10^4$; $8.8 \times 10^4 \pm 2.4 \times 10^4$; $8.9 \times 10^4 \pm 4.3 \times 10^4$), and granulocyte macrophage progenitors (GMPs) ($1.0 \times 10^5 \pm 6.3 \times 10^4$; $1.8 \times 10^5 \pm 6.3 \times 10^4$; $9.3 \times 10^4 \pm 5.3 \times 10^4$) (Figures 1D–1I). We found that 27yoCB, 3yoCB, and frCB also exhibited statistically similar ($p > 0.05$) total numbers of HSC/HPC per CBU, per TNC, per LDCB, and per $CD34^+$ cells (Figures S2A–S2F). These results show that similar numbers of viable immunophenotypically defined HSCs/HPCs can be recovered from long-term cryopreserved CB compared with fresh and more recently frozen units.

Long-term cryopreserved CB retains functional HPCs

Immunophenotyping does not always capture the functional capacity of HSCs/HPCs. Colony-forming unit (CFU) assays were used to examine recovery of functional HPCs. There were no significant differences ($p > 0.05$) in CFU numbers per 10^6 $CD34^+$ enriched cells between 27yoCB and 3yoCB, but there were significantly fewer CFU in cryopreserved CB than in frCB. The 27yoCB contained $1.5 \times 10^4 \pm 4.6 \times 10^3$ and 3yoCB had $5.5 \times 10^3 \pm 5.4 \times 10^3$ functional erythroid progenitors (burst-forming unit-erythroid [BFU-E]) per 10^6 $CD34^+$ enriched cells, while frCB had significantly more ($p = 0.002$) with $3.4 \times 10^4 \pm 9.5 \times 10^4$ (Figure 1J). Similarly, 27yoCB, 3yoCB, and frCB respectively contained $1.6 \times 10^4 \pm 4.0 \times 10^3$, $2.3 \times 10^4 \pm 2.9 \times 10^4$, and $5.8 \times 10^4 \pm 2.7 \times 10^4$ multipotent CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) per 10^6 $CD34^+$ enriched cells and $3.0 \times 10^4 \pm 6.8 \times 10^3$, $3.3 \times 10^4 \pm 3.7 \times 10^4$, and $8.3 \times 10^4 \pm 2.5 \times 10^4$ myeloid biased CFU-granulocyte/macrophage (CFU-GM) per 10^6 $CD34^+$ enriched cells (Figures 1K and 1L). CFU-GM and CFU-GEMM numbers per 10^6 $CD34^+$ enriched cells were statistically similar, but frCB contained significantly more BFU-E/CFU-GEMM/CFU-GM numbers per TNC compared with 27yoCB and 3yoCB (Figures S2G–S2I). This is consistent with previous studies showing that cryopreservation decreases the number of CFUs per CBU.[14] The 27yoCB CFU-GEMM exhibited statistically similar secondary replating capacity as 3yoCB, indicating no loss of limited HPC self-renewal potential (Figure 1M). These data show HPCs from long-term cryopreserved CB have similar functional competency compared with more recently frozen units.

Long-term cryopreserved CB has similar *ex vivo* growth properties compared with fresh and recently cryopreserved CB

Ex vivo expansion has been used successfully to improve numbers of HSCs/HPCs to be used in transplantation, increasing the number and diversity of CBUs that can be used as possible donors. We, thus, examined the expansion capacity of HSCs/HPCs from long-term cryopreserved CB by culturing $CD34^+$ cells in growth stimulating media for 4 days and analyzing immunophenotypic numbers

Figure 1. Long-term cryopreserved CB retains viable immunophenotypic HSCs/HPCs and functional HPC CFUs

(A) Cryopreserved CBU age and TNC numbers.

(B) Viability of TNC after thawing by Trypan blue exclusion.

(C–I) Immunophenotypic labeling of cell surface proteins and FACS to evaluate HSC/HPC numbers. HSC/HPC definitions are in Table S3.

(J–L) CFU numbers from $CD34^+$ enriched cells incubated with FBS in methylcellulose media with indicated growth factors.

(M) Total secondary CFU numbers from individually replated CFU-GEMM. CB397 secondary CFUs were excluded due to contamination. *$p < 0.05$, **$p < 0.01$ ns, not significant. n = 3 biological replicates for 27yoCB and 3yoCB, n = 4–5 biological replicates for frCB. All CFU assays were plated and counted in technical triplicates. Error bars indicate standard deviations. Colors of points: 27yoCB, orange = CB239; green = CB397; blue = CB410; 3yoCB, purple = CB415; red = CB609; pink = CB320.

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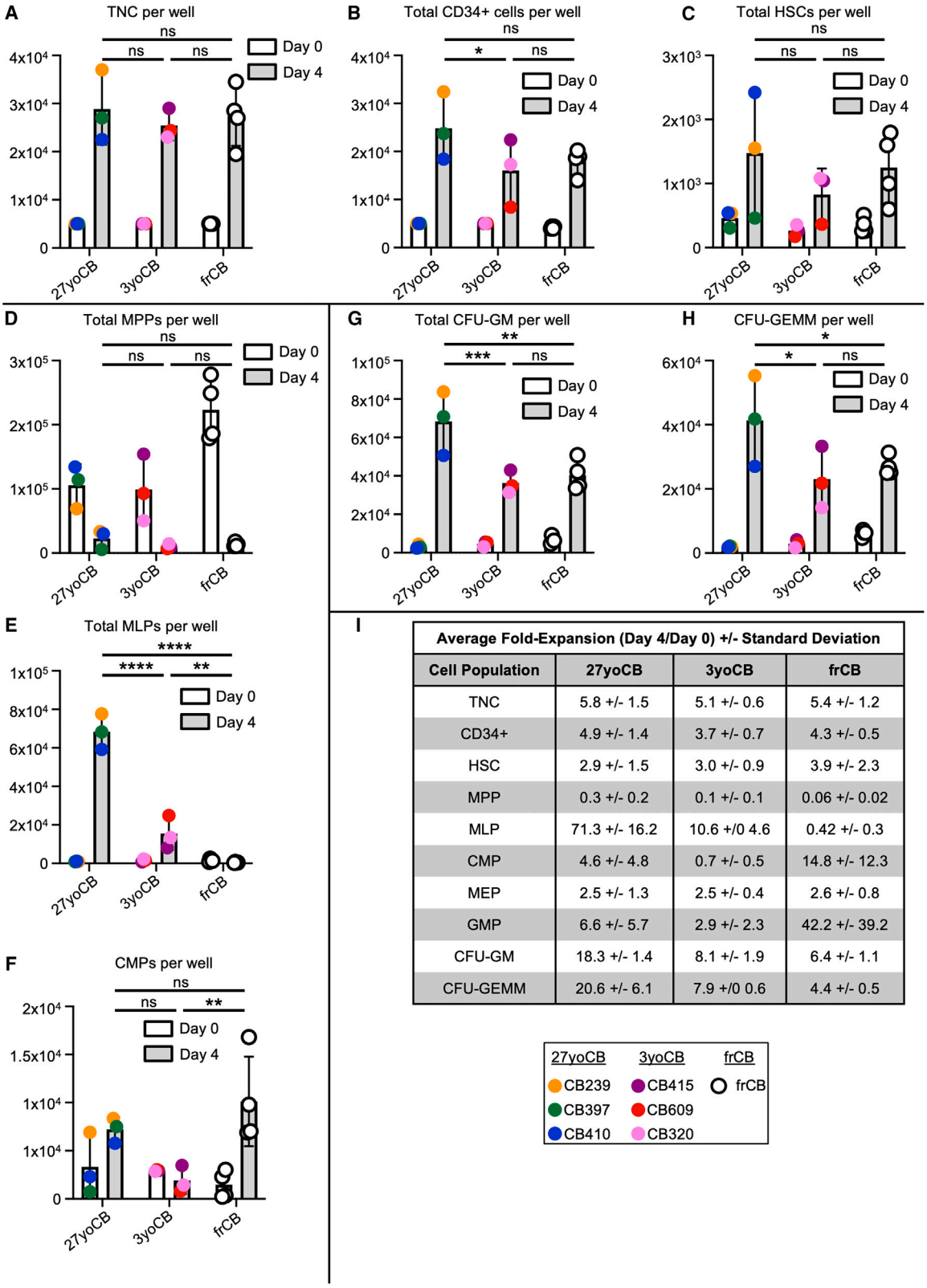

