Uncovering Molecular and Functional Dynamics of Human Hematopoietic Stem Cells During *In Vitro* Culture for Cell Therapy Manufacturing

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INTRODUCTION

- Characterizing long-term engrafting hematopoietic stem cells (LT-HSC) within human CD34+ cells is challenging due to their inherent heterogeneity, particularly when subjected to *in vitro* culturing conditions during cell therapy manufacturing.
- This poses a significant obstacle to adequately monitor the quality of CD34-enriched cell therapies.
- Large scale manufacturing process itself introduces stress, which has the potential to alter the behavior of hematopoietic stem and progenitor cells (HSPCs), consequently impacting the final product.

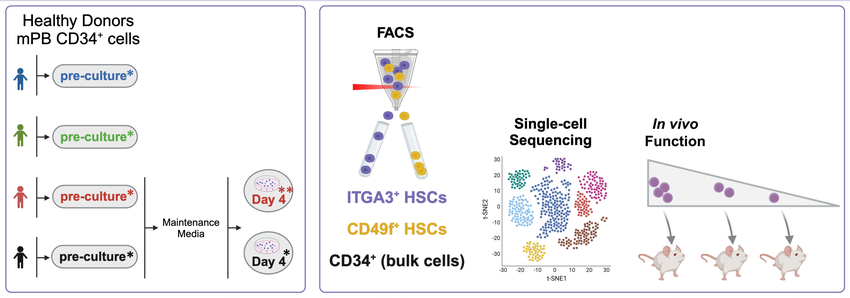
OBJECTIVE

- To gain comprehensive insights into the dynamic changes that can occur during the manufacturing process, we carried out a systematic longitudinal analysis using single-cell transcriptomics to characterize cultured human mobilized peripheral blood-derived HSCs followed by functional assessment through *in vivo* repopulating studies.

METHODS

- Mobilized peripheral blood derived CD34⁺ HSPCs were cultured for 4 days in maintenance media. Due to known down-regulation of CD38, a cell surface antigen commonly included in the HSPC characterization in the CD34⁺ HSPCs, we investigated a new strategy identified in cord blood derived cells that does not rely on CD38 expression to identify LT-HSCs[1]. We therefore conducted a comparative analysis of the commonly used strategy CD34⁺CD38⁻CD90⁺CD45Ra⁻CD49f⁺ (here named as CD49f⁺ HSCs[2]) to an alternative one which bypassed the reliance of CD38: CD34⁺CD90⁺CD45Ra⁻EPCR⁺CD133⁺ITGA3⁺ (here named as ITGA3⁺ HSCs[1]). Furthermore, we investigated whether the ITGA3⁺ HSC panel can allow for better identification and enrichment for LT-HSCs after *in vitro* culture.

