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Clonal Tracking of Hematopoietic Cells: Insights and Clinical Implications

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Summary:

Recent advances in high throughput genomics enable the direct tracking of outputs from many cell types, greatly accelerating the study of developmental processes and tissue regeneration. The capacity for long-term self-renewal with multi-lineage differentiation potential characterizes the cellular dynamics of a special set of developmental states that are critical for maintaining homeostasis. In hematopoiesis, the archetypal model for development, lineage-tracing experiments have elucidated the roles of hematopoietic stem cells to on-going blood production and the importance of long-lived immune cells to immunological memory. An understanding of the biology and clonal dynamics of these cellular fates and states can provide clues to the response of hematopoiesis to aging, the process of malignant transformation, and are key to designing more efficacious and durable clinical gene and cellular therapies.

Keywords

Hematopoiesis; Hematopoietic Stem Cells; Hematopoietic Stem Cell Transplantation; Lineage Tracing; DNA barcoding; T cells; NK cells

Introduction

Cellular specialization and differentiation are important innovations in multicellular organisms. During development, cells proliferate and differentiate in a manner that is highly coordinated both spatially and temporally. Along the developmental pathways from embryo to many of the mature cell types lie metastable states with differing capacities for self-renewal along with multi-lineage differentiation. Many of these developmental waypoints persist in the full developed organism, where they are essential to homeostasis and represent possible targets for gene therapy and reservoirs for regenerative medicine, while at the same time conferring risk to the viability of the organism from dysregulated proliferation or differentiation.

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Recent advances in high throughput genomics and genetic engineering enable tracing clonal outputs from specific individual cells. Due to its ready accessibility, the hematopoietic system is perhaps the best characterized in terms of lineage relationships and differentiation pathways. In this review we focus on insights gained into hematopoiesis from lineage-tracing approaches, including ontogeny as well as the regulation of proliferation and differentiation at various levels along hematopoietic pathways. We discuss how the balance between quiescence and self-renewal versus proliferation and differentiation may be differently regulated during development, homeostasis and regeneration after stress.

Understanding cellular clonal dynamics has many applications in translational hematology. Clonal expansions underlie physiologic immune responses to infections and pathologic neoplastic transformation. Clonal disorders of hematopoiesis run the gamut from clonal hematopoiesis of indeterminate potential to leukemia. Insights into normal and pathologic clonal relationships and output are necessary to understand and treat hematologic cancers and improve the efficacy and safety of gene and cellular therapies, including hematopoietic stem and progenitor cell (HSPC) transplantation.

To provide context, we begin with a brief overview of lineage tracing techniques. The methods divide into two categories depending on whether they track endogenous cellular markings retrospectively or introduce new cellular tags, which can be tracked prospectively. The former enable the study of hematopoiesis in humans under unperturbed native conditions, but generally lack resolution. The latter are, with the exception of human gene therapy trials utilizing integrating vectors, restricted to non-human models, and usually require hematopoietic cell transplantation or immune cell adoptive transfer. Differentiation potential – though not clonal lineages – can be computationally inferred from single cell genomics experiments. The merger of single cell genomics with lineage-tracing techniques promises a mechanistic understanding of clonal dynamics. We move on to discuss insights into hematopoiesis gained from these approaches, starting with development and moving onto post-natal hematopoiesis at steady-state and under stress, such as HSPC transplantation and aging. We describe observations regarding lineage ontogeny, the level of hematopoietic stem and progenitor cells maintaining hematopoiesis, and well as the interaction of HSPCs and the marrow niche at a clonal level. Finally we focus on lineage-tracing experiments in the adult immune system, a composite of long-lived peripherally maintained descendants produced over time from separate waves of HSPCs. Different populations of T cells, B cells and natural killer (NK) cells are functionally and ontologically distinct, fulfilling specific roles in maining self tolerance, rapidly responding to pathogens or tumor cells, and maintaining effective memory.

Techniques for lineage tracing and measuring clonal output from HSPCs

Lineage-tracing has been extensively reviewed elsewhere (Baron and van Oudenaarden 2019, Kester and van Oudenaarden 2018, Lyne*, et al* 2018, McKenna and Gagnon 2019, Wagner*, et al* 2016). Fate mapping is a predecessor to lineage tracing and traces the developmental capabilities of a labeled population of cells sharing some characteristic. While it lacks clonal resolution, it has elucidated many facets of developmental biology on the population level. The history of lineage tracing begins with live microscopy applied to

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visually follow daughter cells arising during mitosis (Sulston*, et al* 1983), which initially limited the technique to the study of transparent, relatively simple organisms or the output of individual mammalian HSPCs cultured *ex vivo*, focusing on the process of symmetric versus asymmetric division (Loeffler and Schroeder 2019). Early approaches to studying clonal dynamics took advantage of the random inactivation of polymorphic X chromosome glucose-6-phosphate-dehydrogenase (G6PD) alleles in heterozygous women to show for the first time that chronic myelogenous leukemia and other myeloproliferative disorders were clonal HSPC disorders involving multiple lineages, and inferring a prodromal Ph-negative stage from which the Ph-positive stage evolved by subclonal expansion (Fialkow*, et al* 1980). This approach was also used to provide some of the first quantitative insights into normal hematopoiesis in G6PD-heterozygous female Safari cats, via low dose HSPC autologous transplantation followed by analysis of varying G6PD allelic contributions over time to infer HSPC numbers and output (Abkowitz*, et al* 1996). Although this method is very low in resolution, it yielded significant insights into the dynamics of hematopoiesis in normal and abnormal states.

