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REVIEW

Leading Edge Techniques in the Quest for Characterizing Rare Hematopoietic Stem Cells

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Abstract

Purpose of Review Hematopoietic stem cells (HSCs) occupy the summit of the intricate hematopoiesis hierarchy, constituting a mere 0.01% of the adult bone marrow. The rarity of these cells poses a challenge in exploring their biological intricacies. Historically, research efforts grappled with this scarcity, often investigating broader cell populations that encompass multipotent progenitor cells, with HSCs representing only a modest 2–5%. Consequently, a crucial necessity emerged for methodologies accommodating the constraints imposed by the low abundance of HSCs.

Recent Findings In the last decade, the landscape has transformed with the advent of multi-omics technologies, such as single-cell-based approaches encompassing proteomics, metabolomics, transcriptomics, and epigenetics. These advancements, coupled with refined ex vivo and in vivo models of stem cells, have ushered in a new era where the independent study of HSCs has become feasible. Notably, strides in humanized models have further enriched the scientific toolkit, enabling the validation of HSC biology within human systems.

Summary This review attempts to spotlight recent breakthroughs and their associated advantages and disadvantages, collectively illuminating the avenues that now exist for delving into the intricate biology of functional HSCs.

Keywords Hematopoietic stems cells · Techniques · Omics · Methodologies

Historically, the groundbreaking realization that blood cells could repopulate the entire bone marrow post-irradiation marked a pivotal moment in scientific history [1, 2]. However, it wasn't until the early 1980s that researchers discovered this regenerative ability was confined to a specific subset of hematopoietic cells. Observations from the popular CFU-S assay revealed that only a select few cells possessed the capacity to sustain colonies over the long term [3]. These exceptional cells, identified by their ability to (i) self-renew, (ii) exhibit trilineage potential producing myeloid, lymphoid, and erythroid lineages, and (iii) give rise to long-term hematopoiesis, were coined hematopoietic stem cells (HSCs). Yet, despite this revelation, the challenge remained: how to identify and isolate HSCs. The subsequent decades of research yielded assays and markers, still in use today, that allow for the identification of both immunophenotypic and functional HSCs [4–7] (as comprehensively reviewed in reference [8]). Phenotypic HSCs and various progenitors were meticulously identified, sorted, and transplanted into lethally irradiated mice. This groundbreaking work unequivocally demonstrated that only HSCs could give rise to all lineages for long-term reconstitution, whereas progenitors exhibited a bias towards specific lineages, sustaining hematopoiesis for a limited duration [7].

Fast forward to our current understanding – HSCs are at the apex of hematopoiesis, constituting a mere 0.01% of the bone marrow. A decade ago, the isolation and study of these rare cells presented significant challenges. However, technological advancements in recent years have facilitated the functional characterization of this elusive population. Focusing on the mouse model system, we delve into the progress made in various technologies that now enable the study of single cells, many of which have already been seamlessly incorporated into HSC and hematopoietic research.

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Transcriptomic Studies

Single Cell Sequencing

The invention of single-cell sequencing has caused an explosion in immunology research as heterogenous immune cell populations in tissues can be analyzed via high throughput techniques [9–12]. Over time, assays have evolved to probe the transcriptome, genome, and proteome of rare cell populations. In single-cell RNA sequencing experiments, the number of cells assayed and detection sensitivity are crucial parameters which influence the number of genes available for meaningful cell-to-cell comparisons [13]. In the context of hematopoiesis and the study of HSCs, a significant challenge for transcriptomic analysis has been the heterogeneity and limited number of obtainable cells. Single-cell sequencing has addressed this, enhancing data resolution, and leading to discoveries unattainable with bulk sequencing alone. Notably, it has revealed that the fate of multipotent progenitor cells is determined early, with transcriptional priming for erythroid/megakaryocyte, lymphoid, or myeloid lineages [13]. Additionally, single-cell RNA sequencing has also unveiled heterogeneity within the HSC population, a subtlety not discernible through flow cytometry based on cell surface expression. Insights into cell cycle dynamics, differentiation, and aging have identified subsets of HSCs exhibiting distinct stemness signatures or expressing lineage-specific genes, suggesting lineage priming at the stem cell level [14–18]. Temporal information on lineage fate decisions has spurred the development of innovative technologies capable of surveying the chromatin landscape, genome, transcriptome, and proteome at the single-cell level [19–22].

While single-cell RNA sequencing is a potent tool for surveying transcriptome, its limitation lies in capturing only about 20% of transcripts, making population differentiation challenging. The introduction of Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq) has mitigated this issue. CITE-seq combines cellular protein and transcriptomic data into a unified read-out, utilizing barcoded oligonucleotide-labeling antibodies that can be amplified through PCR. Unlike traditional flow cytometry, CITE-seq allows the simultaneous assessment of hundreds of antibodies, aiding in distinguishing cell populations with similar transcriptomic signatures based on cell surface markers [23]. This approach has identified an HSC-like population in human pluripotent stem cells, presenting a potential avenue for enhancing therapeutic HSC production from induced pluripotent stem cells [24]. As many as 200 surface protein expression can be used in CITE-seq which identified unique previously unknown surface markers like CD326, CD11a and Tim3 to demarcate early lineage commitments in human immature cell compartment [25]. Another such approach for assaying protein levels along with transcriptomic data is RNA expression and protein sequencing assay (REAPseq) [26]. REAPseq utilizes unidirectional chemistry to create small, stable, covalent bonds between the barcodes and antibodies, this minimizes steric hindrance and may be useful when assaying cell populations of a smaller size (ie HSCs) and for intracellular protein barcoding. Surveying protein abundance on cells is important when it comes to developing drugs to target certain cell types. High-throughput methods like CITE-seq and REAP-seq facilitate the identification of environmental and therapeutic perturbations in heterogeneous cell populations following drug treatment.

