Multi-modal profiling of human fetal liver hematopoietic stem cells reveals the molecular signature of engraftment

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The human hematopoietic stem cell harbors remarkable regenerative potential that can be harnessed therapeutically. During early development, hematopoietic stem cells in the fetal liver undergo active expansion while simultaneously retaining robust engraftment capacity, yet the underlying molecular program responsible for their efficient engraftment remains unclear. Here, we profile 26,407 fetal liver cells at both the transcriptional and protein level including ~7,000 highly enriched and functional fetal liver hematopoietic stem cells to establish a detailed molecular signature of engraftment potential. Integration of transcript and linked cell surface marker expression reveals a generalizable signature defining functional fetal liver hematopoietic stem cells and allows for the stratification of enrichment strategies with high translational potential. More precisely, our integrated analysis identifies CD201 (endothelial protein C receptor (EPCR), encoded by PROCR) as a marker that can specifically enrich for engraftment potential. This comprehensive, multi-modal profiling of engraftment capacity connects a critical biological function at a key developmental timepoint with its underlying molecular drivers. As such, it serves as a useful resource for the field and forms the basis for further biological exploration of strategies to retain the engraftment potential of hematopoietic stem cells ex vivo or induce this potential during in vitro hematopoietic stem cell generation.
The human hematopoietic stem cell (HSC) has been the focus of intense study due to its remarkable regenerative potential and its therapeutic utility in treating a variety of diseases. HSCs reside at the apex of the hematopoietic system and have the capacity to both self-renew and differentiate into all mature blood cell types. As such, these cells, and the hierarchical structure of their progeny, represent an ideal system in which to ask fundamental biological questions and discover key insights into hematopoietic development and disease. These insights could be harnessed to improve ex vivo expansion methods, increase engraftment efficiency, and enable in vitro HSC generation from pluripotent stem cell (PSC) sources.

The first HSCs emerge in the aorta-gonad-mesonephros (AGM) region prior to traveling to the fetal liver (FL), where they undergo expansion to create the HSC pool that sustains hematopoiesis for the lifetime of an individual. The FL remains the main site of hematopoiesis until the bone marrow (BM) becomes competent to serve as the final HSC niche closer to birth1–4. This dynamic transition is coupled to a switch from a proliferative to a predominantly quiescent phenotype postnatally2, reflecting just one of several differences between developmentally distinct HSCs5. A key functional difference between FL-derived and more mature HSCs is the superior engraftment potential of FL when compared to cord blood (CB) and BM cells6. The retention of robust engraftment potential during active expansion highlights a unique feature of FL HSCs, as proliferation and HSC functionality are inversely correlated postnatally7,8. This prompted us to specifically profile FL-derived HSCs to establish a detailed molecular signature of engraftment potential.

To dissect the molecular underpinnings of engraftment potential at the highest possible resolution, we combined several orthogonal single-cell profiling approaches. The emergence of single-cell RNA sequencing (scRNAseq) technologies has led to significant advances in terms of characterization of the larger hematopoietic stem and progenitor cell (HSPC) pool. Transcriptomic profiling of large numbers of single CD34+ cells has resulted in a more nuanced understanding of the postnatal HSPC compartment, revealing a continuum of transcriptional states rather than a succession of clearly demarcated progenitor stages9–12. Recent work has extended such large-scale single-cell transcriptomic profiling efforts to the prenatal stage13, including work in the context of the developmental cell atlas analyzing the dynamic hematopoietic composition of the entire FL and other tissues throughout early human development14. However, despite interrogation of large numbers of total cells, their relative scarcity resulted in the profiling of few truly functional HSCs.

In this study, we specifically focus on this rare HSC fraction by profiling a highly enriched population of FL HSCs that have been confirmed to be functional for engraftment. Several strategies exist to enrich for functional HSCs within a pool of HSPCs15–18, with a combination of GPI-80 and CD133 resulting in one of the highest frequencies described (~1/5)16. Glycophosphatidylinositol-anchored surface protein GPI-80 has been shown to mark a subpopulation of FL HSCs that combine self-renewal ability and engraftment potential17. Using this marker as the basis for functional HSC enrichment as confirmed in transplantation assays, we single-cell profile these highly enriched FL cells to uncover the detailed molecular signature of engraftable FL HSCs. Further dissection of this engraftment profile reveals signatures highlighting the importance of proteome integrity maintenance, as well as the prominent expression of factors linked to aging and the concomitant decline of HSC functionality. This comprehensive characterization of the engraftment signature of FL HSCs using multi-modal profiling to define an essential biological function at a key developmental timepoint will serve as a useful resource for the field.

Results

The hematopoietic landscape of the human fetal liver. To obtain a detailed molecular signature of human FL HSCs, we performed CITE-seq19, a technique that allows for the simultaneous assessment of transcript and cell-surface marker level expression by combining droplet based single-cell RNA sequencing (scRNAseq) and oligo-tagged antibodies (Supplementary Table 1). Following dissociation of a human FL sample, cells were divided into either CD34+–enriched or CD34− flowthrough cells via magnetic bead separation (Supplementary Fig. 1a). The CD34− live cells were further sub-divided into GYPA+ and GYPA− via fluorescence activated cell sorting (FACS) to capture populations of maturing erythroid progenitors that constitute a sizeable portion of the FL at this stage in development (CD34−GYPA+). From the CD34−-enriched compartment, live CD34− cells were sorted (CD34+bulk) to reflect the cell population that is routinely used in a clinical HSC transplantation setting. In a separate fraction, we further enriched this population using GPI-80 expression (GPI-80+), a marker tightly linked to engraftment potential16,17, to focus our analysis on HSCs capable of long-term engraftment. Following data processing and quality control, this resulted in a total of 26,407 FL cells profiled from one fetal liver, divided across the following fractions: 8735 CD34+bulk cells, 7235 GPI-80+ cells, 6793 CD34−GYPA− cells and 3644 CD34−GYPA− cells (Supplementary Fig. 1a).