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Average Fold-Expansion (Day 4/Day 0) +/- Standard Deviation			
Cell Population	**27yoCB**	**3yoCB**	**frCB**
TNC	5.8 +/- 1.5	5.1 +/- 0.6	5.4 +/- 1.2
CD34+	4.9 +/- 1.4	3.7 +/- 0.7	4.3 +/- 0.5
HSC	2.9 +/- 1.5	3.0 +/- 0.9	3.9 +/- 2.3
MPP	0.3 +/- 0.2	0.1 +/- 0.1	0.06 +/- 0.02
MLP	71.3 +/- 16.2	10.6 +/0 4.6	0.42 +/- 0.3
CMP	4.6 +/- 4.8	0.7 +/- 0.5	14.8 +/- 12.3
MEP	2.5 +/- 1.3	2.5 +/- 0.4	2.6 +/- 0.8
GMP	6.6 +/- 5.7	2.9 +/- 2.3	42.2 +/- 39.2
CFU-GM	18.3 +/- 1.4	8.1 +/- 1.9	6.4 +/- 1.1
CFU-GEMM	20.6 +/- 6.1	7.9 +/0 0.6	4.4 +/- 0.5

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of HSCs/HPCs and CFU numbers on day 0 and day 4. Compared with 3yoCB and frCB, 27yoCB showed statistically similar or significantly more expansion of TNC ($p > 0.05$), HSCs ($p > 0.05$), MPPs ($p > 0.05$), MEPs ($p > 0.05$), $CD34^+$ cells (27CB vs. 3yoCB; $p = 0.04$), MLPs (27yoCB vs. 3yoCB [$p < 0.0001$]; 27yoCB vs. frCB [$p < 0.0001$]), CFU-GM (27yoCB vs. 3yoCB [$p = 0.0006$]; 27yoCB vs. frCB [$p = 0.001$]), and CFU-GEMM (27yoCB vs. 3yoCB [$p = 0.01$]; 27yoCB vs. frCB [$p = 0.03$]) (Figures 2A–2G and S2J). CMPs showed similar expansion in 27yoCB compared with both 3yoCB and frCB ($p > 0.05$), but frCB yielded significantly more expansion of CMPs compared with 3yoCB ($p = 0.003$) (Figure 2H). GMPs showed similar expansion in 27yoCB and 3yoCB ($p > 0.05$), but less expansion in cryopreserved units compared with frCB (27yoCB vs. froCB [$p = 0.003$]; 3yoCB vs. frCB [$p = 0.001$]) (Figure S2K). These studies suggest that long-term cryopreservation does not significantly alter the expansion capacity of CB HSC/HPC or CFU *ex vivo* (Figure 2I).

Long-term cryopreserved CB retains highly engraftable HSCs/HPCs

Ex vivo studies do not always recapitulate the *in vivo* functional competency of HSCs/HPCs. To test *in vivo* engraftment, limiting dilution analysis was performed by transplanting three doses of $CD34^+$ enriched cells from 27yoCBUs CB239, CB397, and CB410 to sublethally irradiated NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) immune deficient mice to determine SCID repopulating cell (SRC) frequency. The 27yoCB exhibited early and late human engraftment measured by human CD45 expression in recipient PB at weeks 4, 8, 16, and 20 (Figures 3A, 3B, S3A, and S3B) and BM at week 20 (Figure 3C) at all cell doses. Human cell chimerism in the BM at the highest cell dose was 12.8% ± 19.8%, 0.15% ± 0.065%, and 15.7% ± 17.0% for CB239, CB397, and CB410, respectively, and ranged from low to robust levels of engraftment (0.1%–52.7%) (Figure 3C). At the highest cell dose, recipients of 27yoCB transplantations showed statistically similar ($p > 0.05$) numbers of human HSCs/HPCs in recipient BM (Figures 3D, S3C, and S3D), the expected myeloid bias of donor cells associated with *in vivo* repopulating assays (Figure 3E), and secondary repopulation capacity shown by human chimerism in BM of secondary recipient mice (Figure 3F). CB239 and CB410 had respective SRC frequencies of 327 (95% confidence interval [CI_{95}] = 178–598) and 326 (CI_{95} = 178–597) per 1×10^6 $CD34^+$ enriched cells, while unit 397 had a significantly lower frequency of 44 (CI_{95} = 18–109) (Figures 3G, 3H, and S3G). When corrected for differences in $CD34^+$ purity in the enriched cell fractions, CB397 had a slightly improved SRC frequency that was still significantly lower than CB239 and CB410 (Figures S3E and S3F). SRC frequencies from 27yoCB are statistically similar to six previously published SRC frequencies for fresh or recently frozen CB (composite CI_{95} range, 31–2433 SRCs per 1×10^6 $CD34^+$ enriched cells) (Figures 3H and S3G).[15–20] Out of data from six previously published CBUs used for comparison, CB239 and CB410 showed similar SRC frequencies as four fresh or recently frozen CBUs with significantly lower SRCs compared with only one unit (Figure 3H). In contrast, CB397 showed significantly worse engraftment than several units from the published studies (Figure 3H). These data show that highly functional/engraftable HSCs/HPCs can be recovered from long-term cryopreserved CBUs. Interestingly, one of three (33%) 27yoCBUs in this study yielded low engraftment and SRC frequency and two exhibited robust engraftment and high SRC frequency (Figure 3I). We expect CBUs with low engraftment to be more likely to result in graft failure when used for transplantation, which occurs in 10%–20% of clinical CB transplantations.[21]

Long-term cryopreserved CB and fresh CB are transcriptionally distinct

To examine changes in gene programs induced by long-term cryopreservation, HSCs and MPPs from post-thaw 27yoCB or from frCB were harvested for RNA sequencing (RNA-seq) analyses, revealing differences in transcriptomic profiles between fresh and long-term cryopreserved CB (Figures 4A, 4B, S4A, and S4B; Table S1). RNA-seq comparing 27yoCB to frCB revealed 2,960 and 2,799 differentially expressed genes (DEGs) in HSCs and MPPs, respectively (Figures S4C and S4D). Fast gene set enrichment analysis (FGSEA) revealed that both HSCs and MPPs from frozen CBUs were significantly enriched for genes associated with HSC function ($p_{adj} = 6 \times 10^{-14}$; $p_{adj} = 3 \times 10^{-13}$), quiescence ($p_{adj} = 5 \times 10^{-7}$; $p_{adj} = 1 \times 10^{-8}$), and lymphoid development (Figures S4E–S4G), while HSCs/MPPs from fresh CBUs were significantly enriched for genes associated with oxidative phosphorylation ($p_{adj} = 1 \times 10^{-10}$; $p_{adj} = 1 \times 10^{-7}$) and mitochondrial function ($p_{adj} = 5 \times 10^{-12}$; $p_{adj} = 8 \times 10^{-12}$) (Figures S4H and S4I). Interestingly, this may suggest that HSCs/MPPS from frozen CBUs are enriched for stemness and are less metabolically active. Alternatively, it is possible that HSCs/HPCs with poor stem cell function or high metabolic activity are less efficiently cryopreserved, thus diminishing their effect on the overall transcriptome following thawing compared with frCB. Further, the enrichment for lymphoid-associated genes in HSCs of cryopreserved CB may account for their enhanced expansion of *ex vivo* MLP expansion (Figure 2G), though this enrichment for lymphoid genes does not correlate with functional changes in lymphoid engraftment (Figure 3E). Regardless, we can infer that cryopreservation does not significantly impair pathways associated with HSC/HPC function, in line with our observed functional data (Figure 3). Furthermore, CB397, which had the worst performance in functional assays of the 27yoCB, had a

Figure 2. Long-term cryopreserved CB has similar *ex vivo* growth properties compared with fresh and recently cryopreserved CB

(A–H) We plated 5×10^4 $CD34^+$ cells were plated in RPMI1640 media with SCF, TPO, and FLT3L grown for 4 days. (A–F) Immunophenotypic HSC/HPC numbers at day 0 and day 4. (G–H) CFU numbers from input day 0 or day 4 expanded $CD34^+$ cells plated with fetal bovine serum and rhEPO/SCF/IL-3/GM-CSF in methylcellulose media.