Intriguingly, the progression from surface protein expression analysis to intranuclear cellular indexing of transcriptomes and epitopes (inCITE-seq) has emerged. inCITE-seq allows the simultaneous measurement of intracellular protein levels and the transcriptome in nuclei, providing insights into transcription factors, regulatory proteins, and signaling pathways in hematopoiesis, as well as alterations in complex cellular environments in disease [27], shedding light not only on the abundance of transcript per cell but also downstream functional aspects. This capability to delve into a single-cell level is particularly crucial when studying rare cell populations like HSCs. While these single-cell RNA sequencing methods provide high-resolution transcriptomic snapshots, it alone falls short in delivering functional information or capturing dynamic changes over time. The mouse model Hoxb5-Tom ($Hoxb5^{CreERT2}$;$R26^{LSL\text{-}tdTomato}$) overcomes this drawback by allowing to measure dynamic changes in HSCs over time. Here, tamoxifen treatment specifically stably labels HSCs with 'tomato' which can be inherited by downstream progenies. The bone marrow from the mice is then harvested over 0.5 to 9 months, sorted for tomato + cells and processed for single cell sequencing [28•]. The limitation of this method is that tamoxifen treatment acutely impacts HSCs transcriptome, however this method allows to understand how differentiation of HSCs is impacted with stressors, like environmental cues, aging, cytokine storm, diseases, and over time, which would improve our understanding and identifying stages of therapeutic intervention.

Clonal Tracking

Understanding hematopoietic stem cell (HSC) production, trajectory, and differentiation potential is crucial in unraveling the intricacies of hematopoiesis. Lineage tracing has emerged as a powerful tool, enabling the mapping of cellular fate and the creation of atlases that provide valuable insights into the mechanisms of disease biology. This technique

involves tagging, identifying, and tracing stem cells as they undergo differentiation into mature cell populations in vivo, significantly advancing the field. Several recent studies have outlined prospective and retrospective lineage tracing models using mice [29–32] and zebrafish [33], single cell analyses to determine differentiation hierarchies and lineage fate using cell barcoding [34, 35], reconstructing cell lineages from genome studies at stages of human development [36], and using CRISPR gene editing for barcoding cells [37, 38]. These lineage tracing methods have expanded the field of hematopoiesis research, allowing researchers to ask questions about HSC differentiation and self-renewal.

Seminal models, such as the transposon tagging mouse using the Sleeping Beauty transposase enzyme, have revealed that long-lived progenitors drive steady-state hematopoiesis [32]. This initiates the mobilization and integration of a DNA Transposon into cells undergoing transposition providing an inheritable genetic tag. Using this technique, scientists have discovered that it is the long-lived progenitors which are the drivers of steady-state hematopoiesis. A similar lentiviral-based barcode library (LARRY) identified functionally heterogenous HSC population primed for different lineages as well as either high or low output, where HSCs expressing transcription factor *Tcf* were the most primitive with multilineage potential and required for long-term self-renewal/ quiescence of HSCs [39]. The limited barcoding capacity with lentiviral based models can be overcome by using HSC specific inducible CRISP-Cas9 based mouse models. CARLIN identified that there are only certain clones of HSCs that are responsible for replenishing hematopoiesis after 5-FU induced cytotoxicity. These clones of HSCs had differentially expressed *CD48*, *Plac8*, *Mpo*, *Cdk6* transcripts suggesting inherent transcriptomic differences in different clones of HSCs [40]. A new version of the mouse, DARLIN, which uses Cas-TdT instead of Cas9, is more efficient at lineage recovery as well as introducing barcodes. DARLIN mouse confirmed the fact that HSCs migrate to bones post birth, sharing 80% barcode. Whereas, in adulthood, HSCs remain localized at specific sites and do not migrate from one bone to the other bone, thus sharing only 1–5% of the barcode [41•]. Another system is a multi-fluorescent mouse model (HUe) [29] that tracks fluorescently tagged cells following transplant to understand how cell transcriptomic and epigenetic can infer cell function. With the ability to follow clonal lineages and sort out populations, it was found that HSCs have epigenetic features which impact their functional output. However, transplants can lead to clonal selection of HSCs, providing an artifact in the study compared to transgenic lineage tracing mouse models. Another study replicated this model in zebrafish [33] finding that when hematopoiesis is stressed, either by transplantation or irradiation, clonal diversity is decreased. This information is important for understanding how different cell population change in reference to one another and can inform inconsistencies seen in transplantation experiments.

The use of multi fluorescent reporter strains has aided scientists studying neuronal networks and been adapted to studying intestinal biology, muscle growth and hematopoiesis. In a similar context, the ROSA26 Confetti reporter combined with a tamoxifen regulated Ubiquitin-ERT2-Cre and doxycycline inducible H2B-GFP allele to follow expansion of fetal liver hematopoietic progenitor populations which revealed unique waves of cell proliferation out of the fetal liver. This inducible system allowed tracing of LT-HSCs by way of an inducible promoter, allowing for the controlled monitoring of HSC differentiation and proliferation [42]. Analysis programs for single cell sequencing such as Monocle and RNA velocity allow for the predictive projections of cells based on variance between genes. However, an inherent problem is that spatial and temporal information is lost by single cell sequencing alone. Several groups have developed technologies to work around the knowledge gap. TrackSeq, is one such pipeline which combines imaging, tracking, isolating and transcriptome sequencing of single cells can identify molecular programs controlling cell fate divergence and can be useful in delineating HSC differentiation and expansion of distinct lineages [43]. Another approach – CellTagging allows for the simultaneous capture of clonal history as well as cell identity by performing several round of cell labeling and following sister cells. Importantly, clonally related cells follow similar trajectory patterns which affirms the idea that intrinsic properties determine fate output [44, 45]. Despite the versatility of these tracing techniques, they do not inform how the chromatin and epigenetic landscape are contributing to clonal fate. Recent techniques include single cell lineage tracing which use inherited DNA barcodes to combine cell fate with transcriptomics [40, 45–47]. To this extent, CellTagging approach was advanced to combine single cell lineage tracing and capture of available chromatin (CellTag-multi) [48]. The CellTags are now expressed as polyadenylated transcripts and can simultaneously capture both scRNAseq and scATACseq to capture lineage output fate, transcriptomics, and regions of accessible chromatin. Collectively, high throughput transcriptomic techniques have opened doors to understanding the heterogeneity of the hematopoietic compartment and, in combination with lineage tracing, allowed for the discovery of the fluid nature of hematopoiesis. The ability to model clonal evolution in disease will help answer questions regarding cancer onset, especially in the context of hematopoietic dysregulation and transformation.