Fig. 1. Experimental Design

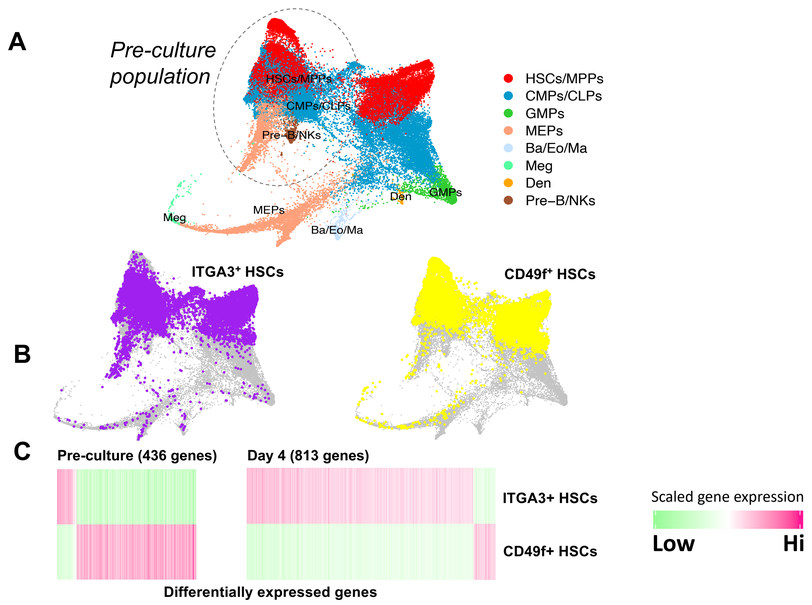


- Mobilized peripheral blood (mPB) human CD34⁺ HSPCs from 4 healthy donors were used for the analysis. Prior to scRNAseq experiments, cells were FACS sorted based on CD34⁺CD90⁺CD45Ra⁻EPCR⁺CD133⁺ITGA3⁺ (ITGA3⁺ HSC) or CD34⁺CD38⁻CD90⁺CD45Ra⁻CD49f⁺ (CD49f⁺ HSC) before culturing ("pre-culture" right after thawing) or after 4 days in maintenance media. For *in vivo* limiting dilution assays, the cells were FACS sorted after 4 days in maintenance media.

* indicates samples used for scRNAseq experiments, ** indicates samples used for *in vivo* experiments.

RESULTS

Fig. 2. scRNAseq of CD34⁺ bulk and populations isolated based on ITGA3⁺ and CD49f⁺ panels reveal cell states seen throughout hematopoiesis. Cell culturing reveal molecular differences between ITGA3⁺ and CD49f⁺ HSCs



- **A.** Cells (including bulk CD34⁺ and enriched HSCs) from all donors and days were aggregated and displayed using force directed layout generated by ForceAtlas[4]. Broad cell states were determined by defining clusters in Seurat and annotating them based on expression of key lineage markers. Cells from pre-cultured samples showed distinct separation from cultured cells, demonstrating the effect of culturing conditions on gene expression. **B.** Cells isolated based on ITGA3⁺ and CD49f⁺ panels are highlighted in purple and yellow, respectively. In pre-cultured conditions, both methods yielded a higher fraction of HSCs compared to that found in CD34⁺ bulk population. Once cultured, ITGA3⁺ cells yielded a higher fraction of HSCs compared to CD49f⁺ cells, with increased expression of *AVP* and *HLF*, both of which are canonical genes observed to be highly expressed in HSC-enriched populations. **C.** Heatmap of genes differentially expressed (Wilcoxon test, absolute FC>1.25, p<1e-5) between ITGA3⁺ and CD49f⁺ HSCs from a single donor across pre-cultured and cultured conditions. The transcriptomes of ITGA3⁺ and CD49f⁺ HSCs diverge as cells are being cultured. MPP: multi-potent progenitor, CMP: common myeloid progenitor, CLP: common lymphoid progenitor, GMP: granulocytic/monocytic progenitor, MEP: megakaryocytic erythroid progenitor, Ba: basophils, EO: eosinophils, Ma: mast cells, Meg: megakaryocytic, Den: dendritic cells, Pre-B: pre-lymphoid B cells, NK: natural killer cells.

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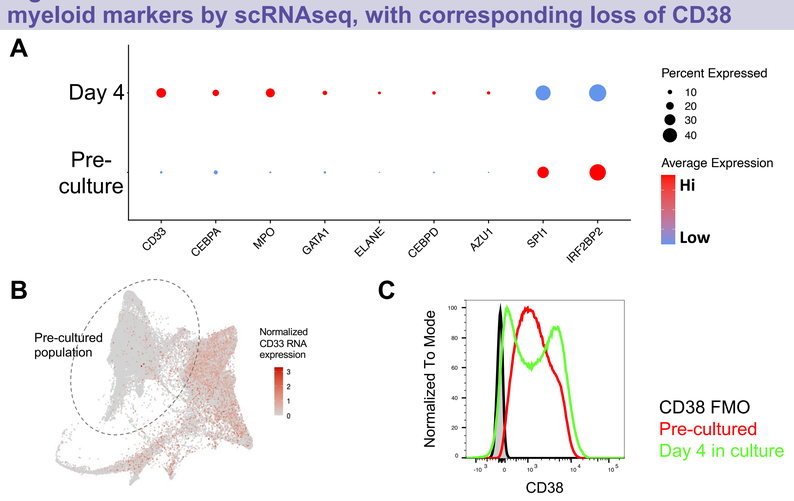
Disclosures