Combinatorial transcriptomic analysis of all four fractions resulted in an overview of the hematopoietic landscape at this developmental stage (Fig. 1a). Figure 1b displays the distribution of the four fractions within the combined dataset, illustrating that the CD34+ HSC/multipotent progenitor (MPP) compartment represents cells belonging to the CD34+ bulk and GPI-80+ enriched fractions. Greater than half of all assayed cells represented CD34+ HSCs/MPPs (Fig. 1a–e), which is further emphasized by the overlay of a previously established HSC/MPP signature14 within the human FL (Fig. 1d) onto our combined data (Fig. 1e). Taking these CD34+ HSCs/MPPs as a starting point, gene expression changes over pseudotime were assessed for each major hematopoietic lineage. This analysis confirmed down-regulation of HSC/MPP marker genes and up-regulation of key lineage identity genes as commitment progresses, reflecting the expected hematopoietic cell types at this developmental stage (Fig. 1e and Supplementary Fig. 1b–e).

Transcriptomic profiling of engraftment potential. In parallel with capture for CITE-seq, cells from these same sorted fractions (with the exception of CD34−GYPA+) were used in simultaneous transplantation experiments to assess engraftment capacity. These experiments revealed superior per-cell engraftment potential of the GPI-80+ fraction as compared to CD34+bulk and CD34−GYPA− fractions, confirming enrichment for bona fide functional HSCs in this population (Fig. 2a). Although the GPI-80+ fraction represented only 2.37% of the cells within the CD34+bulk population (Supplementary Fig. 1a), sorting and enrichment of this fraction enabled the analysis of 7235 GPI-80+ cells, resulting in profiling of the engraftable FL HSC at unprecedented resolution.

In concordance with their superior engraftment potential, we found enrichment for known HSC markers such as ITGA6 (CD49f), PROC (CD201 or EPOR), MLLT3, HLF, MECOM, CLC9A, LMO2, and HMGAA2 in the GPI-80+ fraction compared to CD34+bulk cells (Fig. 2b). Enrichment for these genes, and others with currently unexplored connections to HSCs, distinguishes the GPI-80+ fraction from bulk CD34+ cells, revealing a
detailed transcriptomic signature that marks engraftable FL HSCs. A comprehensive list of all differentially expressed genes (DEGs) that make up this engraftment signature is presented in Supplementary Table S2.

To further analyze engraftable FL HSCs, we first focused on the CD34<sup>+</sup> HSC/MPP population (Fig. 1c) captured within the gray dotted circle in Fig. 1a. Separate clustering of this subset resulted in seven distinct HSC/MPP clusters (HSC/MPP 0–6) in addition to previously identified progenitor clusters (Fig. 2c). To determine which of these clusters best correlated with engraftment potential, we tracked cluster dynamics upon GPI-80<sup>+</sup> enrichment. Using the cluster designations from Fig. 2c, we identified corresponding clusters in the individual fractions making up this CD34<sup>+</sup> HSC/MPP population (Fig. 2d, e). Mapping the changes in cluster proportions between CD34<sup>+</sup> bulk and GPI-80<sup>+</sup> enriched fractions (Fig. 2f), revealed a relative absence of cells...
corresponding to HSC/MPP cluster 1 within the GPI-80+ enriched fraction, suggesting that these cells and their associated transcriptomic signature likely contribute minimally to the engraftment potential of FL HSCs. Emboldening this point, one of the top enriched genes in this cluster is the activation marker CDK6, whose expression marks the transition towards more differentiated progenitors. Similarly, we noted a significant reduction in cluster 5 and those clusters corresponding to pDC/DCpre cells and neutrophil/myeloid progenitors. Conversely, the proportion of cells in clusters 0, 2, and 3 increased in the GPI-80+ fraction. Of these, the most pronounced change was noted for HSC/MPP cluster 0, which showed a substantial fourfold increase. This specific enrichment, resulting in cluster 0 cells accounting for 40% of the total GPI-80+ population, strongly suggests that the transcriptomic profile corresponding to this cluster best represents cells with functional engraftment potential.

To further dissect the gene expression signature specific to cluster 0, we assessed the differential gene expression between this and other GPI-80+ clusters. RGCC and LMNA were identified as the top enriched genes in cluster 0, closely followed by VIM and ID1 and ID3 (Fig. 2g, Supplementary Table 3, and Supplementary Fig. 2a). In addition to ID signaling pathway members, whose
expression appears concentrated along the outer edge of cluster 0, we also found enrichment for KLF6, AHR and TIPARP, encoding key players in the aryl hydrocarbon receptor (AHR) pathway where the latter two make up an AHR-TIPARP-negative feedback axis\(^2\) (Fig. 2g and Supplementary Table 3). Interestingly, LMA\(_N\) was found to be more prominently expressed in FL than postnatal CD34\(^+\) and HSC-enriched fractions (Supplementary Fig. 2b) in line with its previously described inverse correlation with ageing\(^2\).