The ability to track heritable cell labels enabled lineage tracing in more complex organisms. Modern lineage-tracing methods achieve higher resolutions by sequencing high diversity regions of cellular DNA. Retrospective methods rely on detection of endogenous somatic mutations accumulating in either mitochondrial or nuclear genomic DNA. Prospective methods introduce high diversity variable elements into nuclear DNA, which are either invariant or accumulate further changes over time. Modern lineage tracing methods rely on the introduction of genetic tags or mutations, which ethically limits their use in humans to patients who undergo gene therapy with integrating retroviruses.

Retrospective lineage-tracing techniques

Endogenous genetic changes or mutations of various types occur in all cells, and can be used to retrospectively map lineage relationships and clonal output from dividing cells.

Prime examples are the the use of immunoglobin gene or T cell receptor gene rearrangements to study central characteristics of the formation, maintenance and responsiveness of the adaptive immune system or the malignant clonal transformation of B or T cells. However, this approach is not applicable to studying many earlier events in hematopoiesis or lineages not undergoing germline recombination of genes.

Somatic mutations (ie point mutations, frame shift mutations, etc) can be used to retrospectively infer lineages in normal or cancerous tissues via new technologies able to perform single cell whole genome sequencing or whole exome sequencing (Baslan and Hicks 2017) (Figure 1). Over 100,000 spontaneous somatic mutations in genes not associated with cancer could be detected in purified individual HSPC from a healthy adult human and then used to reconstruct the clonal dynamics of hematopoiesis based on sampling of various blood lineages (Lee-Six*, et al* 2018). Sequencing errors complicate the confident identification of somatic mutations, and since somatic mutations are generally sparse, most data obtained from whole genome/exome sequencing is extraneous to retrospective lineage inference. Both factors reduce sensitivities to smaller clones. Advances in targeted error-

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corrected sequencing greatly improved the sensitivity to smaller clones (Acuna-Hidalgo*, et al* 2017, Xie*, et al* 2014, Young*, et al* 2016).

Not all acquired somatic mutations in nuclear genomic DNA are bland clonal markers; some mutations clearly impact on HSPC behavior and clonal output. Clonal expansions magnify with age, which complicates the problem of inferring normal clonal dynamics from endogenous mutations in older adults. In 2014 three groups analyzed exome sequencing data from over 30,000 individuals who were unselected for hematological phenotypes (Genovese*, et al* 2014, Jaiswal*, et al* 2014, Xie*, et al* 2014). They detected somatic clonal hematopoiesis that increased in frequency as well as clone size with age. The expanding and age-related mutations were in a very restricted set of genes, primarily those encoding epigenetic regulators such as *DNMT3A*, *TET2* and *ASXL1* and genes such as *JAK2* or *IDH1* previously linked to myeloid neoplasia.

Mutations in mitochondrial DNA (mtDNA) are a promising set of natural clonal markers (Ludwig*, et al* 2019) for several reasons. The human mitochondrial genome is 16.6 kb in length, allowing deep sequencing at reasonable cost. Mutation rates in mtDNA are estimated to be 10- to 100-fold higher than for nuclear DNA and mitochondrial genomes have high copy number (100 – 1,000). The role of somatic mtDNA mutations as drivers in cancer is somewhat controversial (Schon*, et al* 2012). Moreover, inheritance patterns from mtDNA are more complicated than nuclear DNA, which makes inferring lineage pathways more difficult. Finally, while mtDNA sequences are detected in single cell (sc) assays such as transposase accessible chromatin-sequencing (scATAC-seq) and scRNA-seq, current droplet-based methods have limited coverage of the mitochondrial genome, restricting the ability to combining lineage tracing via mtDNA with single cell "multiomics".

Prospective lineage tracing techniques

Prospective lineage-tracing techniques rely on introduction of exogenous clonal tags into cells of interest, either via integrating retroviruses used to transduce target cells *ex vivo*, or via insertion of cassettes into the germline of a transgenic mouse strain that are then activated via an applied signal to create clonal tags via local recombination or generation of new integration events (Figure 1). In early applications of this approach, replication-incompetent murine retroviral vectors were used to transduce murine HSPCs prior to transplantion. The semi-random and permanent integration of the proviral forms of the vectors into HSPC genomic DNA resulted in integration site (IS)- related restriction fragment length polymorphisms that could be read out via Southern blot, and served as clonal identifiers for output from individual transduced HSPC (Keller*, et al* 1985, Lemischka*, et al* 1986). However, the resolution of this approach was very low, and early transduction methodologies resulted in loss of most engrafting activity, likely accounting for the conclusions that long-term hematopoiesis was oligoclonal and unstable. Subsequent improvements in HSPC culture and transduction as well as the design of far more sensitive PCR-based integration site retrieval methodologies facilitated tracking of hundreds to thousands of individual HSPC clones in murine, non-human primate and even human recipients of autologous cells transduced with integrating viral vectors (Nolta*, et al* 1996, Schmidt*, et al* 2002, Schwarzwaelder*, et al* 2007). However, all IS retrieval approaches are

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relatively inefficient and only semi-quantitative. Introduction of a high diversity random "barcode" library into the integrating vector, allowing retrieval via simple low cycle PCR followed by high throughput sequencing, has allowed more sensitive and quantitative *in vivo* clonal tracking in murine and non-human primate models (Cheung*, et al* 2013, Gerrits*, et al* 2010, Koelle*, et al* 2017, Lu*, et al* 2011, Radtke*, et al* 2017, Wu*, et al* 2018b, Wu*, et al* 2014).