Epigenetic Studies

The evolution of single-cell technologies has significantly enhanced our ability to explore the chromatin environment within the hematopoietic stem cell (HSC) compartment [49–51]. Chromatin immunoprecipitation (ChIP) has long

been a pivotal method for identifying binding sites of DNA-associated proteins, shedding light on crucial gene regulatory mechanisms influencing cell states and responses to environmental stimuli and disease [52]. ChIP was developed more than 30 years ago and allowed for the measurement of transcription factor (TF) binding through the action of crosslinking and fractioning TF bound chromatin [53–56]. However, traditional ChIP has limitations, including potential false positives cross-linking and the requirement for millions of cells, posing challenges for rare cell populations like HSCs. To address these concerns, innovative techniques like CUT&RUN have been developed, eliminating the need for fixation or cross-linking and producing high-resolution output data with low cell input [57]. The use of this method aided in the investigation of chromatin assembly factor-1 (CAF-1) which was found to influence chromatin dynamics and TF binding during lineage differentiation of myeloid stem and progenitor cells [58]. Another method, cleavage under targets and tagmentation (CUT&TAG), offers similar outputs as CUT&RUN but streamlines the workflow by using a transposase loaded with sequencing adaptors, making it more efficient and applicable to fewer cells [59, 60]. scCUT&TAG, in combination with ChromHMM, allows the measurement of chromatin states at a single-cell level, mapping post-translational modifications and identifying DNA sequence motifs corresponding to different chromatin states [61]. Despite these advancements, methods like CUT&RUN and CUT&TAG do not provide information about regions of open chromatin crucial for understanding actively transcribed genes. The Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) has filled this gap, creating an atlas of open chromatin regions that define where transcription factors bind, shaping the transcriptome in different cell types and uncovering changes in gene expression related to hematopoietic genes [62–64]. Combining CUT&TAG with ATAC-seq gives information whether the chromatin is open and if the said transcription factor localizes at the open spot of not. Developing tools for low cell-input, that can combine both these methods could potentially benefit the HSC field.

In recent years, high-throughput multi-omics approaches have emerged, capturing accessible chromatin, protein levels, transcriptomic activity, and gene expression in a single assay. Technologies like single-cell ATAC-seq (scATAC-seq) have been combined with scRNA-seq to reveal that HSCs are not transcriptionally, but epigenetically primed, for lineage commitment [65]. Integrating ATAC-seq with select antigen profiling (ASAP-seq) has enabled the simultaneous detection of chromatin accessibility, active transcription sites, transcription factor binding sites, and intracellular and surface proteins during hematopoiesis [21]. This method has been used to follow changes in chromatin, RNA, and surface proteins during hematopoiesis. scATAC-seq allows for the detection of chromatin accessibility, sites of active transcription and transcription factor binding sites which aids in the detection of chromatin regions of interest that can be further studied with ChIP [21]. ASAP-seq brings us one step forward by providing simultaneous detection of intracellular proteins, surface proteins, epigenome and clonality trough mtDNA. Development of this technique has provided necessary information concerning clonal relationships in hematopoiesis and allowing researchers to gain a multitude of information from rare cell populations. While these techniques have provided valuable insights, challenges persist in the need for a significant number of cells for high-resolution analysis, particularly for rare cell populations. The examination of chromatin landscapes has disclosed that HSCs exhibit more open chromatin regions compared to downstream progenitors. As HSCs age, there is an increase in differentially accessible chromatin regions, potentially influenced by the activity of methylating and demethylating proteins. These regions are enriched in binding motifs of STAT and ATF transcription factor families, suggesting that HSCs retain memory of stressor responses and are primed for self-renewal and myeloid lineage differentiation [66]. A recent technique – Camellia-seq, is single cell nucleosome, methylation and transcription sequencing combined with lineage barcodes. In this method, the cytoplasmic fraction is used for mRNA and barcode lineage analysis, while the nucleus is used to measure open chromatin and methylation state. This method led to the revelation that in HSCs, clonal memory of related cells is retained in genome-wide methylation rather than transcriptome and chromatin accessibility [41•]. Despite the progress made in epigenetic techniques, the ability to link HSC function with chromatin structure heterogeneity remains a challenge. This information is crucial for understanding how chromatin changes over time contribute to or are influenced by age, inflammation, and the presence of oncogenic mutations. Addressing these questions will provide deeper insights into the relationship between HSC function and chromatin dynamics.

Proteomic Studies

Flow and CyTOF

The most widely used method in hematopoiesis to study surface proteins is flow cytometry, which has made it possible to phenotypically characterize HSCs.