Similarly, the expression of several other cluster 0 genes such as ID1, ID3, AHR, TIPARP, and HES1 was more pronounced in FL compared to postnatal HSCs, suggesting the downregulation of these genes over developmental time (Supplementary Fig. 2c). Moreover, gene regulatory network (GRN) analysis using single-cell Regulatory Network Inference and Clustering (SCENIC)\(^3\) revealed that NFE2L2, KLF13 and KLF10 regulons drive a cluster-0-specific transcriptional program (Fig. 2h, i and Supplementary Fig. 2d, e). Notably, KLF13 and KLF10 both regulate LMA\(_N\) expression in addition to KLF10 regulating KLF6, AHR and NFE2L2 expression, with the latter providing increased coverage and an additional layer of information\(^1\) (Fig. 3a and Supplementary Fig. 4a, b).

To functionally validate the engraftment potential of cluster 0, we transplanted CD201\(^+\) and CD201 depleted cells into conditioned NSG (NOD/SCID/IL2r\(^{−}\)-m\(_{mu}\)) mice and compared their engraftment capacity to CD34\(^+\) and CD34\(^-\) depleted cells. These assays highlighted a robust enrichment in engraftable HSCs in both the GPI-80\(^+\) and CD34\(^+\) fractions as compared to the depleted fractions with superior engraftment of the CD201\(^+\) cells as compared to the GPI-80\(^+\) fraction (\(p<0.0001\)) (Fig. 3h). Both CD201 and GPI-80 enriched fractions showed robust multilineage reconstitution when followed for up to 20 weeks post transplantation (Supplementary Fig. 4f, g).

Overall, a strong correlation was found between mRNA and ADT expression, with the latter providing increased coverage and an additional layer of information\(^1\) (Fig. 3a and Supplementary Fig. 4a, b). Figure 3a projects both layers of information onto the CD34\(^+\) UMAP for a selection of HSC markers. Notably, for CD34, CD90 and CD49f, often used in combination to enrich for HSCs, ADT expression patterns were observed that were not readily apparent based on mRNA data alone, indicating co-expression of these markers at the protein level. Importantly, ADT data also highlighted CD201 as a marker for cluster 0 and our engraftment signature (Fig. 3a, b and Supplementary Fig. 4b).

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Notably, 49% of the top CD201 expressing cells within the CD34\(^+\) bulk fraction represented cluster 0 cells versus ~25% when considering markers such as CD49f, CD90 and CD133 (Fig. 3c–f). This level of enrichment for cluster 0 even surpassed that observed when using GPI-80 to sort functional HSCs from bulk CD34\(^+\) cells (Fig. 2f). In stark contrast to CD201, enrichment based on surface expression of CD164, a marker described as an alternative to CD38\(^-\) gating in HSC enrichment strategies\(^1\), was correlated with a decrease in cluster 0 frequency (Fig. 3c, g). Progressive enrichment for HSC markers such as CD90, CD49f and ENG (endoglin, CD105) did not alter the transcriptomic profile of cells (Supplementary Fig. 4c–e).

To functionally validate the engraftment potential of cluster 0 cells and their enrichment based on CD201 surface expression, we transplanted CD201\(^+\) and CD201 depleted cells into conditioned NSG (NOD/SCID/IL2r\(^{−}\)-m\(_{mu}\)) mice and compared their engraftment capacity to CD34\(^+\) and GPI-80 depleted cells. These assays highlighted a robust enrichment in engraftable HSCs in both the GPI-80\(^+\) and CD34\(^+\) fractions as compared to the depleted fractions with superior engraftment of the CD201 cells as compared to the GPI-80 fraction (\(p<0.0001\)) (Fig. 3h). Both CD201 and GPI-80 enriched fractions showed robust multilineage reconstitution when followed for up to 20 weeks post transplantation (Supplementary Fig. 4f, g). Altogether, these
findings illustrate that cell-surface protein expression coupled with transcript-level data provides a powerful approach towards classifying potential enrichment strategies for highly functional HSCs. It also provides a complimentary, yet orthogonal methodology with which to stratify the heterogeneity that exists within the HSC/MPP compartment of the FL.

Multi-dimensional flow cytometry characterization. To further extend the cell-surface marker characterization of FL HSPCs, five additional CD34+ FL samples (post conception weeks 16-22) were profiled by flow cytometry using a 21-marker antibody panel (Supplementary Table 5). To enable comprehensive analysis of the resulting multi-dimensional dataset, marker expression was projected onto a common UMAP scaffold analogous to representations of high-dimensional transcriptomic data. Adult peripheral blood mononuclear cells (PBMCs) were simultaneously phenotyped and projected into the same UMAP space to provide context for less mature FL cell subsets. Phenograph clustering was performed on the combined data (PBMCs and
Multi-modal comparison of HSC enrichment strategies.