Vector integration events themselves can alter fundamental properties of HSPCs via insertional mutagenesis, due to aberrant activation of nearby proto-oncogenes by vector promoter/enhancers or inactivation of tumor suppressor genes (Cavazza*, et al* 2013, Espinoza*, et al* 2018). Modern lentiviral vectors have a markedly decreased likelihood of insertional genotoxicity, with increased safety for clinical gene therapy applications as well as a reduced impact of vector insertions on target cell behavior in clonal tracking studies (Hematti*, et al* 2003, Modlich*, et al* 2009). An additional drawback to use of integrating viral vectors for clonal tracking is the necessity of culturing target cells *ex vivo* during exposure to the vector, potentially impacting on self-renewal, differentiation and/or engraftment. Finally, regardless of the vector utilized, genetically-tagged HSPCs can only be analyzed in the context of the massive proliferative stress imposed by transplantation, which almost certainly alters the self-renewal and even lineage output of individual HPSCs. The heterogeneous output of the multiple daughter HSPCs produced from one HSPC will all carry the same barcode and thus cannot be resolved by these methodologies. Extrapolation of findings from transplantation to steady state "naïve" hematopoiesis may be misleading.

Alternative prospective clonal tracking approaches were therefore designed to avoid the requirement for *ex vivo* transduction of target cells and transplantation. A genetically-modified mouse line inducibly expressing a hyperactive form of the Sleeping Beauty (HSB) transposase acting on an engineered transgenic transposon element was created (Rodriguez-Fraticelli*, et al* 2018, Sun*, et al* 2014) (Figure 1). During a brief period of HSB transposase induction, the transposon element mobilizes and integrates into a new unique location in the genome for each cell, which is then passed on stably to all daughter cells. It is important to note that the transposase is induced ubiquitously, so that all HSPCs are simultaneously transposon-tagged. Insertion site retrieval methodologies can then be applied, as described above. The method is fairly high in resolution, but still suffers from the quantitation challenges of all insertion site retrieval techniques. Transposons are mobilized in both LT-HSCs as well as progenitor cells; the duration over which a specific insertion site is observed characterizes how long a LT-HSC or a progenitor is able to sustain clonal output.

A method aimed at studying native hematopoiesis at defined stages of murine development involves introducing a cassette containing multiple *loxP*-stop codon-*loxP* sites with short intervening DNA sequences (Pei*, et al* 2017). When crossed with a mouse line expressing *Cre* recombinase under the control of a specific promoter, some number of the *loxP-stop codon-loxP* are randomly excised and the resulting combinations of interspersed DNA sequences serve as an essentially unique genetic barcode for each cell. Since *Cre*-expression can be placed under the control of a wide variety of promoters specific to a particular stage of development or cell lineage, the system has wide applicability. This technique exhibits only medium resolution, due to the relatively limited diversity of generated barcodes and the temporally somewhat diffuse expression of the recombinase. A variation on this technique

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places *Cre*-expression under the control of a tamoxifen-inducible promoter, giving more precise control over the time and duration of barcode generation.

Prospective lineage-tracing techniques described thus far track lineages by introducing elements of exogenous DNA that remain invariant over time. Such methods are able to characterize total clonal outputs, but struggle to resolve subclonal dynamics, including cyclical or convergent trajectories or epigenetically-controlled differences in the behavior of daughter cells. In order to overcome this limitation, methods have been devised based on *in vivo* creation of edited insertions or deletions via CRISPR-Cas9 mediated "scarring" of artificial genomic DNA targets introduced into the genome of transgenic mice. Three components are required: a CRISPR-Cas9 endonuclease, an array of DNA target sites and a panel of single guide RNAs (gRNAs) recognizing these targets (McKenna*, et al* 2016). Directed by the gRNA, Cas9 introduces small moderately diverse insertions and deletions ("indels") via non-homologous end joining repair at the DNA target sites, which serve as retrievable clonal tags. With sustained Cas9 activity, subclones are labeled with cumulatively edited tags from which the parental clone can be inferred, along with subsequent unique branches of subclones. However, continuous HSPC exposure to Cas9 can result in DNA-damage-induced perturbations and/or toxicity and thus impact on HSPC function. A mouse line engineered with an doxycycline-inducible Cas9 was recently engineered, allowing simultaneous interrogation of lineage and transcriptomic information from single cells *in vivo* while circumventing the problems of ongoing DNA-damage-induced toxicity (Bowling*, et al* 2020).

Inferring Differentiation Trajectories from Single Cell Gene Expression Data

The recent explosion of methodologies and analytic approaches allowing single cell gene expression profiling of hundreds to many thousands of individual cells has resulted in revolutionary insights into hematopoiesis and other complex branching biologic processes composed of cells existing at multiple points in developmental hierarchies (Pellin*, et al* 2019). High throughput single cell genomics localize the instantaneous state of individual cells in very high dimensional gene expression space, and provide much more information than the same assays performed on bulk highly heterogenous populations of cells such as bone marrow or peripheral blood. It is assumed that developmental processes result from RNA expression and are continuous, and that consequently, differentiating cells follow smooth trajectories through this space. Cells located near each other in RNA expression space are assumed to lie on adjacent differentiation trajectories or differ slightly in how far they have progressed along developmental processes. In this manner, differentiation trajectories, though not direct lineage relationships, may be inferred from single cell RNA-seq data. Computational approaches to infer these trajectories have been recently reviewed (Saelens*, et al* 2019).