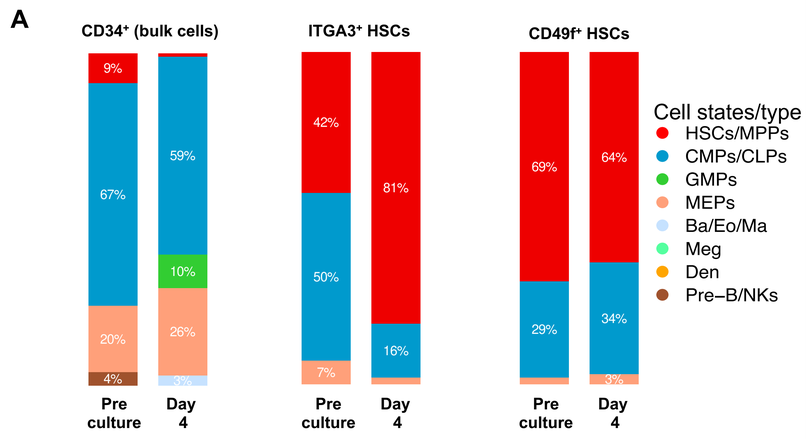
All authors listed here are current employees and equity holders of Vor Bio.

Fig. 3. CD34⁺ HSPCs cultured in maintenance media are enriched for myeloid markers by scRNAseq, with corresponding loss of CD38



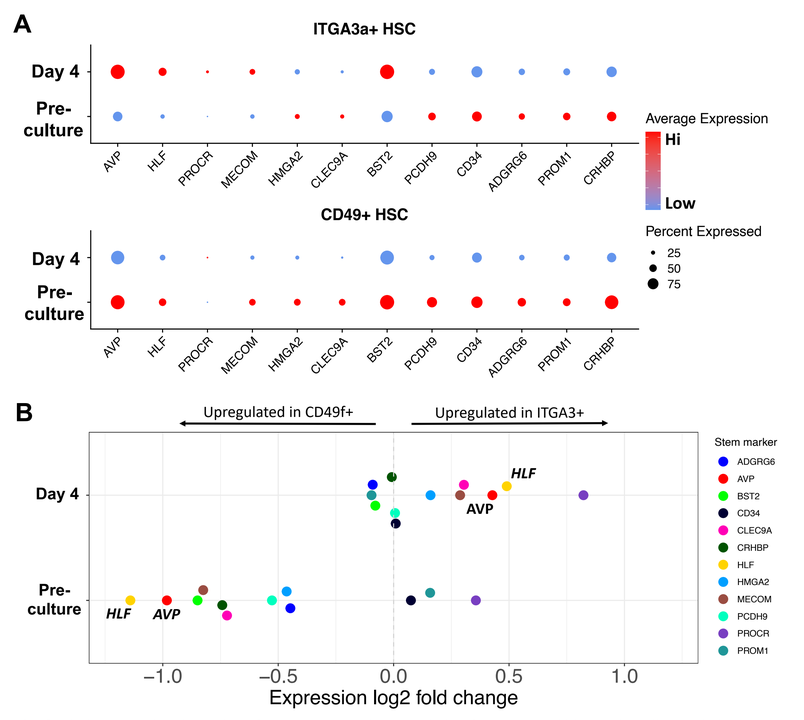
- **A.** Normalized expression intensity of key myeloid markers and the percent of cells expressing them in pre-culture and day 4 samples. *CD33, MPO, SPI1, CEBPA,* and *IRF2BP2* show increase in expression during exposure to culturing conditions. **B.** Cells from all donors and days were aggregated and displayed using force directed layout generated by ForceAtlas[4] . CD33 expression is increased in cells after culturing. **C.** Flow cytometric analysis of CD38 on the surface of CD34⁺ cells over time in culture. Black histogram represents the fluorescence minus one (FMO) control, red represents CD34⁺ cells before cell culture, and green represent cells at days 4.

Fig. 4. ITGA3⁺ and CD49f⁺ HSCs diverge in cell state composition when cultured



- **A.** Distribution of cell state frequencies in CD34⁺ bulk cells and cells FACS sorted ITGA3⁺ and CD49f⁺ HSCs. Cell states were determined by annotating cell clusters using markers derived from literature[5-8]. Consistent with sc transcript analysis (Fig. 3) ITGA3⁺ HSCs yielded a higher fraction of HSCs/MPPs compared to CD49f+ HSCs after culture. This suggests that culturing alters the cell state composition of the bulk sample and renders traditional enrichment strategies such as CD49f⁺ HSCs less effective.