Inspired by the co-expression pattern of HSC markers evident in our data, we next asked if both levels of information (mRNA and ADT expression) could be used to visualize and compare HSC enrichment strategies in silico sorting. This bioinformatic approach harnesses ADT expression values to recreate gating strategies used in FACS sorting. Three sorting strategies were compared by this method: (1) The “classical” HSC enrichment scheme (lin−CD34+CD38−CD45RA−CD90+CD49f+); (2) an HSC enrichment strategy that was recently described to yield a highly purified population of engraftable HSCs from CB (lin−CD34+CD38−CD133+GPI-80+), which we refer to as the “Sumide et al.” signature and corresponds well to the GPI-80-based functional enrichment strategy used in this work; and (3) an “EPCR+” signature (CD34+CD38−CD201+). An overview of the in silico sorting strategy is presented in Supplementary Fig. 4a, b and Supplementary Fig. 5a, b, c. In addition to HSC markers present in our CITe-seq panel, lineage markers were also included to identify committed progenitors within the CD34+ FL fraction. UMAP visualization of the combined flow data from 5 CD34+ FL samples allowed for assessment of the overlap between CD34 expression and that of negative selection markers such as CD38 and CD45RA, as well as more committed lineage markers (Fig. 4e). This process allowed for clear subfractionation of the mature PBMCs (Fig. 4d, e) and demonstrated that certain mature cell markers (CD66c, CD33) are also broadly expressed within the CD34+ population, highlighting the importance of carefully choosing such markers in negative selection strategies. Notably, when looking at the portion of the UMAP that represents CD34+CD38−CD45RA− cells (clusters 1, 3, 8, 13, 30, 31 in Fig. 4d), cells that also co-express CD90 and CD49f (right edge of clusters 1 and 3) were identified as suggested by our ADT data (Fig. 3a). Interestingly, this visualization drew attention to a population of cells at the tip of cluster 3 in which CD49f, CD201 and GPI-80 appeared to be co-expressed (Fig. 4d, e).

To arrive at an in-depth characterization of engraftable FL HSCs, we harnessed the functional HSC enrichment capacity of GPI-80, a marker closely associated with engraftment. Despite representing only 2.37% of total CD34+ cells, sorting thousands of CD34+GPI-80+ cells allowed us to perform parallel profiling of this rare population at the functional, transcriptional and surface protein level. After confirming their superior engraftment potential via xeno-transplantation, we mined the transcriptome of these rare cells, resulting in a molecular signature of engraftable FL HSCs at unprecedented resolution.

Given the transcriptionally distinct clusters we discerned within this highly purified population, we sought to understand which of these transcriptomic profiles exhibited the strongest correlation with engraftment potential. By analyzing cluster proportions before and after functional enrichment, we identified several clusters that were significantly enriched, thus corresponding to putative engraftable HSCs.

One such cluster (HSC/MPP cluster 3) was characterized by a prominent unfolded protein response (UPR) signature, represented by an abundance of genes encoding heat shock proteins (HSPA1A, HSPB1B, HSPA6, HSPB1). Interestingly,
Fig. 4 Multi-dimensional flow cytometric characterization across multiple FL samples validates antibody-derived tag (ADT) expression patterns.

a, b Individual representation of peripheral blood mononuclear cells (PBMCs) (a) and CD34^+ FL cells (b) in the same UMAP space. Color indicates plot density. c UMAP representation of both PBMCs (pink) and CD34^+ FL cells (green). Major mature blood cell subsets are indicated on the PBMC sample for orientation purposes. d UMAP representing the different Phenograph clusters identified in the combined dataset (PBMCs and CD34^+ FL cells). e Expression patterns of individual markers and light scatter cytometric parameters overlaid onto the UMAP representation of the combined data. Color indicates fluorescence or light scatter signal intensity.
Fig. 5 In silico comparison of HSC enrichment strategies reveals considerable transcriptomic overlap between signatures. a Overlays of in silico sorted HSC enrichment signatures onto the CD34+ bulk UMAP, 270, 207 and 184 cells were sorted for the classical, Sumide et al. and EPCR+ signature, respectively, of which 15 cells are common to all three signatures. b Venn diagrams showing overlap of differentially expressed genes (DEGs) between the different HSC enrichment signatures. For each signature, the number of DEGs between cells corresponding to that signature based on in silico sorting and the background (other) is depicted. c Overview of top 25 enriched genes corresponding to the Sumide et al. signature and their rank in the different HSC enrichment signatures.

protein quality control has been linked to the ability of HSCs to maintain their undifferentiated status29–31. FL HSCs possess the unique capacity to tolerate significant proliferation without impacting multilineage engraftment potential and this has been associated with a heightened DNA damage response in mouse FL versus postnatal HSCs32. Similarly, there appears to be a role for the maintenance of proteome integrity in shielding HSC function during expansion in the FL. While bile acids have been described to serve as chaperones alleviating unfolded protein stress in expanding mouse FL HSCs33, our data suggest that heat shock proteins may take on this role in the human FL.

The most highly represented cluster upon functional enrichment (HSC/MPP cluster 0) exhibited increased expression of RGCC, LMNA, ID genes and members of the AHR pathway, including TIPARP, the product of which has been identified as a negative regulator of AHR activity32,34. AHR inhibitors such as SR-1 have been described to expand CD34+CB cells35 and we and others have linked AHR inhibition to enhanced endothelial-to-hematopoietic transition and HSPC expansion during in vitro differentiation from PSCs36,37, highlighting a role for AHR inhibition in HSPC biology. LMNA was identified as the second most enriched gene in this cluster and was a part of both KLF10 and KLF13 regulons executing a cluster 0-specific transcriptional program. LMNA encodes the nuclear lamina protein Lamin A/C and is expressed in postnatal HSCs where it shows a decline in expression throughout ageing22,28. Interestingly, comparing LMNA expression between our FL CD34+ cells and existing scRNAseq datasets representing postnatal CD34+ and HSC-enriched fractions9,10,39, revealed more prominent LMNA expression in the FL (Supplementary Fig. 2b), suggesting that the decline in LMNA expression might begin in utero. Given the superior engraftment potential of FL HSCs compared to postnatal HSCs6, this might suggest a role for LMNA in endowing FL HSCs with this remarkable capacity. Consistent with this hypothesis, LMNA has been described to be more highly expressed in LT-HSCs than in less potent ST-HSCs in mice38, a finding that is reflected in our data by the enrichment of LMNA in the GPI-80+ compared to the CD34+ bulk fraction (Supplementary Table 2). Altogether these findings support a link between LMNA expression and HSC functionality.