However, trajectory inference methods provide only indirect information on the true lineage relationships between cells, i.e. cells along the same path are not necessarily clonally related. Therefore, merging of single cell transcriptomics together with prospective genetic tagging proving clonal relationships is particularly powerful and is being actively pursued. For instance, CRISPR-scarring arrays of DNA target sites have been moved within

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constitutively transcribed regions so that the scarring-related barcodes can be retrieved via single cell RNA-seq (Alemany*, et al* 2018, Raj*, et al* 2018, Spanjaard*, et al* 2018), allowing investigation of gene expression profiles associated with specific clonal and subclonal behavior. Similarly, a lentiviral expression cassette containing a barcode expressed at high levels constitutively has allowed simultaneous barcode retrieval along with single cell transcriptomics to investigate HSPC differentiation and self-renewal pathways both *in vitro* as well as *in vivo* following transplantation (Weinreb*, et al* 2020).

Insights into Clinically-Relevant Aspects of Hematopoiesis from Lineage-Tracing Studies

Developmental hematopoiesis

Transplantation experiments support the notion that a population of long-term hematopoietic stem cells (LT-HSCs) continually replenishes mature lineages and maintains homeostasis in adult organisms. In mice, three distinct waves of non-persistent hematopoietic progenitor cells emerge prior to and independently of *bona fide* LT-HSCs during fetal development (Dzierzak and Bigas 2018, Ghosn*, et al* 2019, Palis*, et al* 1999). A number of different lineage-tracing approaches established that some cellular descendants from the initial waves of embryonic or fetal hematopoiesis survive into adulthood and play discrete roles in the immune system. For instance, several types of tissue-resident immune cells are produced from progenitors during fetal development and are maintained into adulthood by *in situ* self-renewal, including a wide variety of types of tissue-resident macrophages such as skin Langerhans cells, as well as a population of innate-like B cells (B1 cells) producing a class of “natural” antibodies without antigen stimulation (Gentek*, et al* 2018, Ghosn*, et al* 2016, Hashimoto*, et al* 2013). Some IL-17-producing γδ T cells in adults also appear to have an embryonic origin (Haas*, et al* 2012).

Fate mapping experiments demonstrated that murine neonatal T cells arise from distinct progenitors and persist into adulthood (Smith, et al 2018, Yang, et al 2015). Compared to T cells produced later in life, neonatal T cells are poised to more quickly differentiate into regulatory or effector T cells. Neonatal Tregs are more effective at maintaining self-tolerance, with greater proliferative and immunosuppressive capacities (Yang, et al 2015). In response to pathogens, neonatal CD8+ T cells rapidly proliferate and differentiate into effector cells, whereas adult CD8+ T cells preferentially give rise to memory cells. These properties may account for some of the unique clinical properties of cord blood transplants, such as less graft-versus-host disease while retaining anti-tumor effects (Hiwarkar, et al 2017, Hiwarkar, et al 2015, Lee, et al 2011, St John, et al 2016). Moreover cord blood-derived neonatal T cells can be stimulated by cytokines to proliferate vigorously and generate human memory stem T cells (Cieri, et al 2013), making them ideal for adoptive immunotherapies.

Insights into the relative clonal output of HSCs versus their descendants

Notions about the number of HSCs that are concurrently actively cycling or differentiating have evolved over time (Bystrykh*, et al* 2012). Clonal succession theories, proposed initially on theoretical grounds (Kay 1965), posit that HSCs have limited repopulation potential and

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that, over any time span, only a few HSCs are actively self-renewing or differentiating. On the other hand, clonal stability theories, initially proposed on the basis of observations from experiments performed under transplant conditions (Gerrits, *et al* 2010, Jordan and Lemischka 1990, Lemischka, *et al* 1986, Naik, *et al* 2013) holds that a large fraction of HSCs are actively self-renewing and maintain mature lineages through sustained clonal outputs. The conditions of transplant and antecedent *ex vivo* manipulations impose stresses on HSCs and progenitors, which can conceivably impact on their potentitia.

Stem cells under conditions near homeostasis

Transposon tagging of murine HSCs was among the first methods to study hematopoiesis under unperturbed conditions (Rodriguez-Fraticelli, *et al* 2018, Sun, *et al* 2014). The dominant roles of progenitor cells in blood production is suggested by the finding that few IS tags are detected at successive time points, consistent with the initial repletion of mature lineages with the descendants of short-lived and uni- or oligopotent progenitors. The limited senstivity of this study does not rule out stable contributions from long-term hematopoietic stem cells (LT-HSCs), but suggests that, in relative terms, they are minor in comparison to the contributions of multipotent transient progenitors during steady-state hematopoiesis.

A subsequent analysis using the murine polylox recombination system (Pei, *et al* 2017) to label embryonic cells found that, after birth, the hematopoietic system is derived from the composite output of at least hundreds of embryonic clones, a lower bound limited by the resolution of this model. The analysis of spontaneous mutations in the bone marrow, peripheral and cord blood of healthy donors revealed multiple non-nested clones ubiquitous across lineages and time points, consistent with highly polyclonal blood production from continuously active LT-HSCs (Lee-Six, *et al* 2018, Osorio, *et al* 2018). The number of active HSCs at various time points was estimated via capture-recapture sampling to be between 50,000–200,000 (Lee-Six, *et al* 2018), numbers within the same order of magnitude calculated via NHP barcoding or Safari cat G6PD modeling approaches (Abkowitz, *et al* 2002, Koelle, *et al* 2017). During embryogenesis and early childhood there is a rapid growth in the number of active HSCs, reaching a plateau in adulthood.