Flow cytometry has been a cornerstone method in hematopoiesis research, enabling the phenotypic characterization of HSCs using Cluster of Differentiation (CD) markers [67]. HSCs are currently defined as Lineage negative, $cKit^{Hi}Sca1^{+}Flt3^{-}CD48^{-}CD150^{+}$, which allows for the identification and isolation of these cells [5]. A subpopulation

in the HSC compartment defined as negative for CD34 and positive for EPCR surface markers has been recently found to remain stem-like even after exposure to inflammation [6]. However, the utility of flow cytometry is limited by the number of available markers and complex compensation, hindering a more comprehensive understanding of HSC heterogeneity throughout differentiation and maturation. To overcome this limitation, single-cell mass cytometry, also known as "Cytometry by Time-of-Flight" (CyTOF), has emerged as a powerful alternative. CyTOF employs heavy-metal ion-tagged antibodies that can detect over 40 cellular markers, encompassing surface proteins, intracellular signaling targets, transcription factors, and cytokines. This technology enables a simultaneous analysis of 89 immune cell populations, providing a more nuanced view of perturbations in frequency and phenotype across multiple cell subsets [68, 69].

For instance, in the context of hematopoietic stem cell transplantation (HSCT), CyTOF analysis revealed distinct NK cell and T cell phenotypes in patients who experienced Human cytomegalovirus reactivation compared to uninfected patients [68]. Here, analysis of 40 peripheral blood mononuclear cell surface markers using CyTOF uncovered that HSCT patients who experienced Human cytomegalovirus reactivation had high levels of HLA-C (human leukocyte antigen) with distinct NK cells and T cells compared to uninflected patients [68]. In another study, CyTOF has proven particularly valuable in capturing the dynamic progression and hierarchical relationships of hematopoietic cells along the erythroid trajectory using temporal barcoding. This approach allows for the comparison of key protein levels between different stages of erythropoiesis, providing insights into the differentiation process [70]. The barcoding allows for comparison amongst the levels of key proteins between the stages which of erythropoiesis defined by older literature [70]. Such studies showcase the unique capability of CyTOF to simultaneously analyze multiple parameters and generate comprehensive data, making it an invaluable tool for studying hematopoiesis under various conditions, including HSCT and cell differentiation.

Apart from analyzing surface protein and intracellular protein expression, traditional flow cytometry can also be combined with measuring up to 4 transcripts using 'Prime Flow' [71]. This method has been widely used in immunology, but has yet to be explored for studying HSCs [72, 73]; Prime Flow allows correlating abundance of RNA with surface proteins also analyze kinetics of RNA and respective protein in the same cell.

Mass Spectroscopy

While CyTOF and flow cytometry are powerful tools for studying hematopoiesis, they are inherently biased as targets must be chosen prior to analysis. In contrast, mass spectrometry (MS) offers an unbiased proteomic analysis by measuring proteins based on their mass-to-ion ratios, allowing for a comprehensive examination of the entire cellular proteome. Recent advances have increased the sensitivity of MS, enabling the detection of proteins from as few as 1000 cells to even single-cell resolution [70, 74–78]. MS based proteomic analysis of pure bulk HSC population has revealed that compared to progenitors, HSCs are enriched in glycolytic enzymes, cellular ion homeostasis and hypoxia, with less abundance of DNA damage response and cell cycle proteins compared to the progenitors, which correlates to the known HSC biology [74, 75, 79]. Interestingly, the transcriptional diversity detected in HSCs does not always correlate to proteomic diversity, suggesting that the HSCs are transcriptionally primed to translate protein as needed, but at homeostasis, the protein abundance is kept in check by miRNAs [74]. This was found to be specific to HSCs, with transcript and protein being concordant in progenitor cells. Although bulk MS analysis of HSCs provided us with insight into the proteome of HSCs – which form the basis of cellular function, it fails to take into consideration heterogeneity.

Recognizing the limitations of bulk MS analysis in capturing heterogeneity, single-cell MS methods have been developed, such as true single-cell-derived proteomics (T-SCP), Single Cell ProtEomics (SCoPE2), and SCoPE2-based SCeptre. SCoPE2, a multiplexed MS analysis, allows the identification of proteins with a high limit of detection by sorting and labeling single cells before pooling them for MS [76–78]. T-SCP, on the other hand, enables sensitive identification of proteins at a single-cell level by directly sorting single cells into lysis buffer before MS analysis [76]. These methods can be of great value to study the heterogenic HSC proteome, HSCs are known to translate less protein compared to progenitors, mature cells in the marrow and even fetal HSCs [80, 81]. This is because HSCs produce less misfolded and unfolded proteins owing to high proteome quality to maintain proteostasis and in turn self-renewal potential of HSCs [81]. In this context MS will assist in identifying if the differences just lie in overall protein translation, but what proteins get transcribed and are important to maintain HSC function. Although single-cell based MS analysis have yet to be used to study HSCs, they have been applied in leukemia biology and has helped to uncover that there are two distinct differentiation stages of leukemia stem cells to blast cells indicating heterogeneity at protein level, which ultimately impact treatment outcomes [77].

Recent advancements in resolution, robustness, reproducibility, and speed make single-cell MS-based proteomics a promising tool for clinical studies and understanding the importance of the HSC proteome. However, it is essential to note that MS provides static data, and complementary assays are necessary to elucidate the dynamic nature of proteins and their significance in HSC function.

Proximity Assays

In addition to identifying proteins and their abundance, understanding protein function often involves studying protein–protein interactions. Mass spectrometry (MS) allows for unbiased global protein interaction analyses but typically requires millions of cells, posing challenges for low cell count samples like HSCs. The Proximity Ligation Assay (PLA) is a flow cytometry and imaging-based technique that can study protein–protein interactions on low cell count populations. However, it is limited to known interactions and requires strong antibody probes [82–84]. Both methods can thus be used hand-in-hand for discovery, validation and functional studies.