(I) Summary table of expansion expressed as day 4/day 0 ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. ns, not significant. n = 3 biological replicates for 27yoCB and 3yoCB, n = 4 biological replicates for frCB. Expansion assays were plated without technical replication. All CFU assays were plated and counted in technical triplicates. Error bars indicate standard deviation. Colors of points: 27yoCB, orange = CB239; green = CB397; blue = CB410; 3yoCB, purple = CB415; red = CB609; pink = CB320.

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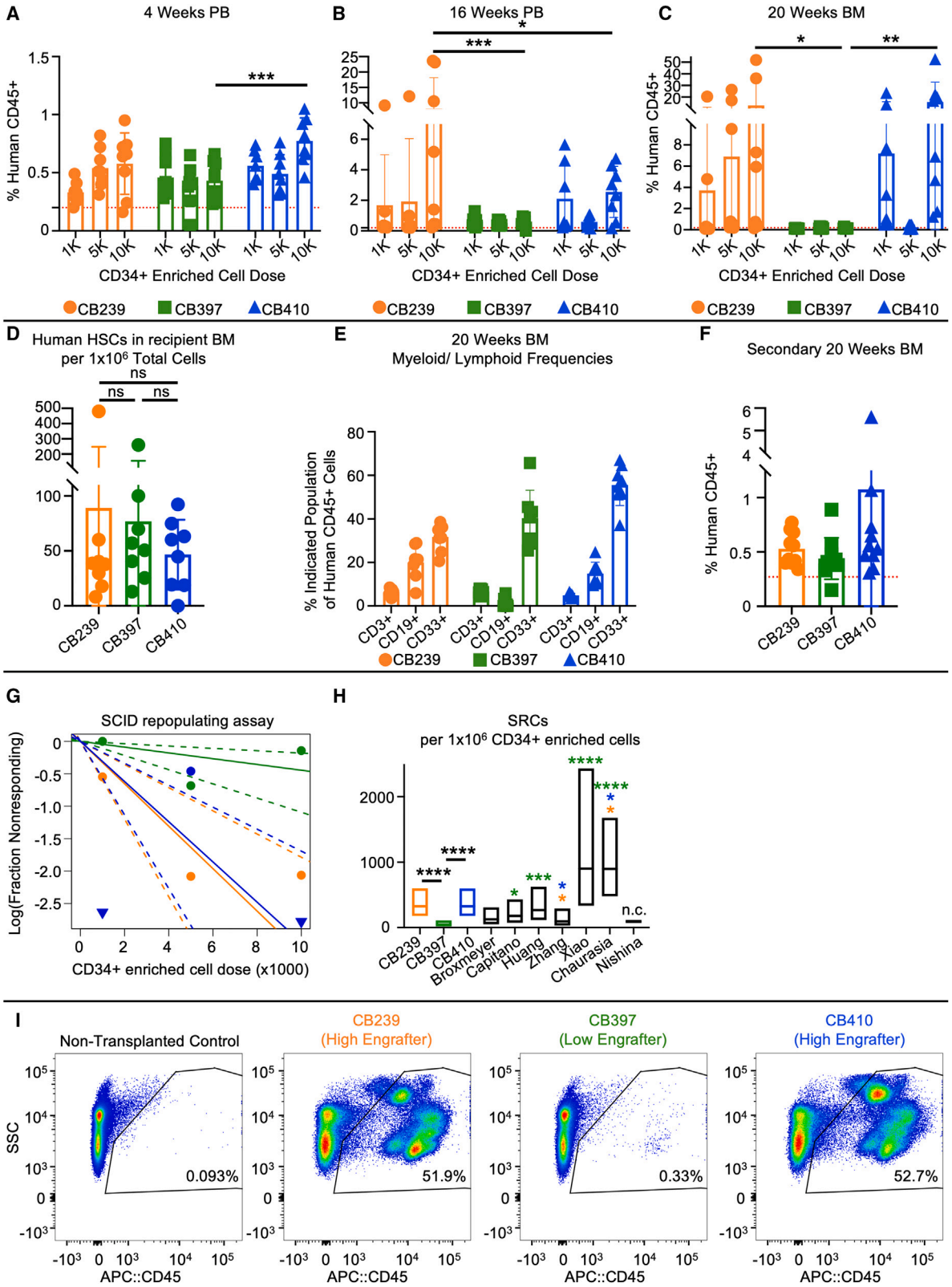

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more similar transcriptome to frCB than either CB239/CB410 based on Euclidean distance analyses (Figures 4A, 4B, S4A, and S4B), underscoring discordance between cryopreservation and function.

Transcriptomic analysis of highly engraftable CB reveals gene programs associated with HSC/HPC functional competency

Differences in repopulating capacity of the 27yoCB in this study were not well predicted by immunophenotyping, expansion, or CFU numbers. For example, CB397 had 1.4-fold to 1.75-fold fewer CFU numbers relative to CB239/CB410 (Figures 1J–1L), but yielded almost 10-fold fewer SRCs (Figures 3G and 3H) and nearly 100-fold less average human chimerism in the BM of recipient mice (Figures 3C and 3I). To identify gene programs predictive of successful engraftment for use as clinical potency markers we performed transcriptomic analyses and examined DEG patterns of immunophenotypically defined HSCs and MPPs from 27yoCB comparing two high engrafting CBUs (CB239/CB410) and the single low engrafting unit (CB397). This revealed 22 genes and 20 genes significantly enriched ($p_{adj} < 0.05$) in high engrafting HSCs and MPPs respectively, while 48 genes and 39 genes are enriched in low engrafting HSCs and MPPs (Figures 4C and 4D). FGSEA revealed that targets of HOXA5, a gene program associated with self-renewal and lineage determination, and the hypoxia inducible factor-1α transcription factor pathway, which is associated with responses to oxidative stress, are significantly enriched in high engrafting HSCs ($p_{adj} = 6 \times 10^{-8}$ and $p_{adj} = 1 \times 10^{-3}$, respectively) (Figures 4E and 4F). Genetic programs associated with negative regulation of quiescence are enriched in low engrafting cells ($p_{adj} = 2 \times 10^{-8}$) (Figure 4G). In high engrafting MPPs, significantly enriched gene programs in high engrafting CB include genes associated with protein localization ($p_{adj} = 1 \times 10^{-7}$) and translation initiation ($p_{adj} = 1 \times 10^{-5}$), while low engrafting MPPs are enriched for genes associated with catabolic/metabolic programs ($p_{adj} = 3 \times 10^{-4}$) (Figures 4H–4J). For gene level analysis, DEGs were further filtered for increased stringency because of the low sample number (see STAR Methods), revealing 17 genes and 18 genes highly enriched in high engrafting HSCs and MPPs, respectively, and 32 genes and 26 genes highly enriched in low engrafting HSCs and MPPs (Figure 4K; Table S2). Genes associated with proliferation and HSC/HPC function such as *MYC* and *CXCL8* are highly enriched in high engrafting HSCs (Figure 4K; Table S2),[27,28] while genes encoding post translational modifiers and epigenetic regulators are highly enriched in low engrafting HSCs, such as *STYK1* and *KAT14* (Table S2). Through comparative analysis using frCB, we inferred that some DEGs may be due to the effects of cryopreservation, while others may be enriched based on donor variation (Table S2). Although few DEGs were statistically significant in both HSCs and MPPs, 18 genes that were significantly enriched in high engrafting HSCs or MPPs also exhibit an increase in expression of more than 2-fold high engrafting units compared with low engrafting in the other subpopulation (Figure 4L). These include genes with known importance in hematopoiesis such as *MALT1* and *MAP2K1*.[29,30] Thus, candidate genes of potential importance to CB engraftment are revealed by transcriptome profiling of HSCs/HPCs from CBUs with varying engraftment capacities.