The murine transposon studies highlight the dominant role that a diverse and numerous pool of progenitors play in conditions near homeostasis, though they presumably lack the sensitivity to detect sustained contributions from LT-HSCs. The studies of the murine polylox system and human endogenous mutations make it evident that hematopoiesis is highly polyclonal and stable. None of the studies support clonal succession or exhaustion of LT-HSCs.

Stem cells under conditions of transplantation stress

Bystrykh's group studied clonal repopulation and long-term dynamics of murine HSPCs after transplantation (Gerrits, *et al* 2010) using a barcoded vector library of modest diversity. They were able to track a limited number of clones (<30 per animal) over time post-transplantation. Some clones persistently and appreciably contributed to either the granulocyte or T cell compartments at all time points. Other clones were shared between both compartments. The results suggested that, after transplantation, hematopoiesis can

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be stably maintained by a few HSC-derived multi-potent clones. The moderate barcode diversity limited clonal resolution, while the lack of sequencing depth limited sensitivity to smaller clones. Weissman's group utilized a lentivirally-delivered barcode library of very high diversity and used it in a murine model (Lu*, et al* 2011), documenting more polyclonal and multipotent stable contributions from LT-HSCs.

These clonal tracking studies in mouse models have provided insights into the complexity of HSPC biology impossible to achieve with bulk population-level studies or low throughput technologies studying only a few HSCs at a time, such as non-limit dilution transplantation. However, the physiology of mice and humans differs in significant ways. The shorter lifespan of mice limits the duration over which clones can be followed and smaller body size limits the amount of blood that can be obtained and therefore clonal sampling sensitivity. Moreover, human and murine hematopoiesis differ in phenotype, frequency and function of HSPCs (Catlin*, et al* 2011, Larochelle*, et al* 2011). *In vitro* single cell culture assays and xenotransplantation animal models elucidated functional features and ontogeny relationships of human HSPCs subsets and changes during human development (Doulatov*, et al* 2010, Miyawaki*, et al* 2017, Notta*, et al* 2011, Notta*, et al* 2016). Barcoded human cord blood HSPCs engrafted in primary and secondary xengrafts were followed over time, with demonstration of multipotential clones and tracking of some individual clones long-term in both primary and secondary animals (Cheung*, et al* 2013). However, the inefficiency of xenografts and lack of release of many human blood cells into the circulation imposed sampling constraints. Extrapolating results from *in vitro* single cell culture studies or xenografted mice to the behavior of human HSPCs in a normal homologous bone marrow microenvironment may be misleading.

Non-human primates models overcome some of these limitations, with results likely to be of greater relevance to human HSPC transplantation and adoptive cell and gene therapies. We and others applied barcoding technology in a rhesus macaque model for clonal tracking of hematopoiesis (Fan*, et al* 2020, Kiem*, et al* 2004, Kim*, et al* 2014, Koelle*, et al* 2017, Radtke*, et al* 2017, Wu*, et al* 2018b, Wu*, et al* 2014). In this model, several thousand barcoded HSPC clones per macaque were tracked over time following autologous transplantation. Clones that were detected at 1–2 months after transplantation were lineage-restricted and transient, and were supplanted over the next several months by multilineage clones, initially myeloid-B and eventually myeloid-B-T cell clones. Stable polyclonal multilineage long-term repopulating clones were observed for at least several years after transplantation (Koelle*, et al* 2017, Wu*, et al* 2014). Stable long-term clonal lineage biases toward certain cell types were observed, both in our macaque study, as well as in human gene therapy clinical trials (Koelle*, et al* 2017, Six*, et al* 2020). The faster and closer clonal relationships between B and myeloid cells than between B and T cells supports the existence of a common myelo-lymphoid progenitor (CMLP) versus a common lymphoid progenitors (CLP) in primates (Kawamoto*, et al* 2010), although T cell output in this macaque model is delayed and impaired due to the impact of total body irradiation on the thymus. Insertion site retrieval from a number of recent clinical HSPC gene therapy studies also came to similar conclusions regarding reaching stable clonal output from multipotent LT-HSC after several months (Scala*, et al* 2018).

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Clinical relevance of HSPC tracking studies

Both NHP and human gene therapy clinical trial clonal tracking studies suggest that following a several month period of transient contributions from lineage-restricted short-term progenitors, LT-HSCs can contribute stably for many years following transplantation. The stability of LT-HSC contributions were also illuminated by somatic mutation tracking. These conclusions have a number of clinical implications. Current gene therapies based on modification of LT-HSCs are likely to result in stable therapeutic outcomes, with levels of correction observed by 6–9 months post-transplantation likely to persist indefinitely. Quantitation of LT-HSC frequencies from both approaches uncovers the rarity of *bone fide* LT-HSCs, and the importance of focusing on the impact on this cell population linked to interventions such as *ex vivo* HSPC expansion or treatment with HSPC-toxic therapeutics. While LT-HSCs cycle slowly and much of hematopoietic output results from faster proliferating progenitors, any non-deleterious mutations that occur in LT-HSCs will likely persist indefinitely in the hematopoietic pool, and even minimal positive impact on self-renewal will result in expansion and a higher risk for acquiring secondary mutations, and thus eventual malignant transformation.

HSPC clonal tracking has a number of additional direct translational applications. Beyond the scope of this review, retrieval of IS has been critical to understanding the genotoxic events linked to activation of proto-oncogenes via integration of clinical gene therapy vectors (Hacein-Bey-Abina*, et al* 2003, Nienhuis*, et al* 2006). Modeling the likelihood of such events via quantitative clone tracking in animal models, via detection of pre-malignant clonal expansions, is an approach to predict genotoxicity of new vectors prior to clinical introduction (Montini*, et al* 2009, Yabe*, et al* 2018). In addition, clonal tracking can be used to understand the impact of *ex vivo* expansion on HSPC function or numbers, or associate engraftment with particular phenotypic classes of HSPCs (Radtke*, et al* 2017).