We also observed enrichment for members of the inhibitor of DNA binding (ID) gene family along the top edge of cluster 0. Expression of ID1, ID2 and ID3 has been reported higher in HSCs compared to downstream progenitors in CB and reduction in their expression coincides with loss of quiescence and in vivo repopulating capacity40. Moreover, ID1 has recently been implicated in controlling the balance between dividing and resting neural stem cells by promoting quiescence41. It is tempting to speculate that ID family members could play a similar role in FL HSCs, especially when taking into consideration co-enrichment for genes encoding other factors involved in cell cycle control in this cluster such as RGCC or “regulator of cell cycle” and the predominantly quiescent profile of cluster 0 cells. A similar function has been reported for NFE2L2 in mouse HSCs24, in addition to its more widely described role as master regulator in the antioxidant response pathway24,42,43. The fact that we find this factor regulating expression of a cluster 0-specific transcriptional program in human FL HSCs suggests that NFE2L2 might be involved in balancing quiescence and self-renewal here as well.

Notably, a recent study profiling PSC-derived HSPCs at the single-cell level, identified ID2 as enriched in what was considered the most naive, in vitro-derived HSPC fraction, highlighting an encouraging overlap in gene expression with engraftable HSCs in vivo44. In this work, the transcriptomic profile of PSC-derived
HSPCs was compared to that from FL HSPCs, identifying additional commonalities but also dissimilarities that might suggest how to further improve PSC-based HSC generation. In line with this goal, we believe that our comprehensive characterization of engraftable HSCs and the detailed enrichment signature that cluster 0 represents will be of particular interest to the field to further optimize this process. Moreover, comparison to postnatal HSC-enriched fractions indicated that the expression of several cluster 0 enriched genes was higher in the FL compared to the BM, suggesting that the signature identified in this work specifically represents HSCs at a stage where they display superior engraftment potential. Given that this prenatal stage presents a developmentally more relevant comparator than postnatal HSC sources when it comes to in vitro generation of HSCs starting from PSCs, we consider our dataset especially valuable in pursuit of this goal for regenerative medicine applications.

Beyond in-depth transcriptional characterization, this study also provides linked cell-surface marker expression data of engraftable FL HSCs. Comparing both transcript and protein expression data, we found that ADT read-out offered complementary insights as they allowed for the identification of marker co-expression that was not readily apparent based on mRNA expression alone. Importantly, ADT but not mRNA expression data suggested that EPCR (CD201) could be used as a cell-surface marker to specifically enrich for cluster 0 cells and thus engraftable FL HSCs, which was confirmed by the superior engraftment potential of CD201+ selected cells in transplantation experiments in immunocompromised mice. While this marker has been proposed as a useful addition to existing enrichment strategies to purify FL HSCs,18 the linked mRNA and ADT data in this work would suggest that EPCR may serve as a viable single enrichment marker for functional FL HSCs.

Using flow cytometry, we further extended our protein-level characterization of FL HSPCs through simultaneous assessment of 21 cell-surface markers and confirmed the expression patterns observed based on ADT expression data as representative across multiple biological replicates of FL. This dataset can be accessed and further interrogated via the following repository (http://flowrepository.org/id/FR-FCM-Z32M). The UMAP representation of the multi-dimensional flow data highlighted a region characterized by co-expression of several prominent HSC enrichment markers, suggesting that it could represent the apex of the hematopoietic hierarchy where engraftable HSCs reside. This interpretation was further supported by the almost exclusive localization of CD201 expression in this region, which we have functionally validated to further enrich for engraftment potential as suggested by its ADT expression profile. These observations are in line with the recent molecular characterization of mouse HSCs where Procr (Epcr) was shown to be enriched in the most primitive subset of functional long-term repopulating HSCs. While the CITE-seq profiling was performed on a single biological FL sample, which could be seen as a limitation of this study, the confirmation of expression patterns by multiparameter flow cytometry as well as functional validation of the predictions regarding superior engraftment potential of the CD201+ fraction resulting from our integrated transcriptomic and cell-surface level expression analysis, highlight the biological accuracy of our dataset and analysis.

Lastly, to illustrate the added value of cell-surface marker expression data linked to transcriptomic data we integrated ADT and mRNA information to compare three well-established HSC enrichment strategies. Through in silico sorting, we gated populations of interest based on ADT expression and compared transcriptomic profiles of the resulting cells. Here, a GPI-80-based enrichment strategy showed very high similarity to enrichment strategies driven by either CD90+CD49f− or EPCR+ enrichment. This led us to conclude that the enrichment signature that we identify in this work is representative of engraftable FL HSCs irrespective of the markers used during isolation. Using our dataset, the same in silico sorting approach can be harnessed to query other markers without the need to physically sort out cell fractions and subject them to transcriptomic profiling.