Expression of CD22 and MYC proteins in CBUs with known early engraftment potential

In a small-scale validation study, we examined the protein expression of targets identified by transcriptomics in three independent CBUs with known early engraftment outcomes in a mouse model of transplantation. We selected CD22, which is upregulated in high engrafting MPPs (Figure 4L); as a cell surface protein, its expression is easily measured by FACS. We also examined expression of MYC, which is upregulated in high engrafting HSCs (Figures 4K and 4L), because it is widely studied and there are highly validated tools for its analysis. We demonstrated that CD22 and MYC protein expression levels could be assessed on small (200 μL) aliquots of cyropreserved whole blood that were retained prior to transplantation using antibody staining and FACS (Figures S4J and S4K). In the three CBUs we tested, the CBU with the lowest levels of CD22 and MYC expression had the poorest early engrafting cell frequency, but expression levels did not differentiate the CBU with the highest SRC frequency from that with the intermediate SRC frequency (Figure S4L). Thus, post thaw FACS analysis of molecular markers identified by transcriptomic studies is a feasible method to assess protein expression levels of a CBU, and low expression of CD22 and MYC may be candidates for exclusionary criteria of particularly poor engrafting CBUs. Further studies are required to identify a panel of genes/proteins that delineate all levels of engraftment potency.

Figure 3. Long-term cryopreserved CB retains highly engraftable HSCs/HPCs

(A–C) Human CD45 chimerism of sublethally irradiated NSG mice transplanted with the indicated cell doses of $CD34^+$ enriched cells in PB at 4 weeks (A) and 16 weeks (B) post transplant and in BM at 20 weeks post transplant (C). Red line = fluorescence in non-transplanted control mice. Significance comparisons between same cell doses are shown.

(D) Number of immunophenotypically defined HSCs in recipient mouse BM at week 20. Human origin was confirmed by human $CD34^+$ positivity.

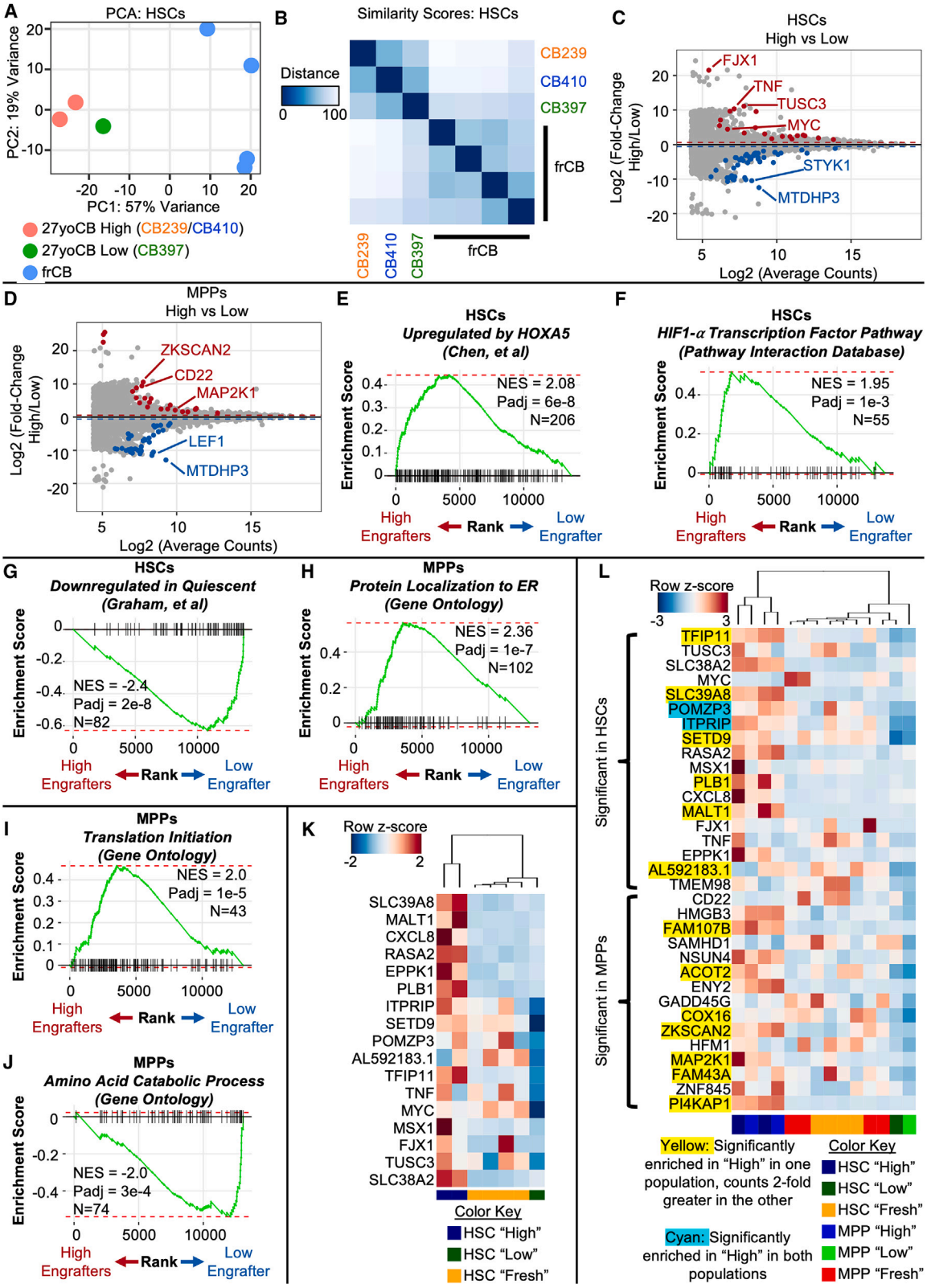
(E) Percentages of the indicated lymphoid ($CD3^+$, $CD19^+$) and myeloid ($CD33^+$) cell populations in the BM of recipient mice.

(F) Shown is the Human $CD45^+$ chimerism in the BM of secondary recipient mice 20 weeks post-transplantation of 5×10^6 total BM cells from pooled primary recipients.

(G) Extreme limiting dilution analysis Poisson modeling[22] to reveal predicted SCID-repopulating cell (SRC) frequency based on number of recipient mice engrafted at the various cell doses using 20 weeks BM chimerism.

(H) SRC frequencies of 27-year cryopreserved CBUs compared with published data. For published data, the graph label is the last name of the first author of the study.[15–20] Likelihood ratio tests were used for inter-unit comparisons. Asterisks are color coordinated for published data comparisons to the different 27yoCBUs (orange = CB239; green = CB397; blue = CB410). Box plots show 95% confidence interval with frequency estimation as the middle line. n.c., confidence intervals not reported.

(I) FACS plots for primary recipient mice BM showing most robust engraftment from each CBU. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns, not significant. n = 3 biological replicates of 27yoCB. Each CBU were transplanted to n = 7–8 mice per cell dose. Mined published data used n = 3–8 mice per cell dose per group. Error bars indicate standard deviations. Colors of points: orange = CB239; green = CB397; blue = CB410.