Clonal dynamics of aged HSPCs

Numerous clinically-relevant changes in the hematopoietic and immune systems occur with aging, such as a diminished capacity to regenerate following stress, impaired adaptive immunity, shifts in lineage output, expansion of somatically-mutated HSPC, and increased risk of malignant transformation (Geiger*, et al* 2013, Verovskaya*, et al* 2019). In both autologous and allogeneic human HSPC transplantation, aged HSPC have been correlated with adverse outcomes, including delayed blood count recovery (Kollman*, et al* 2001, Woolthuis*, et al* 2014). The relative contribution of intrinsic HSPC changes versus extrinsic effects of an aged microenvironment in the marrow and thymus are a matter of some debate (Dorshkind*, et al* 2020), however, both compartments appear to play a role, as demonstrated by bidirectional transplant studies between young and old mice. Limit dilution transplantation studies as well as *in vitro* assays have demonstrated maintenance or even an increase in phenotypic HSPCs with age, however, decreased functional engraftment and a marked myeloid lineage bias, linked to epigenetic changes (Beerman*, et al* 2013, Cho*, et al* 2008, Dykstra*, et al* 2007). A murine transgenic model incorporating a clever mitotic clock suggested that true LT-HSCs can only undergo a relatively limited number of symmetric self-renewal divisions, resulting in increased output from committed myeloid HSPCs with age (Bernitz*, et al* 2016). A transgenic clone tracing system was used to compare the clonal

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complexity of aged versus young murine hematopoiesis independent of transplantation. Overall, clonal complexity declined with age, and transplantation resulted in further loss of clonal diversity (Ganuza*, et al* 2019). Of note, whole exome sequencing revealed somatic mutations in potential growth-control genes in the oligoclonal aged mice, albeit in a set of genes that did not overlap with those mutated in human age-related clonal hematopoiesis.

The vast majority of mechanistic and experimental investigations into the impact of aging on hematopoiesis have thus far been carried out in murine models. However, there are numerous differences between mice and humans likely to impact on HSPC aging, including lifespan, size, telomere length, and susceptibility to malignant transformation (Doulatov*, et al* 2012). Therefore, we focused on studying HSPC aging in rhesus macaque, given their extended lifespans and similar HSPC characteristics to humans. Transplantation of autologous barcoded HSPCs was performed in aged macaques and compared to results in young adult animals. Aged macaques had markedly delayed emergence of multipotent LT-HSC, as well as higher contributions from both myeloid-biased as well as lymphoid-biased clones (Yu*, et al* 2018), in contrast to murine models, which have uncovered primarily myeloid-biased clones. Strikingly, clonal expansions emerged over time in the aged macaques, suggesting a process akin to clonal hematopoiesis in humans. We have now found acquired somatic mutations in aged macaques in the same genes found in aged humans (unpublished data).

The impact of the hematopoietic microenvironment on HSPCs at a clonal level

The majority of clonal dynamics and lineage tracing studies have focused on elucidating intrinsic properties of HSPC. However, the marrow microenvironment controls not only localization, but also self-renewal versus differentiation. Increasingly, a variety of niches within the marrow have been identified as controlling hematopoiesis via metabolic, cytokine and adhesive signals (Morrison and Scadden 2014). Even at steady state, a very small number of HSPCs are present in the circulation, increasing with stress or recovery from marrow suppression, as well as in various pathologic states such as myeloproliferative syndromes. However, the physiologic role and eventual fate of circulating normal HSPCs are poorly understood. The very slow mixing of marrow HSPCs in parabiotic pairs of mice with shared blood supplies suggests that marrow niches are closed to circulating HSPC entry, and that exit from the marrow into the blood may normally be a death pathway (Abkowitz*, et al* 2003).

HSPCs transplanted following myeloablative conditioning home to the marrow and proliferate rapidly, regenerating the HSPC pool (Pawliuk*, et al* 1996). This process might be expected to be accompanied by continued release of HSPC into the circulation, due to local niche occupancy and high levels of stimulating cytokines, resulting in rapid mixing of the daughter cells of transplanted HSPC throughout the marrow. However, comparison of chimerism levels between bones following transplantation revealed spatial differences (Nilsson*, et al* 2015). Short-term live imaging studies have revealed localized proliferation and nearby retention of daughter HSPCs (Lo Celso*, et al* 2009, Wang*, et al* 2013). Confocal imaging of the marrow folloing transplantation of murine HSPCs tagged with an array of fluorescent proteins revealed marked long-term geographic restriction of proliferating HSPC

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clones (Malide, *et al* 2012). In rhesus macaques transplanted with barcoded HSPC, there was marked geographic segregation of CD34$^+$ HSPCs, followed by very gradual clonal mixing between marrow sites over months to years (Wu, *et al* 2014). Neutrophils, B cells and immature NK cells were clonally-related to CD34$^+$ HSPCs from the same marrow site, implying local production, and surprising, early following transplantation, some local production of mature T cells was also uncovered. A similar study in mice documented geographic marrow segregation, with acceleration in equilibration across marrow sites with cytokine mobilization (Verovskaya, *et al* 2014), a manuever that also sped mixing in parabiosis experiments (Chen, *et al* 2006).