The unprecedented resolution at which the engraftable HSC fraction is interrogated in this study, together with the multi-modal profiling of these cells at the functional, transcriptomic and protein-level, makes our in-depth characterization unique among prior studies interrogating HSPCs. We envision that this openly shared resource, which has been made available in an interactive format at https://engraftable-hsc.cells.ucsc.edu, will enable new biological insights into engraftment potential, including how it can be retained during ex vivo culture and how it can be induced to generate functional HSCs from PSCs.

Methods

Ethics statement

The reported research complies with all relevant ethical regulations. All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Massachusetts General Hospital (MGH) (Protocol #2009N000136). Human fetal liver tissues were obtained from participants who consented in writing to the use of tissues resulting from termination of pregnancy for research. Participants received no compensation.

Human peripheral blood mononuclear cells were obtained from a commercial provider as de-identified discarded human material. This study was reviewed by the Mass General Brigham Institutional Review Board (IRB Protocol #2016P001106) and was determined to be exempt as it does not constitute human subjects research given its use of de-identified, discarded material.

Processing of fetal liver (FL) samples.

The stage of the FL samples is indicated as post conception weeks (pcw). The FL sample used for CITE-seq was collected at 21 pcw (sex NA). For the multiparameter flow cytometry the following 5 samples were used: FL1 (21 pcw, sex N/A), FL2 (22 pcw, F), FL3 (16 pcw, sex N/A), FL4 (17 pcw, sex N/A), FL5 (17 pcw, F). FL samples were mechanically dissociated into small pieces and incubated in Liver Digest Medium (Fisher Scientific, 17703034) at 37 °C. Mononuclear cells were isolated over a Ficoll gradient (Lymphoprep: Stem Cell Technologies, 7851) prior to separation into CD34+ and flowthrough (CD34−) cells using magnetic beads (CD34 Microbead Kit: Miltenyi Biotec, 130-064-702).

CITE-seq sample preparation.

FL cells were thawed and allowed to recover at 37 °C for an hour prior to staining. Cells were blocked with TruStain FcX (BioLegend, 422301) and stained with TotalSeq A antibody mix containing 1ug of each TotalSeq A antibody per condition (BioLegend, see Table S1 for a list of antibodies). Anti-human CD235a-APC antibody (BD Biosciences, 553136) was added to the CD34− fraction. The CD34+ fraction was stained with anti-human CD34-APC antibody (BD Biosciences, 555824) and anti-human GPI-80-PE antibody (MBL International, D087-5). All samples were stained with calcein blue (Invitrogen, C34853) for live/dead exclusion and cell populations were sorted (Beckman Coulter MoFlo Astrios) as shown in Fig. S1A prior to loading onto the 10x Genomics platform. Chromium Single-Cell 3′ Reagent Kit v3 with Feature Barcoding technology for Cell-Surface Protein was used and the recommendations from the manufacturer were followed as specified in 10x Genomics user guide document number CG000185, Rev B.

Transplantation experiments.

NOD/SCID/IL2Rγnull (NSG) mice (JAX NSG 005557 - NOD.Cg-Prkdck-scid Il2rgtm1Wjl/Sjl) were given sub-lethal irradiation (200 cGy) and cells were injected into the retro-orbital sinus. Cells from the same sorted fractions as used in the CITE-seq experiment were transplanted into NSG mice (14–16 weeks old, F). Five mice were transplanted per condition with 10,000 cells each (4000 cells for the GPI-80− condition). Engraftment was assessed after 16 weeks by determining the ratio (%) of peripheral blood cells expressing human CD45 versus total CD45 expressing cells. Cells were stained with APC-conjugated anti-human CD45 antibody (BioLegend, 304012) and anti-mouse CD45-PacBlue antibody (BioLegend, 103126). To assess engraftment of the CD34+CD201+ sorted fraction, six NSG mice (13–15 weeks old, F) were transplanted per condition with 2500 cells each. Engraftment of the different sorted fractions shown in Fig. 3h was checked at weeks 4, 8, 12, 16, and 20 post transplantation and multilineage reconstitution potential was assessed 20 weeks post transplantation by checking for expression of human lineage markers in the human CD45+ fraction of peripheral blood using the following antibodies (1:50 dilution): BV421-conjugated anti-human CD4 antibody (BioLegend, 300532), BV605-conjugated anti-human CD29 antibody (BioLegend, 300460), BV650-conjugated anti-human CD20 antibody (BioLegend, 300470).
CITE-seq: transcriptomic analysis. Fastq files were generated and counts extracted from each of the three runs separately for expression libraries and antibody-derived tag (ADT) libraries using bc2fastq v. 2.2 and cellranger v. 3.0.2. The expression libraries were mapped to a combination of the human and mouse genome reference entries (GRCh38 and GRCm38), in order to detect both human and mouse cells. The ADT counts were summarized using CITE-Seq-Count v 1.4.2. We used Seurat v 3 to further process the data. The proportion of cells which included both human and mouse tags was used as a threshold to obtain quality control units. As expected based on the 10X Chromium guidelines, the doublet rate was proportional to the density of cell loading, but was found to be half the rate originally calculated. Cells with more than a 25% of reads mapping to mitochondrial genes were filtered out. The transcriptomic and ADT assays were aggregated into an integrated multi-assay analysis. The CD34+ fraction generated 16,253 cells at a depth of 33,334 reads/cell. The CD34+ GYPA− (CD34N-GD355AN) fraction generated 5,957 cells at a depth of 42,728 reads/cell. The CD34+ bulk (CD34P-Bulk) fraction generated 10,082 cells at a depth of 29,308 reads/cell. The GIPI− (CD34N-GP180P) fraction generated 8,529 cells at a depth of 33,017 reads/cell. Quality control was performed with the singleCellExperiment package45. We observed 9904, 4901, 9870.5, 10187 median UMIs and 2681, 1401, 2808, 2923 median genes detected for fractions CD34+ GYPA+, CD34+ GYPA−, CD34+ bulk, and GIPI−, respectively (Supplementary Fig. 7). The median levels of contamination estimated by DecontX was <1% for all fractions46. For the combined analysis, the four samples were merged and then normalized using SCTransform. Cell cycle scores were generated using the default S and G2M genes detected for fractions CD34+ GYPA+, CD34+ GYPA−, CD34+ bulk, and GIPI−, respectively (Supplementary Fig. 7). The GPI-80 module score and approximate GPI-80 protein expression in the CD34+ HSC/MPP compartment, clusters from one of the four lineages were grouped into one HSC/MPP cluster for the representation in Fig. 1a. For the extended transcriptomic signature of the GPI-80+ cells and used to retroactively label putative GPI-80 expressing cells within the HSC/MPP populations and the clusters from one of the four lineages that would be obtained via analogous FACS sorting (Supplementary Fig. 6f). To further dissect the transcriptional signature of the GPI-80+ HSC/MPP compartment, the Single-cell Regulatory Network Inference and Clustering (SCENIC) pipeline was applied to our SCTransform normalized data. This pipeline first identifies regulons (sets of genes regulated as a unit that are co-expressed with transcription factors in our dataset), then selects only the regulons with significant motif enrichment, and finally uses an AUCell algorithm to assign a score to each regulon for each cell. UMAP embeddings/coordinates from our transcriptomic analysis were used to display the AU regulon scores and a Regulon Specificity Score (RSS) was used to identify regulons important in each of the GPI-80+ clusters.