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DISCUSSION

Long-term cryopreservation does not significantly alter numbers and function of HSCs/HPCs found in CBUs

As CB transplantation evolves, the field faces a critical question of whether to use (and continue to preserve) continually aging units. To examine this question, we performed comprehensive *ex vivo* and *in vivo* analyses of the oldest CBUs to be tested to date. We showed that similar numbers of viable TNC, $CD34^+$ cells, and stringently defined HSCs/HPCs are found in long-term cryopreserved CB compared with more recently frozen and fresh CB. Cryopreserved units also had similar CFU numbers, regardless of the duration of storage. Long-term cryopreserved CB had similar *ex vivo* expansion capacity as more recently preserved units and fresh units and were capable of short- and long-term engraftment as well as secondary engraftment *in vivo*. In summary, these studies demonstrate that functionally competent HSCs/HPCs can be recovered from CB that has been cryopreserved for at least 27 years.

Differential gene expression reveals promising candidates for molecular potency markers for CB transplantation

Two of three long-term cryopreserved CBUs exhibited robust engraftment capacity in mouse models with similar SRC frequencies as published fresh or recently cryopreserved units. The third unit showed poor engraftment and a relatively low SRC frequency that was still within the range of published SRC frequencies. One possibility for these differences that cannot be ruled out by the analyses we performed in this study are effects of cryopreservation. However, we speculate that long-term cryopreservation itself is unlikely the cause of the lower engraftment efficiency based on the performance of the other two units and the similarity scores between the transcriptomic profiles of the low engrafting unit compared with fresh CB. Alternatively, the low engraftment efficiency may have been caused by the intrinsic biological properties of the CBU, such as high or low expression of specific gene programs that drive engraftment, resulting in less functionally competent HSCs/HPCs. Regardless of the cause of engraftment efficiency differences, improved criteria could help to refine selection of stored CBUs for clinical or research use. Our analysis of divergent molecular profiles between CB of varying engraftment potential demonstrated that global profiling approaches may reveal potential predictors of cryopreserved CB engraftment potency. Many of the genes and programs enriched in high engrafting HSCs play a role in self-renewal and proliferation, including *MYC*, *TNF*, and *MAP2K*, among others, suggesting that HSCs/MPPs may be primed for better engraftment by the expression of genes associated with cell growth. Further, several genes that we identified as being enriched in high engrafting HSCs have understudied roles in hematopoiesis, such as *FJX1* and *AL592183.1* (Figure 4K), and may indicate roles for these genes in regulating HSC function and engraftment. The genes and gene programs identified in this study represent a set of promising target candidates for detectable potency markers for early and late reconstitution for more effective CBU selection.

Important implications for the use of long-term cryopreserved CB

CB is an important source of HSCs/HPCs for life-saving transplantations. CB is also a potential source of cells to be used in gene editing and engineering, as well as other immunotherapies, such as gene editing and chimeric antigen receptor T cell therapies to treat both hematologic and non-hematologic diseases and is an excellent resource for the study of human hematopoietic stem and progenitor cells in laboratory settings.[31] Long-term cryopreserved CB is a mostly untapped resource that could provide higher chances of finding acceptable units for patients lacking a match in other registries or more recently stored CB, provide cells for other therapeutic strategies, and provide cells for research purposes. Taken together, our data suggest that the duration of cryopreservation alone is not a valid exclusionary criterion for CBU selection and should not define currently accepted criteria. We also propose and demonstrate the feasibility of a method for identifying molecular markers of CB potency using transcriptomic profiling. Utilizing this approach may allow for the development of accurate potency assays to determine the best CBUs to use clinically, such as targeted FACS analysis of one or several proteins or targeted gene expression analysis of a panel of genes. These assays could be performed on the small volumes already retained for quality analysis of CBUs prior to transplantation, as demonstrated by our small-scale validation study (Figures S4J–S4L), and could be measured using high sample numbers of CBUs with known engraftment capacities to determine appropriate expression levels to use as potency thresholds. Further comprehensive bioinformatics studies are warranted to refine markers that can be used to select the best CBUs regardless of age and to delineate the potency of stored CBUs for clinical selection.

Figure 4. Transcriptomic analysis of highly engraftable CB reveals gene programs associated with HSC/HPC functional competency

(A) RNA-seq principal component analysis (PCA) plot of 27yoCB and frCB HSCs.

(B) Heatmap showing similarity scores in Euclidean distances between CBUs.

(C and D) Log ratio vs. mean average (MA plot) plot showing DEGs in high engrafting CB vs. low engrafting CB for HSCs (C) and MPPs (D). Red dots indicate significantly enriched in high engrafting, blue dots indicate significantly enriched in low ($p_{adj} < 0.05$).

(E–J) Gene set enrichment analyses revealing gene programs[23–26] enriched or negatively enriched in high engrafting CB compared with low in HSCs (E–G) and MPPs (H–J). Positive NES indicates enrichment in high engrafting CB.

(K) Heatmap showing genes highly enriched in high engrafting CB HSCs.

(L) Heatmap showing genes that are significantly enriched in either HSCs or MPPs. Highlighted genes are also significantly enriched in the other subpopulation in high engrafting CB or have 2-fold increases in counts in both high engrafting units vs. the low engrafting unit for the other subpopulation. For K and L, only DEGs using stringent threshold are considered (see STAR Methods). NES, normalized enrichment score; p_{adj}, p value adjusted for multiple comparisons; N, number of genes tested in the differential expression analysis that are also in the indicated gene set. For both HSCs/MPPs: n = 3 biological replicates for 27yoCB; n = 4 biological replicates for frCB; n = 2 biological replicates for high engrafting 27yoCB; n = 1 biological replicate for low engrafting 27yoCB.