These findings may help explain the highly varied cellularity in human bone marrow biopsies, particularly following recovery from transplantation or myeloablative chemotherapy. Notably, similar geographic heterogeneity has been reported for barcoded human leukemia cells following xenotransplantation into immunodeficient mice (Belderbos, *et al* 2017). Reliance on single marrow samples for quantitation of chimerism, cytogenetic abnormalities, or acquired somatic mutations may be skewed by geographic heterogeneity, and thus analysis of blood as opposed to marrow would better reflect overall hematopoiesis, particularly important for comparisons over time.

Conditioning to ablate endogenous HSPCs is necessary to permit engraftment following transplantation, but these regimens may damage or modulate functions of marrow niches, potentially impacting on HSPC regeneration and even long-term function following transplantation (Dominici, *et al* 2009, Pietras, *et al* 2015). Clone tracking approaches have begun to offer some insights into the impact of various conditioning regimens. More homogeneous contributions of individual murine HSC clones were detected following transplantation of large doses of HSCs into non-conditioned hosts, with little lineage bias (Lu, *et al* 2019). In contrast, following either TBI or antibody-mediated anti-CKIT conditioning, clonal contributions were more heterogeneous in size and more likely to show lineage bias, thus HSPC properties may vary depending on the impact of conditioning regimens on the microenvironment.

Insights into the ontogeny and life histories of natural killer cells

Natural killer (NK) cells have traditionally been defined as innate immune effector cells able to produce cytokines and kill virally-infected or malignant targets independent of antigen recognition or MHC restriction. The absence of somatic recombination of germline antigen receptors has precluded clonal tracking based on analysis of endogenous receptor sequences, in contrast to B or T cells, limiting direct insights into NK cell development and life histories. The assumption that NK cells lack specificity or the capacity for memory has been challenged over the past decade, based on a series of studies demonstrating that murine NK cells can respond to a viral or hapten challenge with long-lasting specific responsiveness, persisting upon adoptive transfer and re-challenge (O'Sullivan, *et al* 2015).

There are marked differences between the phenotype and function of mouse versus human NK cells. Human NK cell ontogeny has been inferred based on comparative phenotyping of NK cells in the marrow, lymph nodes, and blood; kinetics of recovery after transplantation; in vitro culture, and most recently, imputation of differentiation from single cell RNA

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sequencing (Pfefferle*, et al* 2019). The classical human model suggested that precursor or immature CD56^{bright} NK cells develop from HSPCs in the marrow, then migrate to lymph nodes or secondary lymphoid tissues, where continuous maturation to CD56^{dim}CD16^{+} NK cells occurs, followed by release into circulation. In humans, subsets of CD56^{dim}CD16^{+} NK cells expand and show persistent functional characteristics following cytomegalovirus (CMV) infection (Beziat*, et al* 2013). Epigenetically, these putative "adaptive" or memory NK cells approximate CD8^{+} effector T cells (Lee*, et al* 2015, Schlums*, et al* 2015). Epigenetically imprinted "clonal" expansions in humans have been inferred by the presence of NK cells expressing distinct combinations of diverse cell surface receptors termed KIR following CMV reactivation (Beziat*, et al* 2013). However, mechanisms conferring persistent functional NK cell diversity and memory, in the absence of somatic receptor rearrangements, have remained mysterious.

Human NK cells are scantly produced in immunodeficient mice, and robust *in vitro* clonal assays are lacking; thus, mechanistic studies of human NK cell development and function have been challenging in experimental models. Relative to rodents, rhesus macaque (RM) NK cells are well studied, are evolutionarily close to humans, and share phenotypic and functional characteristics with human NK cells (Webster and Johnson 2005). Functional evidence for antigen-specific NK cell memory in RM after simian immunodeficiency virus (SIV)/HIV vaccination has been reported (Reeves*, et al* 2015). Following autologous transplantation of barcoded macaque HSPCs, immature CD56^{bright}CD16^{-} NK cells showed similar clonal derivation to myeloid, B cell and eventually T cell lineages, with eventual stable derivation from LT-HSCs (Wu*, et al* 2018a, Wu*, et al* 2014). In contrast, mature circulating NK cells developed marked oligoclonal expansions following engraftment, with a waxing and waning pattern over time, suggestive of peripheral self-renewal of NK cells in response to environmental stimuli, such as viruses. Expression of specific KIR cell surface receptors tracked with barcoded clones, linking clonal dynamics with expression of molecules known to be critical for response to viruses in the context of non-classical MHC molecules such as HLA-E (Hammer*, et al* 2018). Following CMV infection of barcoded macaques, NK cell clonal patterns changed, supporting the concept of clonal adaptive NK responses (Truitt*, et al* 2019).

An understanding of NK cell life histories should enhance the development of effective NK cell clinical therapies. Mature adaptive NK cells able to self-renew peripherally may be particularly attractive targets for adoptive transfer targeting tumors, resulting in improved persistence for approaching including CAR-NK cell therapies.

Insights into the ontogeny and roles of T-cell subsets

B and T lymphocytes classically form the core of the adaptive immune system, whose hallmarks include clonal expansion and memory. Recently it has become clear that subsets of innate lymphocytes (see above) and even myeloid cells share some of these features (Dai*, et al* 2020, Lee*, et al* 2015). Moreover senescent T cells have been noted to functionally resemble NK cells (Pereira*, et al* 2020). The blurring of boundaries between the adaptive and innate immune systems begs for a deeper understanding of the developmental relationships between different lymphocyte subsets.