Fig. 5. Increased expression of canonical stem markers in ITGA3⁺ HSCs relative to CD49f⁺ HSCs over time in culture from a single donor

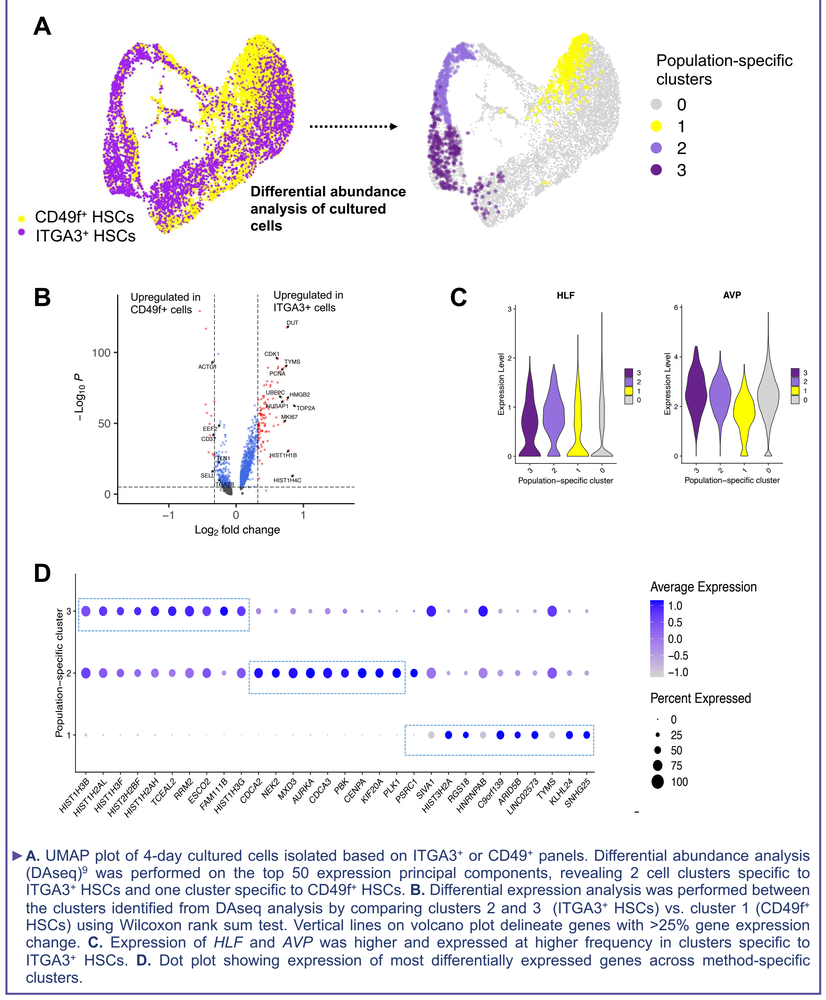


- **A.** Scaled expression intensity of stemness markers and the frequency within each cell population across different days. **B.** Expression $\log_2$ fold change between ITGA3⁺ HSCs and CD49f⁺ HSCs were computed for 12 stemness markers at pre-culture and 4 days in culture timepoints for one donor. *AVP* and *HLF* expression was significantly higher in ITGA3⁺ HSCs relative to CD49f⁺ HSCs at days 4 of culture but not in pre-cultured cells.