PLA has revealed critical protein interactions in HSCs, such as the Cdc42-Borg4-Septin7 interaction's importance in HSC polarity and function. Other interactions, like DEK-NCoR1 and CD53-DREAM, play roles in maintaining quiescence and protecting HSC function under stress [85]. Another protein important to maintain quiescence of HSCs is DEK. Nuclear DEK interacts with NCoR1, a histone deacetylase which regulates quiescence associated genes like *Akt1/2*, *Ccnb1*, *p21*; restricting mTOR signaling and subsequently mitochondrial metabolism to maintain quiescence [86]. Inflammatory and proliferative stress also force HSCs into cycling, interaction of CD53 with DREAM-associated proteins is important to bring the HSCs back into quiescence thus protecting HSC function under stress. Disruption of this interaction leads to continued HSC cycling and ultimately exhaustion [87]. The understanding of such protein interactions can thus help harness the HSC function to maintain stemness for instance in transplant therapy.

PLA is a widely used method, however it is limited to identify interaction within a 40 nM and is also unable to identify all molecules due to stearic hinderance. To overcome this limitation, PLA was adapted for large volume 3D in situ imaging-based quantification [88]. This adaptation has identified that CXCL12, an important chemokine for HSC support in the bone marrow is globally distributed in the bone marrow, but also has rich localized small pockets [88]. Being able to look at tissue wide distribution can thus help understand HSCs interaction with the niche. Another limitation of PLA is that ligation reaction may not work in all biological fluids, and leads to recovery loss [89]. To overcome this, Proximity Extension Assay (PEA) was developed which combines the specificity of antibody detection with the quantitative power of PCR [90]. Two matched antibodies labeled with unique oligonucleotides simultaneously bind to a target protein in solution. The DNA oligonucleotides hybridize, extend, and create a double stranded DNA "barcode" unique to the specific antigen and quantitatively proportional to the initial concentration of target protein. This is different from the PLA where, oligonucleotides from two matched antibodies are connected together by a DNA ligase reaction, which can then be amplified and detected for detection [89]. Because PEA technology applies PCR amplification properties, a wide range of proteins can be measured with an extremely small sample volume (around 3uL, for 1536 assays). The method is high throughput such that 92 cytokines, chemokines, and growth factors can simultaneously be quantified using just a few microliters of serum [69]. The biggest limitation of PEA technology, similar to PLA is selection bias since proteins are pre-selected for analysis and antibody probe performance, but this bias can be mitigated by using MS concurrently. MS provides a broad scope of protein expression in a sample while PEA/PLA can be utilized to analyze proteins in lower abundance [90]. Both methods provide new avenues for studying HSCs, but the two methods combined would produce a more complete and thorough analysis from a single sample. In addition, developing sensitive methods to identify protein function in low cell counts populations can help the field move forward to understanding HSC function at a global proteomic level.

CyTOF and PEA are great tools for observing cell-to-cell heterogeneity, but their outputs are limited by antibody probe performance [91]. Western blot is a technique commonly used to confirm protein expression without the possibility of antibody cross-reactivity and can provide a clear discrimination between on-target and off-target signals [91]. Traditional Western blot can only be used to look at protein expression in a bulk cell population, masking single-cell behaviors that are crucial to look at when studying cell populations that are complex and plastic in nature such as HSCs. Unlike Western Blot gels, scWestern uses a slide covered in photoactive polyacrylamide gel with microwells that single cells can settle into; from there the cells undergo lysis in situ, gel electrophoresis, photoinitiated blotting to immobilize proteins and antibody probing [91]. A common theme with CyTOF, PEA, and scWestern is the ability to observe protein expression in different cell clusters from a single population. Single cell analytics have been critical in observing various conditions simultaneously in each cell population and generating multi-perspective data sets in a concise amount of time. Using these novel methods in unison will further aid to the development of personalized medical care in cancer therapy.

Metabolic Assays

Similar to proteomics, mass spectrometry (MS) is a commonly employed technique for metabolic studies. While many metabolic assays traditionally require large cell numbers, advancements in mass spectrometer sensitivity and metabolite extraction techniques now enable the study of

steady-state metabolite enrichments with as few as 10,000 cells. This approach provides robust data on global metabolite abundance in bulk HSCs. A more recent MS-based method called, NPELDI-MS (nanoparticle enhanced laser desorption/ionization-MS) enables analysis of 100 + features, even those in the low mass range. This method led to the identification that oxidative phosphorylation is essential for self-renewal and activation of HSCs by protecting against oxidative stress [92•]. However, the method measures static levels of metabolites and thus may not reveal insights into whether metabolite enrichment is due to accumulation, excessive use, or production [93–96]. To add a functional dimension to metabolic analysis, the single-cell flow-based method SCENITH has been developed. SCENITH correlates protein translation to ATP generation, enabling the determination of glycolytic and mitochondrial capacity while considering cellular heterogeneity. However, this method is dependent on correlation of ATP from either glycolysis or oxidative phosphorylation with protein synthesis, which may not stand true for HSCs, since quiescent HSCs, do not rely on oxidative phosphorylation for energy. Mitochondrial reporter mouse models like MitoTag and mt-keima allow the study of mitochondrial distribution and proteins across various cell types. Additionally, advancements in flow cytometry and CyTOF-related panels have been designed to specifically observe changes in metabolic enzymes, while CRISPR screens targeting metabolic genes help identify metabolic vulnerabilities [97–104] (Fig 1).