Limitations of the study

This study was conducted using a small sample size of long-term cryopreserved CB, due to the rarity of CBUs that have reached this duration of cryopreservation (these are likely three of the oldest units in existence based on the dates of the first recorded banked samples). Further, this study was limited to CBUs preserved and stored in a single CB bank. When more cryopreserved units of 27 or more years of age become available at this and other CB banks, a larger scale, multi-bank study is warranted to confirm the functional competency of long-term cryopreserved CB. Additionally, while transplantation with immune deficient mice is considered a gold standard for modeling engraftment, there are no definitive studies correlating outcomes in mice with outcomes in patients; thus, further studies are necessary to confirm human engraftment. Prior to clinical use, it may also be useful to confirm engraftment of long-term cryopreserved CB in non-human primate models, given the noted differences between these models of transplantation and SCID repopulating assays.[32] Additionally, the low sample size limited the power of the transcriptomics studies. Future studies with increased sample sizes (including CBUs of varying ages) will allow for the generation of a more comprehensive database of gene expression correlated with CB functional competency.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2023.101259.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.E.B., J.R., and S.C.; methodology, H.E.B., J.R., and S.C.; investigation, J.R. and S.C.; formal analysis J.R. and S.C.; resources, C.D.H., L.L., R.S.W., A.J., E.M.F., W.v.H., M.L.C., and M.H.K.; data curation, J.R.; writing – original draft, J.R. and S.C.; writing – review and editing J.R., S.C., C.D.H., L.L., R.S.W., A.J., E.M.F., W.v.H., M.L.C., and M.H.K.; visualization, J.R.; supervision, J.R. and S.C.; funding acquisition, H.E.B., J.R., M.H.K., and M.L.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Human CD34-APC (Clone 581)	BD Biosciences	Cat#555824; AB_398614
Anti-Human CD38-FITC (Clone HIT2)	BD Biosciences	Cat#555459; AB_395852
Anti-Human CD135-PE (Clone 4G8)	BD Biosciences	Cat#558996; AB_397175
Anti-Human CD45RA-PECF594 (Clone HI100)	BD Biosciences	Cat#562298; AB_11154413
Anti-Human CD10-PE-Cy7 (Clone HI10a)	BD Biosciences	Cat#565282; AB_2739153
Anti-Human CD90-BV421 (Clone 5E10)	BD Biosciences	Cat#562556; AB_2737651
Anti-Human CD49f-PerCP-Cy5.5 (Clone GoH3)	BD Biosciences	Cat#562475; AB_11151910
Anti-Human CD45-APC (Clone HI30)	BD Biosciences	Cat#561864; AB_11153499
Anti-Human CD22-PE (Clone S-HCL-1)	BioLegend	Cat#363503; AB_2564609
Anti-Human MYC-AlexaFluor488 (Clone 9E10)	SantaCruz Biotechnologies	Cat#sc40 AF488 AB_2892598
Biological samples		
Cryopreserved Umbilical Cord Blood	New York Blood Center	N/A
Fresh Umbilical Cord Blood	Cleveland Cord Blood Center	N/A
Fresh Umbilical Cord Blood	Cryo-Cell International	N/A
Chemicals, peptides, and recombinant proteins		
RPMI1640 (Gibco)	Thermo Fisher	Cat#11875135
StemSpan SFEM II	STEMCELL Technologies	Cat#09655
Fetal Bovine Serum (Corning)	Fisher Scientific	Cat#35015CV
Zombie Yellow Viability Dye	BioLegend	Cat#423104
Ficoll-Paque Plus	Cytiva	Cat#17144003
Epogen (Amgen)	McKesson Medical Surgical	Cat#419193
Recombinant Human Stem cell factor	R&D Systems	Cat#255-SC
Recombinant Human Flt-3 ligand	R&D Systems	Cat#308-FK
Recombinant Human Interleukin-3	R&D Systems	Cat#203-IL
Recombinant Human Granulocyte-macrophage colony stimulating factor	R&D Systems	Cat#215-GM/CF
Recombinant Human Stem cell factor	R&D Systems	Cat#255-SC
Critical commercial assays		
RNeasy Micro Plus Kit	Qiagen	Cat#74034
Deposited data		
Raw and analyzed data	This paper	GEO: GSE225699
Human reference genome GENCODE version 41, GRCh38	GENCODE	https://www.gencodegenes.org
Experimental models: Organisms/strains		
Mouse: NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG)	Indiana University *In Vivo* Therapeutics Core	N/A
Software and algorithms		
Prism 9	GraphPad	https://www.graphpad.com
FlowJo 10.8.1	BD Biosciences	https://www.flowjo.com/solutions/flowjo
FastQC	Babraham Bioinformatics	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cutadapt	Martin[33]	https://cutadapt.readthedocs.io/en/stable/installation.html
STAR	Dobin et al.[34]	https://github.com/alexdobin/STAR
HTSeq	Putri et al.[35]	https://htseq.readthedocs.io/en/master/install.html
DESeq2	Love et al.[36]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
fgsea	Korotkevich et al.[37]	http://bioconductor.org/packages/release/bioc/html/fgsea.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for raw and analyzed data should be directed to and will be fulfilled by the lead contact, James Ropa (jropa@iu.edu).

Materials availability

This study did not generate unique reagents.

Data and code availability

- All raw and processed data will be made available by the lead contact upon reasonable request. Raw and processed data from RNA-sequencing have been deposited in the NCBI Gene Expression Omnibus database, GEO: GSE225699.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Umbilical cord blood primary samples

Cord blood units (CBUs) were cryopreserved at the New York Blood Center or were freshly collected and distributed under the supervision of Cleveland Cord Blood Center or CryoCell International. Three 27-year cryopreserved CBUs and three 3-year cryopreserved CBUs were shipped in a dry shipper to Indiana University School of Medicine, where all experiments were performed. Two to six fresh CBUs from Cleveland Cord Blood Center or CryoCell international were used for each experiment. All cord blood units were de-identified prior to receipt by investigators at Indiana University, as is common practice for research use and clinical use when using unrelated donors from public cord blood banks. Thus, sex, ancestry, socioeconomic background, health status and history and other identifying information could not be controlled for. For RNA-seq analysis, sex of the infant was inferred using expression of X- and Y-linked genes and was controlled for in the transcriptomic model.

In vivo mouse modeling

Female NOD-scid IL2Rgamma^{null} (NSG) mice were purchased in-house from the *In Vivo* Therapeutics Core at Indiana University School of Medicine. Because of the limited sample size of 27-year cryopreserved CBUs, only female mice were used to control for sex-dependent differences in engraftment. Future studies should confirm that there are no sex-dependent effects on engraftment for long-term cryopreserved CBUs. Mice were 8–10 weeks of age when used for transplantation experiments. Mice were housed in groups of 5 according to age, sex, and genotype under light and temperature-controlled conditions (12h light/12h dark cycle) in a biosafety level 2 room maintained with extra precautions for sterility for immune compromised mice. 1 week prior to transplantation, mice were placed on a diet of antibiotic-dosed food (Uniprim) and were given acid water to prevent gastroenteritis. 24 h prior to transplantation, NSG mice were given a sublethal (350rads) whole body dose of radiation. Irradiated mice were transplanted by tail vein injection with varying cell doses of $1x10^3$, $5x10^3$, or $10x10^3$ CD34$^+$ enriched cells, 200uL per injection. Each group (CBU + cell dose) contained 7–8 mice. Every 4 weeks for 20 weeks, $\leq$ 100uL of peripheral blood was collected from each mouse in heparin by submandibular bleeding.

METHOD DETAILS

General experimental details

Experiments were not randomized or blinded. Each experiment was performed with 2–6 biological replicates indicated in the following subsections and figures. Colony forming unit assays and expansion assays were also performed in technical triplicates.

No replicates were excluded except in the case of culture contamination, which occurred in the secondary colony forming unit assays for a single 27-year cryopreserved CBU.

Cord blood cryopreservation

Cord blood (CB) units were cryopreserved at the New York Blood Center following standard procedures. Collected CBUs were partially red cell- and plasma-depleted and volume reduced to 20mL, then supplemented with DMSO to a final concentration of 10% DMSO and final total volume of 25mL. The units were then frozen at a controlled rate of 1 °C/min until the units reached a temperature of −50°C. The units were then transferred to liquid nitrogen and cryopreserved for the indicated length of time (Figure 1A). Cryopreserved CBUs were shipped from the New York Blood Center to Indiana University School of Medicine for processing in a liquid nitrogen dry shipper and immediately submerged in liquid nitrogen until further processed.

Thawing and recovery of cryopreserved cord blood

A hybrid method of thawing and reconstitution was developed out of necessity due to the unavailability of Dextran40 (national shortage) and other clinical supplies and equipment in the research lab. In short, the cryo-storage bags were placed inside a Ziploc bag and then placed in a 37°C water bath with gentle agitation until a slushy liquid state was achieved. The cryo-storage bag was removed from outer bag and quickly transferred to laminar flow hood. Ports were sterilized with alcohol wipes. Over a 5 min period, 25mL of chilled, sterile expander solution consisting of equal volumes of 6% hetastarch (Hospira, Inc) and 5% human albumin (Albuked) (Grifols Therapeutics) in sterile saline was slowly introduced through one of the bag ports to the 25mL CB sample with constant mixing. This mixture was then transferred to 2- 50mL conical tubes and kept on ice. An additional 25mL volume expander was used to rinse the bag and equally combined with first collections. Tubes were centrifuged at 400xg for 10 min at 4°C. Supernatant was removed and cell pellets were reconstituted with cold 50% expander solution and 50% sterile saline and centrifuged as before. Pellets were resuspended in cold DPBS for further processing and analysis. Based on CFU numbers of 27 year old CB (27yoCB) and 3 year old CB (3yoCB) compared to fresh CBUs (frCB), similar numbers of CFUs were recovered as expected.[14]

Fresh cord blood units

Fresh CB samples were either purchased from CryoCell International or were provided by the Cleveland Cord Blood Center. CBUs are only considered fresh if they were received and processing begun within 48 h of birth. CBUs were collected in 35mL of Anticoagulant Citrate Phosphate Dextrose Solution and shipped to Indiana University at ambient temperature. Only CBUs with total volume $\geq$ 60mL were used. CB was transferred to sterile 50mL conicals and cords were washed with 2X volume DPBS. Tubes were centrifuged at 400xg for 10 min at 4°C. Supernatant was removed and cells were resuspended in cold DPBS for further processing.