CITE-seq: ADT processing and analysis. ADT data transformation and background removal. Prior to sorting, cells were stained with a panel of oligo-tagged antibodies (Table S1) so that the antibody-derived tags (ADTs) corresponding to the cell-surface markers present on each cell would also be captured in the sequencing data. Non-specific staining using oligo-tagged antibodies was minimal and was corrected based on background staining levels detected in mouse cells (mESCs) that were spiked into the profiled fractions to account for noise. ADT centered-log-ratio (CLR) transformation and background removal was performed as reported with adaptations19. Specifically, 8735 CD34+ bulk cells and 207 mouse cell spike-ins, raw ADT count data were CLR-transformed for each of the ADTs, using the function NormalizeData with normalization method = “CLR”, log10 transformation = 1 in Seurat V3.2.1. The value of one standard deviation greater than the average CLR-transformed ADT counts from mouse cells were defined as the background cutoff and were subtracted from the 8735 human CD34+ bulk cells. The same procedure was applied to 7235 GIPI− cells and 188 mouse cell spike-ins.

Dimension reduction using UMAP. Log-normalized mRNA data and CLR-transformed ADT data were grouped into 10 bins and colored according to their feature expression levels, respectively. For CLR-transformed ADT data, the top 1 percent feature values were labeled outlier and removed by specifying max.cutoff = “<99” in FeaturePlot function in Seurat V3.

In silico gating strategy. For in silico sorting purposes, cells were first gated based on CD34+CD38− criteria and subsequently underwent signature-specific gating (schematic overview presented in Supplementary Fig. 6a). This process was guided by the average percentages obtained for the different marker combinations in flow cytometry experiments on 5 individual FL samples to ensure that the in silico gated populations that would be obtained via analogous FACS sorting (Supplementary Fig. 6a, b). As the CD38 oligo-tagged antibody in our CITE-seq panel did not result in ADT signal due to technical issues specific to this antibody, we based CD38− selection in the first gating on mRNA values for CD38 (See Supplementary Fig. 6c, d for surface level CD38 expression and its correlation with CD34 and GIPI− surface level expression). While in this study we used a GIPI− selection step to enrich for engraftment potential resulting in the profiling of 7235 GIPI− cells and establishment of a detailed engraftable FL HSC signature, an oligo-tagged GIPI− antibody was not included in our CITE-seq panel. Given the poor correlation between GIPI− protein expression and the corresponding mRNA expression of its encoding gene VNN2 (Supplementary Fig. 6e), we consulted the extended transcriptomic signature of the GIPI− cell population to derive a GIPI− module score and approximate GIPI− protein expression in the CD34+ GIPI− gated population. This module score was obtained by generating an AddModuleScore based on the top 30 enriched genes within the GIPI−/HSC/MPP fraction and used to retroactively label and margin GIPI− in Seurat V3.2.1. The value of one standard deviation greater than the average CLR-transformed ADT counts from mouse cells were defined as the background cutoff and were subtracted from the 8735 human CD34+ bulk cells. The same procedure was applied to 7235 GIPI− cells and 188 mouse cell spike-ins.