These techniques, while providing valuable information, generally capture cellular heterogeneity in a static state. Researchers have utilized these methods to characterize HSC metabolism, revealing that under steady-state conditions, HSCs primarily rely on glycolysis compared to progenitors and mature cells [105–107]. They exhibit reduced respiratory capacity and mitochondrial turnover rate but maintain a high mitochondrial mass [108, 109]. Fatty acid metabolism is also crucial for HSC maintenance, proliferation, and differentiation, influencing asymmetric or symmetric cell division and the self-renewal process [110–112]. Specifically, deletion of sphingosine kinase 2 gene, responsible for generation of lipid metabolite, leads to stabilization of HIF1a, which is important for maintenance of stemness and limit exhaustion of HSCs [113]. As opposed to young HSCs, aged HSCs have increased metabolic activity with accumulation of dysfunctional mitochondria [112, 114]. However, increasing the mitochondrial membrane potential using mitochondrially targeted coenzyme-Q10 or supplementing with nicotinamide riboside, a form of Vitamin B3 reverses the aging HSC metabolic phenotype [114, 115]. Despite these advancements, there is a need for methods that can study the dynamic correlation between HSC function and cellular energetics, shedding light on whether metabolic changes are a cause or consequence of HSC function. This ongoing exploration will contribute to a deeper understanding of hematopoietic stem cell biology.

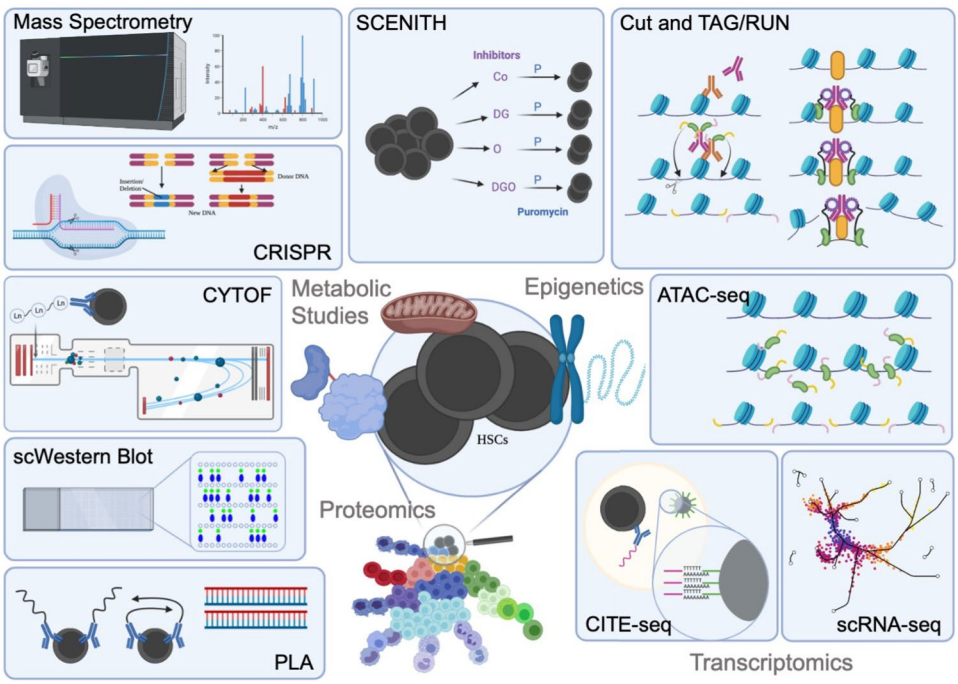

Fig. 1 Methods for the detection and investigation of hematopoietic stem cells. The advent of multi-omics technologies, that allow single-cell-based studies encompassing proteomics, metabolomics, transcriptomics, and epigenetics have revolutionized the field of hematopoietic stem cells, providing a better functional understanding of this rare cell population

Modeling – Hematopoietic Stem and Progenitor Cells

Ex vivo Expansion of HSCs

Culturing HSCs in vitro has historically been challenging, as the culture conditions often support differentiation over self-renewal. One strategy to maintain the stem-like state of HSCs is the immortalization of stem and progenitor cells. *Hoxb8*, a member of the Hox gene family highly expressed in hematopoietic stem and progenitor cells, has been cloned into whole bone marrow, primarily used in neutrophil and dendritic cell biology [116]. Hoxb8-immortalized cells retain myeloid and lymphoid differentiation potential but lose the ability to differentiate into megakaryocyte and erythroid lineages [117–119]. Another gene, LH2 (LIM-homeobox), has been used for the immortalization of HSCs, and cells immortalized with LH2 retain engraftment and tri-lineage potential [120]. Transducing stem cells with *Lhx2* gene immortalized them, an example being the murine embryonic stem cell derived hematopoietic cell line HPC7 [121]. Murine stem and progenitor cells immortalized using the same approach retain engraftment as well as tri-lineage potential, not seen with Hoxb8 immortalized cells [122]. This approach would a good tool for molecular biology studies and cloning where proliferative status of the cell does not impact the underlying biology.