Low density (LD) and CD34$^+$ cell enrichment

Cell suspensions were brought to 50mL using sterile DPBS. 10uL were removed from each unit for cell counting and Trypan Blue viability analysis. 25mL of CB was gently layered over 15mL of Ficol Paque (GE Healthcare/Cytiva) and centrifuged for 25 min at 400xg at room temperature with the brake off. Low density bands were collected, combined for each unit, and washed 2 more times in DPBS at 400xg for 10min. Final pellets were resuspended in DPBS. 10uL were removed for cell counting. Further enrichment for CD34$^+$ cells were performed using magnetic anti-CD34 monoclonal antibody and magnetic selection columns (Miltinyi). Enrichments were performed per manufacturer instructions with one exception-for each unit, a maximum of 300uL of selection beads were used, resulting in some units having lower than expected CD34$^+$ purity. For this reason, all phenotyping data is examined as total number in unit and as fractions of total nucleated cells and low density cells in order to confirm any phenotypic differences were not due to differences in CD34$^+$ purity. After collection of CD34$^+$ fraction, cells were washed 1x in DPBS and resuspended in DPBS. 10uL were removed for counting and the rest were used for further analysis.

Primary ex vivo colony forming unit (CFU) assays

Because purity and cloning efficiency can vary widely from sample to sample, CD34$^+$ enriched cells were plated at various concentrations. In short, CFU-GM, BFU-E, and CFU-GEMM colonies were derived by plating 250, 500, and 2000 CD34$^+$ cells in 1% methylcellulose/Iscove's Modified Dulbeccos Medium (IMDM) with 30% FBS (Corning, prescreened), 2 U/mL recombinant human (rh) Epogen (EPO) (Amgen), 10ng rh Interleukin-3 (IL-3) (R&D Systems), and with or without 50ng rh Stem cell factor (SCF) (R&D Systems), and 10ng rh Granulocyte/macrophage colony stimulating factor (GM-CSF) (R&D Systems). Cultures were incubated at 37°C in a humidified environment containing 5% CO_2/5%O_2. Colonies were scored 12 days after plating. CFU-GM and BFU-E colonies were scored on plates containing only EPO and IL-3. CFU-GM and CFU-GEMM were scored on plates containing EPO, IL-3, SCF, and GM-CSF.[38] For frequency of bulk cell population calculations, CFUs were first assessed as a fraction of the viable CD34$^+$ enriched cells used for plating. The remaining calculations were performed based on these numbers and the fraction of CD34$^+$ enriched cells found in the other bulk populations.

Secondary colony replating assays

Well defined, individual CFU-GEMM colonies were harvested from plates containing EPO, IL-3, GM-CSF, and SCF using an inverted microscope in a clean bench. In short, colonies were identified and harvested by simple aspiration in approximately 20ul using a micro-pipettor. Each colony was transferred to a tube containing 50ul IMDM and dispersed into a single cell suspension with repeated up down pipetting. After all colonies had been harvested, 1mL of complete methylcellulose culture medium, matching that of the primary culture, was added to each tube and vortexed. 1mL of each culture suspension was transferred to a 35mm culture dish and cultured as before. Plates were scored at 12 days after plating and were scored for total colonies (CFU-GM+CFU-GEMM). Secondary CFU are reported as 0, <10, 11–50, or >50 new colonies per plate.

Immunophenotyping by cell surface staining and fluorescence-activated cell sorting (FACS)

Cells were analyzed for cell surface expression of the proteins indicated in Table S3 to enumerate the immunophenotypically defined subpopulations of hematopoietic stem (HSCs) and progenitor cells (HPCs) defined in Table S3.[39] 1-5x10^6 $CD34^+$ enriched cells were incubated in PBS with 1:500 Zombie Yellow Fixable Viability dye for 15 min at room temperature. Antibodies against cell surface markers were added to each sample and cells were incubated for 15 additional min at room temperature. Cells were washed 2x with cold DPBS +0.5% BSA + 2mM EDTA. Cells were resuspended in cold DPBS +0.5% BSA + 2mM EDTA for FACS. Gates were drawn based on unstained and single stained controls. Concurrent to this analysis, 5x10^4 viable $CD34^+$ cells were sorted directly into 12-well untreated plates in 1mL RPMI1640 + 10% FBS +100 ng/mL recombinant human TPO/SCF/FLT3L cell growth media for expansion assay (see below). Also concurrent to this analysis, immunophenotypically defined HSCs and MPPs were sorted separately directly into RLT Lysis Buffer (Qiagen) for RNA-sequencing (see below). Immunophenotypically defined cell subpopulations were enumerated using FlowJo software. For frequency of bulk cell population calculations, immunophenotypically defined subpopulations were first assessed as a fraction of viable $CD34^+$ enriched cells using dead cell and debris exclusion with Forward/Side Scatter and Zombie Yellow gating. The remaining calculations were performed based on these numbers and the fraction of $CD34^+$ enriched cells found in the other bulk populations. Cell subpopulations were defined as follows (Table S3): Live: Zombie Yellow negative (ZY-); CD34+: ZY-$CD34^+$; HSC: ZY-$CD34^+CD38^-$CD45RA-CD49f+$CD90^+$; MPP: ZY-$CD34^+CD38^-$CD45RA-CD49f-$CD90^-$; MLP: ZY-$CD34^+CD38^-$CD45RA + $CD10^+$; CMP: ZY-$CD34^+CD38^+CD10^-$CD45RA-CD135+; MEP: ZY-$CD34^+CD38^+CD10^-$CD45RA-CD135-; GMP: ZY-$CD34^+CD38^+CD10^-$CD45RA + CD135+.

Ex vivo expansion assays

For cryopreserved 27yoCB and 3yoCB, pure $CD34^+$ cells were sorted directly into 12-well untreated plates. For CBUs that were never frozen (frCB), unsorted $CD34^+$ enriched cells ($CD34^+$ purity $\geq$85%) were plated in 12-well untreated plates. All cells were grown at a density of 5x10^4 cells/mL in 1mL RPMI1640 + 10% FBS +100 ng/mL recombinant human TPO/SCF/FLT3L cell growth media, media that has previously been utilized for *ex vivo* culture of HSCs/HPCs.[40–44] Cultures were incubated at 37°C in a humidified environment containing 5% CO_2/5%O_2. On day 4 after plating, cells were collected and total viable nucleated cellularity was counted by Trypan Blue staining. Cells were stained as described above for immunophenotyping or were plated for expanded CFU assays as described above. HSC/HPC subpopulations and CFU numbers were enumerated for Day 4 and total fold-expansion was analyzed by comparing to Day 0 totals for each 5x10^4 cells plated. It is noteworthy that RPMI1640 + 10% FBS is not used clinically. To compare this method with clinical expansion methods, we expanded fresh CBUs independent of the cryopreservation studies in RPMI1640 + 10% FBS or SFEM II (STEMCELL Technologies) serum free media for four days. After 4 days, we measured expansion of TNC and $CD34^+$ cells by cell surface staining and flow cytometry and show that, while SFEM II yields more expansion of $CD34^+$ cells than RPMI1640 + 10% FBS, differences between biologically distinct CBUs are consistent regardless of the culture media used (Table S4). Thus, we can infer that the expansion results for the 27yoCB would have been similar using serum free media and that long-term cryopreserved CBUs can be expanded for clinical utility.