Reference

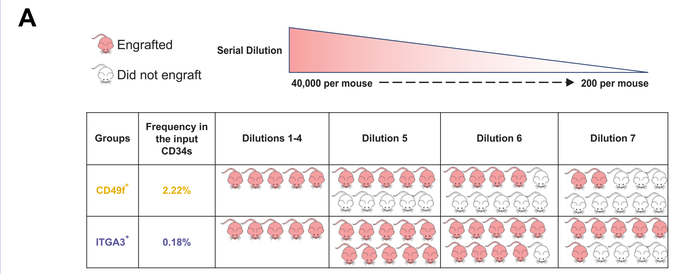
1. Tomellini E, et al (2019). Integrin-a3 Is a Functional Marker of Ex Vivo Expanded Human Long-Term Hematopoietic Stem Cells. Cell Rep.
2. Notta, F et al (2011). Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science.
3. Hu, Y, and Smyth, GK (2009). ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays. Journal of Immunological Methods.
4. Butler, A, et al (2018). Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nature Biotechnology.
5. Hay SB, et al (2028) The Human Cell Atlas bone marrow single-cell interactive web portal. Exp Hematol.
6. Lehnertz B, et al (2021). HLF expression defines the human hematopoietic stem cell state. Blood.
7. Pellin D, et al (2019). A comprehensive single cell transcriptional landscape of human hematopoietic progenitors. Nat Comm.
8. Ranzoni AM, et al (2021). Integrative Single-Cell RNA-Seq and ATAC-SeqAnalysis of Human Developmental Hematopoiesis. Cell Stem Cell.
9. Zhao, J, Jaffe, A, Li, H, Kluger, Y (2021). Detection of differentially abundant cell subpopulations in scRNA-seq data. PNAS, 118(22):e2100293118.

Fig. 6. A subset of cell states found only in ITGA3⁺ HSCs shows increased expression of histones, stem cell markers and genes involved in cell cycle regulation



- **A.** UMAP plot of 4-day cultured cells isolated based on ITGA3⁺ or CD49⁺ panels. Differential abundance analysis (DAseq)[9] was performed on the top 50 expression principal components, revealing 2 cell clusters specific to ITGA3⁺ HSCs and one cluster specific to CD49f⁺ HSCs. **B.** Differential expression analysis was performed between the clusters identified from DAseq analysis by comparing clusters 2 and 3 (ITGA3⁺ HSCs) vs. cluster 1 (CD49f⁺ HSCs) using Wilcoxon rank sum test. Vertical lines on volcano plot delineate genes with >25% gene expression change. **C.** Expression of *HLF* and *AVP* was higher and expressed at higher frequency in clusters specific to ITGA3⁺ HSCs. **D.** Dot plot showing expression of most differentially expressed genes across method-specific clusters.

Fig. 7. ITGA3⁺ HSCs yields 10x more long-term repopulating cells than CD49f⁺ HSCs

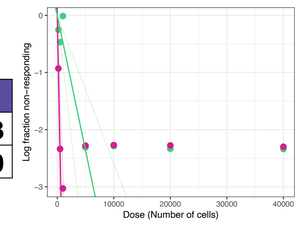


B

1 / Stem Cell Frequency (SRC) at day 4 in culture

With Lower/Upper bounds at 95% confidence interval

Method	Lower	Estimate	Upper
ITGA3⁺	359	210	123
CD49f⁺	3862	2117	1160



- **A.** Xenotransplantation study was conducted to quantify functional HSCs by transplanting limiting dilutions of ITGA3⁺ HSCs or CD49f⁺ HSCs into immunocompromised mice. Analysis of multilineage chimerism in transplanted mice after 16 weeks revealed a substantial difference in the long-term repopulating HSC content between the two enrichment strategies. Positive engraftment was considered as >0.02% of hCD45⁺ together with myeloid (CD33⁺) and lymphoid (CD19⁺) positive cells; n=180 mice. **B.** The stem cell frequency (or SCID-repopulating cells, SRC) was calculated using the ELDA software[3]. ITGA3⁺ HSCs exhibited a 10-fold higher number of HSCs compared to CD49f⁺ HSCs.

CONCLUSION

- We observe a global myeloid-biased shift with upregulation of *CD33* and loss of CD38 surface antigens across all cell states during culture in maintenance media.
- **Molecular profiles of ITGA3⁺ HSCs and CD49f⁺ HSCs diverge as they are cultured.**
- ITGA3⁺ HSCs shows higher RNA expression of canonical stem markers compared to CD49f⁺ HSCs in culture.
- **ITGA3+ HSCs are enriched in HSC-like cell states compared to CD49f⁺ HSCs in culture.**
- Cell states specific to ITGA3⁺ HSCs in culture show enrichment of cell cycle pathways.
- **ITGA3+ panel is more precise to enrich for HSCs (confirmed transcriptionally and functionally) than CD49f+ panel, partially due to the lack of using CD38 as one of the markers.**
- ITGA3+ panel has the potential to more accurately identify HSCs after cellular and genomic manipulation.

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