To overcome artifacts associated with cell lines, several conditions have been explored to maintain HSCs both phenotypically and functionally in vitro. Conditions such as fatty acid supplementation, serum-free media, minimal cytokines with low amounts of stem cell factor (SCF) and high amounts of thrombopoietin (TPO), reduced oxygen levels, and inhibition of protein translation have been identified to help maintain HSCs [110, 123–125]. These conditions have been successful not only for murine HSCs but also for human HSCs, allowing ex vivo expansion with the addition of valproic acid, aryl hydrocarbon receptor inhibitors, sphingolipid metabolism inhibitors, or UM171 [124, 126–128]. HSCs expanded in serum-free media with low SCF and high TPO maintain engraftment and tri-lineage potential even after 2 weeks of culture, in both irradiated and non-conditioned transplant scenarios [123, 124, 129]. Expanded HSCs exhibit phenotypic and transcriptional heterogeneity, with EPCR + cells maintaining long-term repopulating capacity and sharing similarity with freshly isolated HSCs [123, 130]. Additionally, assays that used to be difficult in carrying out with HSCs due to limited number, are now more feasible post expansion ex vivo [123]. One such area is metabolomics. Expansion of HSCs has made metabolic tracing possible on a pure stem cell population instead of using a broader stem and multipotent progenitor population, off which HSCs are only 10–15%. It was thus possible to identify that HSCs specifically use synthesized aspartate during regeneration and reduction of aspartate levels impaired HSC function [131]. Moreover, expansion cultures have also made genetic manipulations of HSCs possible, without losing engraftment potential [129]. CRISPR-knock out screen identified Tada2b and Taf51, members of the SAGA complex, as important regulators of HSC lineage commitment, where Tada2b and Taf51 is reduced in aging and subsequently upregulating interferon response gene expression [132]. Importantly, CRISPR-Cas9 mediated gene correction can also be carried out on expanded HSCs. Transplantation of corrected beta-hemoglobin gene in sickle cell disease expanded HSCs normalized red blood cells as well as myeloid chimerism [133].

While the ability to work with HSCs in vitro has opened new avenues of research, it is crucial to consider the caveat of forcing stem cells into proliferation, which may not fully reflect their in vivo biology. Therefore, findings obtained through culture conditions should be validated in pre-clinical in vivo models.

Humanized Mouse Models AND 3D Scaffolds

Although there have been advancements in transgenic mouse models to mimic normal and malignant human hematopoiesis, it is essential to validate findings in a human hematopoietic system. This led to the development of immunosuppressed mouse models like the nude mice, $Rag^{-/-}$ mice and NOD-scid mice. Nude mice were the first immunosuppressed mouse model lacking T-cells that was established in 1966 [134]. However, the presence of B-cells and an active immune system led to rejection of transplanted human cells [135]. After 3 decades, the $Rag^{-/-}$ mice and NOD-scid mice were developed [135]. The $Rag^{-/-}$ mice have a knockout in the recombinant activating gene (Rag) which impairs V(D)J recombination and hence lack a proper adaptive immune system including B and T-cells [136]. On the other hand, the NOD-scid mice are non-obese diabetic mice with a scid mutation causing defective NK and myeloid cell function, further improving their compatibility with human HSCs [137]. However, human HSC engraftment remained low. The next revolution of immunocompromised mice came in the early 2000s, when NSG and NOG mice were developed [135]. These mice are made on the NOD background, but in addition also lack interleukin-2 receptor common γ-chain (IL2rg) expression, eliminating murine NK cells and further improving human HSC engraftment [138, 139]. These immunocompromised models are highly used in the field today especially for malignant hematopoiesis studies and have seen a tremendous improvement in engraftment of

malignant HSCs. However, the problem with engraftment of healthy HSCs as well as development of human hematopoiesis in mice persists, mostly due to lack of cross-reactivity of murine cytokines. Lack of human cytokines, such as IL3, IL4, IL7, IL15, SCF and TPO reduced the development of a functional human lymphopoieses causing bias towards myelopoiesis [135].

This led to generation of humanized mouse models, in which, to support human hematopoiesis, murine cytokines genes were replaced with human coding sequences. The NOG-EXL mice express human GM-CSF and IL3, while the NSG-SGM3 (NSGS) also expressed human SCF in addition to GM-CSF and IL3 [140, 141] =. Both these models have stable HSC engraftment and maintenance of monocytes, macrophages, and dendritic cells [142, 143]. MISTRG mice on the other hand express human GM-CSF, IL3, M-CSF, TPO and SIRPa leading to an additional improvement in human NK cell development compared to NOG-EXL and NSGS mice, but also see reduced life-span due to severe anemia post engraftment of human HSCs [144]. To overcome this drawback of MISTRG mice and reproduce the effect of human SIRPa expression in the more robust NSG/NSG-S mice, macrophages can be depleted using clodronate. This enabled improved engraftment potential of human CD34 + with maintained function of reconstituting the hematopoietic system [145]. All of these humanized mouse models need to be irradiated or busulfan conditioned in order to make space in the marrow for engraftment of human HSCs. However, these conditioning have its own drawbacks inducing inflammation that could perturb studies focusing on impact of inflammation. The NBSGW mice, carry mutation in the gene expressing murine cKit, which is important for the maintenance of HSCs, thus supporting human hematopoietic expansion without pre-conditionaing [146]. Comparing the NSGS, MISTRG and NBSGW mice, they all support multilineage immune maintenance, where NSGS mice show engraftment starting two months, and all the mice engraft HSCs > 85%, 4 months post-transplant [147]. However, NSGS mice lead to exhaustion of human HSCs, where MISTRG and NBSGW maintain functional HSCs at frequencies found in the human marrow [147]. Combining the NBSGW mouse with expression of human cytokines could produce next generation of humanized mouse models with improved capability of transplanting healthy HSCs in an irradiation independent system.

HSCs are supported and maintained by cues from the bone marrow niche and its interaction with stromal cells. This environment lacks in humanized mouse models. Although further studies need to be carried out to identify if engrafted human HSCs interact with the murine bone marrow niche, humanized 3D scaffold niche models were developed as an answer to this limitation in 2017 [148]. Where BM chip is an in vitro model to recapitulate the BM microenvironment, 3D scaffolds provide an in vivo system. Here, human stromal cells are first injected into a gelatin based porous scaffold. Next, human hematopoietic cells are injected and allowed to adhere to the stromal cells. This scaffold is then implanted onto the back of the mouse and complete hematopoiesis was maintained from 8–24 weeks post implantation [148, 149]. Although, cell output at the endpoint would be low to carry out cell-intensive downstream analysis with HSCs, 3D scaffolds can be a great tool to study human HSC-stromal interaction, and also for pre-clinical drug toxicity studies.