In vivo SCID repopulating cell frequency assay and analysis

Female NSG mice were purchased in-house from the *In Vivo* Therapeutics Core at Indiana University School of Medicine. 1 week prior to transplantation, mice were placed on a diet of antibiotic-dosed food (Uniprim) and were given acid water to prevent gastroenteritis. 24 h prior to transplantation, NSG mice given a sublethal (350rads) whole body dose of radiation. Irradiated mice were transplanted by tail vein injection with varying cell doses of 1x10^3, 5x10^3, or 10x10^3 $CD34^+$ enriched cells, 200uL per injection. Each group (cord blood unit + cell dose) contained 7–8 mice. As these mice are sublethally irradiated with 350rads, no helper cells are included because the host hematopoietic system provides enough support to survive engraftment failure (no mice were lost to hematopoietic failure during these experiments, even in mice exhibiting little to no human chimerism). Every 4 weeks for 20 weeks, $\leq$ 100uL of peripheral blood was collected from each mouse in heparin by submandibular bleeding. Peripheral blood was incubated in red blood cell lysis buffer (BioLegend) for 10 min, washed with DPBS twice, stained with anti-human CD45 fluorophore conjugated antibody, and analyzed by FACS to measure human cell chimerism. 20 weeks following transplantation, mice were sacrificed and bone marrow from each mouse was collected by flushing 1 femur with PBS using a 25g needle. Bone marrow cells were counted. 3x10^6 cells were then stained with antibodies targeting cell surface markers to analyze the immunophenotypes indicated in Table S3. For SCID repopulating cell (SRC) frequency calculations, mice with >0.2% human chimerism in the bone marrow of recipient mice at week 20 after transplantation were considered responders. This value is 2.15-fold higher than background observed in an untransplanted

control. SRC frequencies and comparisons between different CBUs were determined using Extreme Limiting Dilution Analysis (ELDA) software available from Walter+Eliza Hall Bioinformatics (https://bioinf.wehi.edu.au/software/elda/).[22] These SRC calculations and comparisons also included SRC frequencies from previous studies mined from published data.[15–20,45] SRC frequencies were also back-calculated based on $CD34^+$ purity of the $CD34^+$ enriched cells used for transplantation to account for differences. The remaining bone marrow cells from the groups of mice that received $10x10^3$ $CD34^+$ enriched cells were pooled for each cord blood unit group. $5x10^6$ total bone marrow cells from the pooled primary recipients were then transplanted as described above to sublethally irradiated NSG mice for secondary transplant analysis. Secondary recipients were monitored as described for primary recipients.

RNA-sequencing

Immunophenotypically HSCs and MPPs were collected directly in RLT Lysis Buffer by FACS as described above for both 27yoCB and frCB. After collection of $\geq$ 500 cells, tubes were vortexed for 30 s, snap frozen, and stored in -80°C. For collection of RNA, frozen tubes were gently thawed at 4°C. Tubes were then vortexed again for 30 s and allowed to sit at room temperature for 5 min to ensure complete lysis. RNA was then harvested per manufacturer instructions using the Qiagen RNeasy Micro Plus Kit. High quality RNA (RIN => 7.9) was submitted to the Indiana University School of Medicine Center for Medical Genomics for library preparation using the SMART-Seq v4 Ultra Low Input RNA Kit (Takara). mRNA libraries were sequenced on a NovaSeq v1.5 S4 (200 cycles kit) for paired end sequencing. Raw fastq files passing quality filters were obtained from the Center for further analysis.

Correlation of protein expression with early SRC frequency

Three independent CBUs were obtained within 48 h of birth. Prior to any processing, 200uL aliquots of whole cord blood were cryopreserved by adding a final concentration of 90% FBS and 10% DMSO then freezing at a rate of $\sim 1^\circ$ per minute using a Mr. Frosty device. Aliquots were transferred to liquid nitrogen within 72 h of freezing and were stored for less than 6 months. The remainder of the CBU was processed as described above to obtain $CD34^+$ enriched cells with >90% $CD34^+$ purity. $CD34^+$ enriched cells were cryopreserved using the same method as the whole cord blood aliquots. For assessment of early SRC frequency, $CD34^+$ were thawed rapidly in a 37°C water bath and washed with serum free media. Cells were resuspended in PBS and delivered to sublethally irradiated NSG mice as described above at doses of $1x10^4$, $2.5x10^3$, and $5x10^2$. One month following transplantation, peripheral blood from the recipient mice was obtained and analyzed for human chimerism as described above. Separately, the 200uL aliquots of whole cord blood were thawed rapidly in a 37°C water bath, subjected to red blood cell lysis using RBC lysis buffer (Biolegend), were washed, and were then stained with anti-human CD34 antibody. Some aliquots were simultaneously stained with anti-CD22 antibody. Alternatively, following anti-CD34 staining, cells were permeabilized using Fix/Perm solution (Biolegend) and were stained intracellularly with anti-human MYC antibody. Stained cells were washed three times and were analyzed for protein expression levels using FACS.

QUANTIFICATION AND STATISTICAL ANALYSIS

RNA-sequencing analysis

Fastq files were analyzed for quality using FastQC. Reads were trimmed of adapters and low-quality reads using Cutadapt with the arguments -a CTGTCTCTTATA -A CTGTCTCTTATA –nextseq-trim = 20 –minimum-length 50. Raw genome files downloaded from GENCODE (GRch38 version 41) were used as input to generate genome indices with STAR alignment software using the arguments –sjdbOverhang 99. Trimmed reads were aligned to the genome using STAR with the arguments –readFilesCommand zcat –outSAMtype BAM SortedByCoordinate –outSAMunmapped Within. Aligned reads were assigned to exons and annotated by gene using HTSeq with the arguments -s no -r pos -f bam. Gene counts were normalized and differential expression was analyzed using DESeq2 R package. Sex of the baby that the CB came from was inferred by expression of *XIST* (female specific transcription) and *DDX3Y* (male specific transcription) so that sex can be controlled for in the model. The design used to examine differential expression between "High engraftment/SRCs" and "Low engraftment/SRCs" was ~Sex + Response. The design used to examine differential expression between cryopreserved units and fresh units was ~Sex + Condition. Due to the low sample size, significant differential expression was analyzed with heightened stringency. First, differential expression was analyzed with an alpha cutoff of p_{adj} = 0.05. Next, genes were only further explored at the individual gene level if there was a 2-fold change or greater between each "High" unit and the single "Low" unit. For example, a gene with an adjusted p value of 0.03, normalized counts of 25 and 30 for the "High" units, and 10 for the "Low" unit would be further explored. In contrast, a gene with an adjusted p value of 0.03, normalized counts of 25 and 30 for the "High" units, and 14 for the "Low" unit would not be further explored because one of the "High" units is less than 2-fold greater than the "Low". Gene set enrichment analysis was performed using test statistics generated by DESeq2 against curated datasets from MSigDB c2, c5, and c6 (downloaded from Walter+Eliza Hall Bioinformatics resource https://bioinf.wehi.edu.au/MSigDB/v6.1/) with fgsea R package with the arguments minSize = 50, maxSize = 500 and adjusted p value cutoff of 0.01.

Statistical analyses

All appropriate statistical analyses were determined prior to performing experiments. For studies examining 27yoCB, 3yoCB, and frCB, one-way ANOVA was performed with post hoc Tukey's multiple comparisons. For studies examining expansion, two-way ANOVA was performed with post hoc Tukey's multiple comparisons only examining differences between CB group controlling for Day of the expansion assay. For *in vivo* studies, two-way ANOVA was performed with post hoc Tukey's multiple comparisons only examining differences between CB group controlling for cell dose. To determine differences in SRC frequency, pairwise differences are analyzed using the likelihood ratio test implemented by ELDA software from Walter+Eliza Hall Bioinformatics (https://bioinf.wehi.edu.au/software/elda/).