Comparison to postnatal datasets. In order to investigate LMNA expression in prenatal HSCCs versus postnatal HSPCs we obtained raw count data from publicly available datasets47,48. These three datasets were merged with the analysis of the CD34+ bulk fraction. CD45+ cells were processed separately for the transcriptomic analysis of our four fractions combined in order to compare expression of LMNA. To specifically compare expression of cluster 0 DEGs between HSC-enriched fractions, we compared gene expression in our GIPI−+ fraction with that of postnatal CD45+− gated index-sorted cells that underwent single-cell transcriptomic profiling.

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approximate what was sorted for the CITE-seq analysis based on GPI-80 cell-surface marker expression, the top 2.37% of CD34+ cells positive for GPI-80 identity. Based on this GPI-80 score were considered (corresponding to 3% of CD34+CD38− gated cells, Supplementary Fig. 6a). Those GPI-80 score+ cells that were also CD133+, were gated to reflect the Sumite et al. signature for comparison to the “classical” (CD90+CD49F−) and “EPICR+” (CD201+) signature, which were both gated based on ADT expression values.

For two-dimensional density plots showing in silico gating thresholds, Density was estimated using two-dimensional kernel density estimation with an axis-aligned bivariate normal kernel, evaluated on a square grid. Function kde2d in R package MASS V7.3-51.6 was used with number of grid points in each direction equals 500 (n = 500).

Processing of peripheral blood mononuclear cells (PBMCs). PBMCs from a 65-year-old individual (M) were used as a control for the flow cytometry experiments. Mononuclear cells were isolated from a commercially available healthy blood leukapheresis pack (New York Biologics Inc) over a Ficoll gradient (Cytiva, 17144003).

Multi-dimensional flow cytometry. All antibodies were purchased from commercial vendors (Table S5) and were pre-titrated using PBMC and FL cells. For the 22-color analysis, PBMC and FL cells were thawed, washed, blocked with Human FcBlock (BioLegend, 422301) and stained with Live-Dead Blue amine dye (Thermo Fisher, L34961). A cocktail of antibodies was prepared fresh and supplemented with Monocyte Blocker (BioLegend, 426102) and Brilliant Buffer Plus (BD Biosciences, 555483). Cells were stained in the dark at 4°C and washed twice. A sub-panel of brightly expressed markers was assessed as a separate stain to serve as a fluorescence-minus-multiple control for further data interpretation. PBMC cells and single-stain Ultracomp beads (Thermo Fisher, 01-2222-42) were stained to provide single-stain spectral controls. Cells and single-stain controls were analyzed on a 5-color Aurora spectral flow cytometry (Cytek Biosciences) and raw fluorescence data from 64 channels were unaligned using ordinary least square algorithm in Spectroflo v2 (Cytek Biosciences).

Fluorescence cytometry data analysis. Data analysis pipelines were built using cloud-based OMIA analysis platform (Omics). Briefly, single-cell data were asinh transformed (cofactor 6000) and 100,000 (as defined by least size FL sample) live single-cell events from each sample were selected for analysis. All fluorescence data (excluding live-dead dye staining intensity) from 5 FL samples and 1 PBMC sample were projected into two-dimensional space with UMAP algorithm34 (neighbors = 15; minimum distance = 0.4; learning rate = 1; epochs = 200). For analysis, intensities of each fluorescence parameter were overlaid on UMAP maps to represent expression levels. Same dataset was clustered with Phenograph27 (K = 20, distance metric = euclidean) and clusters were color-coded and overlaid over UMAP maps. Phenograph clusters were also plotted as heatmaps to represent cell abundance over multiple clusters and median fluorescence intensity across multiple surface protein markers.

Quantification and statistical analysis. Specifics about the statistical tests and replicates used in each experiment are available in the figure legends or specified in the text. The p-value threshold to determine significance was set at p = 0.05. p-value annotations on graphs are as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and are based on a one- or two-sided analysis of variance (ANOVA) test with Tukey’s multiple comparisons adjustment. Data for quantitative experiments is typically represented as the mean with error bars representing the standard error of the mean, as specified in the figure legends.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The raw CITE-seq data generated in this study has been deposited in the GEO database under accession number GSE160251. The human genome (GRCh38) sequence data used in this study is available through the Ensembl genome browser [http://ftp.ensembl.org/pub/release-104/fasta/homo_sapiens/dna/]. The mouse genome (GRCm38) sequence data used in this study is available through the Ensemble genome browser [http://ftp.ensembl.org/pub/release-104/fasta/mus_musculus/dna/]. Source data are provided with this paper.

Code availability

The code used in this work is available at [https://github.com/CReM-BU/Vanuytsel_hPSC25]. Multi-parameter flow cytometry code (unmixed fluorescence cytometry dataset with integrated UMAP and Phenograph parameters) is available at [http://flowrepository.org/id/FR-FCM-Z32M].

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**Author contributions**

K.V., G.J.M., and A.B.B. designed research; K.V., W.F.G.-B., V.V., D.P., E.C.L., T.W.D., S.K., A.K.Y., G.M., and A.C.B. performed research; G.V.-M., J.L.V., Z.W., T.M.M., M.L., F.W., J.D.C., R.D., and A.C.B. performed computational analysis; A.C.B. designed the multi-dimensional flow cytometry panel; K.V., G.J.M., and A.B.B. analyzed the data and wrote the manuscript. K.V. (kimvan@bu.edu), G.J.M. (gjmurphy@bu.edu), and A.B.B. (abalazs@mgh.harvard.edu) are co-corresponding authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information**

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