While humanized mouse models and 3D scaffolds serve as valuable tools for pre-clinical studies, there are challenges, including low engraftment potential of human HSCs compared to murine HSCs. Continuous efforts are needed to improve these models to better mimic the complexity of human hematopoiesis and to validate findings in a human system.

Bone Marrow-on-a-Chip (Mouse and Human)

Working with in vitro/ex vivo expanded cells neglects the impact of the microenvironment and niche on HSCs. Bone marrow is a complex environment composed of the hematopoietic and non-hematopoietic parts. Where the niche, consisting of stromal cells, endothelial cells, osteocytes, adipocytes, neural cells, and the microenvironment consisting of cytokines influence the function of HSCs [9, 150].

In order the study hematopoiesis while taking into consideration the complexity of the bone marrow, 'bone marrow-on-a-chip' was devised [151, 152]. Here, an artificial bone marrow is created subcutaneously in vivo and then the explanted marrow is maintained in vitro in a microfluidic chamber. A poly(dimethylsiloxane) chamber with a central cavity, sealed at the top is filled with type-I collagen demineralized bone powder and bone morphogenetic proteins – BMP2 and BMP4. Eight weeks post subcutaneous implantation, histologically, the device resembled a femur and had the presence of trabecular network and CXCL12 expressing endosteal and perivascular niches, nestin + cells, leptin receptor + cells as well as CD31 + vascular endothelial cells, which are all known to be supportive of HSCs [153]. The artificial bone marrow also has the presence of lymphoid cells, myeloid cells, erythrocytes and hematopoietic stem and progenitor cells in the normal frequencies found in the femur. Additionally, maintaining this explanted artificial bone marrow in a microfluidic chamber for additional 7 days, retained hematopoiesis and functional HSCs without any additional supplementation like cytokines, usually required to maintain in vitro cultures [151]. This 'bone marrow-on-a-chip' can hence be used to test for drug toxicity studies when it enters phase I clinical trials. This approach will need surgical expertise as well as a microfluidic set-up to maintain the explanted artificial bone marrow.

A similar bone marrow on a chip has also been developed for human cells. The difference being that in this case, the human CD34 + HSPCs are directly maintained in a poly(dimethylsiloxane) two-channel microfluidic chamber without implantation on a mouse [154]. The top channel is filled with HSPCs and stromal cells, while the bottom 'vascular' channel is lined with endothelial cells. The chamber is then perfused through the vascular channel with media rich in HSC supportive cytokines – SCF, Flt3 and TPO. In case, of studying myeloerythroid differentiation, GCSF and EPO are other cytokines that are fed through the bottom channel. Within 2 weeks, hematopoiesis and the niche in the BM chip resembles that in a living BM [154]. In another case, BM chip is constructed using a hydroxyapatite coated zirconium oxide scaffold [155]. Here, human mesenchymal stromal cells are allowed to form an environment resembling human BM for a week before adding CD34 + HSPCs in the cavity to culture for an additional 4-weeks. During these entire 5 weeks, the chip is attached to a microfluidic chamber for constant supply of nutrients and oxygen, resembling the vasculature of human BM [155]. These BM-on-a-chip based organoids have been shown to mimic HSC not only transcriptionally, but also through interaction with the niche [152]. Because the human BM chip can also be used for infusion-based drug delivery, this model works as a great tool for drug efficacy and safety test, as well as impact of radiation therapy on BM. Pharmacokinetic and pharmacodynamic properties of a drug can vary from species to species, thus making the human BM chip a more appropriate pre-clinical tool to study drug behavior. Additionally, the human BM chip can serve as a good pre-clinical system to study disorders in which animal models recapitulate only some aspects of human disease, for example, Shwachman-Diamond Syndrome, Polycythemia Vera and Myelodysplastic Syndrome [152, 156–158]. Similarly, murine bone marrow-on-a-chip model, the human BM chip also needs a specialized microfluidic set-up, making it difficult to establish in labs.

In summary, bone marrow-on-a-chip technology provides a unique and physiologically relevant approach to study hematopoiesis and the bone marrow microenvironment in vitro. However, its limited accessibility due to cost and complexity poses challenges for widespread use and reproducibility.

Discussion

The study of HSCs has indeed seen significant progress, especially with the advent of single-cell technologies that have revealed the heterogeneity within this critical cell population. However, challenges persist, including the need for assays that allow the study of HSCs in their native environment to avoid biases introduced by isolation processes. Moreover, most studies have been conducted in mouse models, necessitating efforts to validate findings in human HSCs.

HSCs are crucial for the continuous production of blood cells and have been extensively studied, with recent advancements shedding light on their heterogeneity. However, many studies involve isolating HSCs from their native environment, potentially introducing biases. Additionally, the predominant use of murine models poses challenges in extrapolating findings to human HSC biology. To address these issues, it is essential to develop assays that enable the study of HSCs within their natural niches, allowing for a more comprehensive understanding of their behavior and regulation. Moreover, efforts to validate findings in human HSCs are crucial for translating research outcomes into clinical applications.

Similarly, in the context of leukemia, leukemia stem cells (LSCs) represent a subset of cells responsible for driving the disease. Understanding LSC biology is essential for developing targeted therapies and improving patient outcomes. Applying advanced assays that have enhanced our understanding of HSCs to the study of LSCs holds great promise. By uncovering the unique characteristics and vulnerabilities of LSCs, researchers can identify potential targets for therapeutic intervention, ultimately leading to more effective treatments for leukemia patients.

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Compliance with Ethical Standards

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state. This method helps gather a global picture of transcriptome and epigenetic changes along the path of development and differentiation.

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