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Granulocytes and lymphocytes share a common ancestry in hematopoiesis. T (and likely NK) lymphocytes diverge from B lymphocytes and granulocytes early in hematopoiesis. At 8 weeks after transposase induction, the activity of clones with multilineage potential included erythrocyte, granulocyte and B lymphocyte progenitors (Rodriguez-Fraticelli*, et al* 2018). The analysis of endogenous mutations found that most of the early mutations, at the top of the phylogenetic tree, were found in all cell lineages (Lee-Six*, et al* 2018). However, some adult stem cell clones contributed to detectable numbers of granulocytes and B lymphocytes, but not to T lymphocytes. The sensitivity of both studies leave it unclear whether the T cell clonal output derived from these HSCs added to a large long-lived pool of mature T lymphocytes, diluting below the detection threshold, or whether it signals the existence of a progenitor with lineage output restricted to erythrocyte, granulocyte and B lymphocyte progenitors.

Recently it was reported that 5 X-SCID patients infused with gammaretrovirally engineered autologous CD34+ cells maintained vector positive T and NK lymphocytes 15 years after the loss of their HSC graft (Izotova*, et al* 2020). Significantly, a vector positive subpopulation of naïve T cells could be detected. Mathematical modelling of integration site data supports the existence of a long-term lymphoid progenitor. About 40% of the insertion sites observed in CD3- CD56+ NK cells were shared with naïve T cells, consistent with a long-term lymphocyte progenitor with T/NK-restricted potential. Identification and exploitation of this population could benefit adoptive cell therapies, suggesting a focus on transfer of these cells could result in very long-term impact on immunity without the need for ablative HSPC transplantation.

The long-term persistence of memory T cells *in vivo*, allowing expansion of specific memory clones with re-exposure, is critical to durable protection from pathogens, vaccine efficacy, and cancer relapse in the case of CAR-T cells. In an elegant analysis of 10 patients transplanted with a T cell depleted HSPC allograft along with an infusion of genetically-tagged donor T lymphocytes containing a suicide gene allowing T cell depletion in the setting of severe graft-versus-host disease (Oliveira*, et al* 2015), it was shown that tagged T cells could be detected for up to 14 years post-infusion, including within the entire spectrum of memory T cell subsets.

Chimeric antigen receptor T (CAR T) cells provide effective treatments for several hematological malignancies, including large cell lymphoma and pediatric B-ALL. Durable remissions are associated with higher peak expansion and longer persistence of CAR T cells (Fraietta*, et al* 2018a, Porter*, et al* 2015). Recently, insertion site data from patients treated with CAR T cells was analyzed with the aim of understanding potential impact of CAR T vector insertion sites on clone persistence in responders (Nobles*, et al* 2020). Differences in the distribution of vector ISs were noted in patients with positive clinical responses, though the study could not definitely resolve whether these differences were secondary to *a priori* differences in the transcriptional activity of the initially transduced cell pool or changes in function linked to specific insertions. Of note, insetions near genes involved in cell signaling and chromatin modification were enriched in responders. A single CLL patient with a profound and lasting remission following CAR T infusion was found to have expansion of a single CAR T cell clone containing a vector insertion inactivating the TET2

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gene, potentially accounting for the clonal persistence and clinical outcome in this patient (Fraietta, *et al* 2018b). Genes and pathways involved in the proliferation of CAR T cells uncovered in this manner may be targeted to improve CAR T cell function and persistence in future clinical applications. Besides the impact of insertion site location on CAR-T function, studying the relationships between CAR-T clonal diversity and clonal dynamics on clinical outcomes may provide clues to factors impacting on clinical efficacy.

Insights from the study of stem cells into regenerative medicine

One of the ultimate goals in stem cell biology is to precisely understand and control homeostasis and regeneration in complex tissues. Hematopoietic stem cells and their niches are widely studied due to their ready accessibility and centrality to the immune system. Organoids derived from embryonic stem cells, induced pluripotent stem cells or tissue-resident adult stem cells are a recent technological breakthrough which model many features of the three-dimensional organization of complex tissues (Fatehullah, *et al* 2016). Investigatons of organoids via lineage tracing and single cell genomics have revealed insights into the differentiation of stem cells into more mature cells types with concomitant ability to self-organize into complex tissue architectures (Danahay, *et al* 2015, Guo, *et al* 2020, Subramanian, *et al* 2019). In combination with perturbations such as cell ablation and conditional knock-outs, the study of organoids have uncovered the mechanisms determining decisions between symmetric versus asymmetric divisions of stem cells as well as some of the prerequisites for maintaining stemness (Pardo-Saganta, *et al* 2015). Although beyond the scope of this review, the cross-fertilization of ideas from the study of non-hematological stem cells will likely hace great impact hematology, oncology and regenerative medicine.

Conclusions

We hope this review has provided readers with an appreciation for the value of clonal and lineage tracking technologies to study the life histories, lineage relationships, spatial characteristics and chronologic dynamics of hematopoietic cells, including properties of stem and progentior cells, immune cells, and tumor cells. The insights gained from these studies have direct clinical implications for the development of gene therapies, HSPC transplantation, and adoptive immune cell or engineered anti-tumor cell therapies. Insights into the clonal behavior of normal HSPCs provides essential knowledge for optimizing outcomes of HSCTs as well as understanding the changes in hematopoiesis with aging or other stressors. Insights into subclonal relationships in hematologic malignancies are crucial for the design and optimization of new treatments. In the future, maturation and wider availability of approaches combining lineage tracing with single gene expression or epigenetic profiling should provide even more interesting insights, as well as combining such approaches with advanced imaging or targeted genetic manipulations to further test hypotheses regarding hematopoiesis and immunity.

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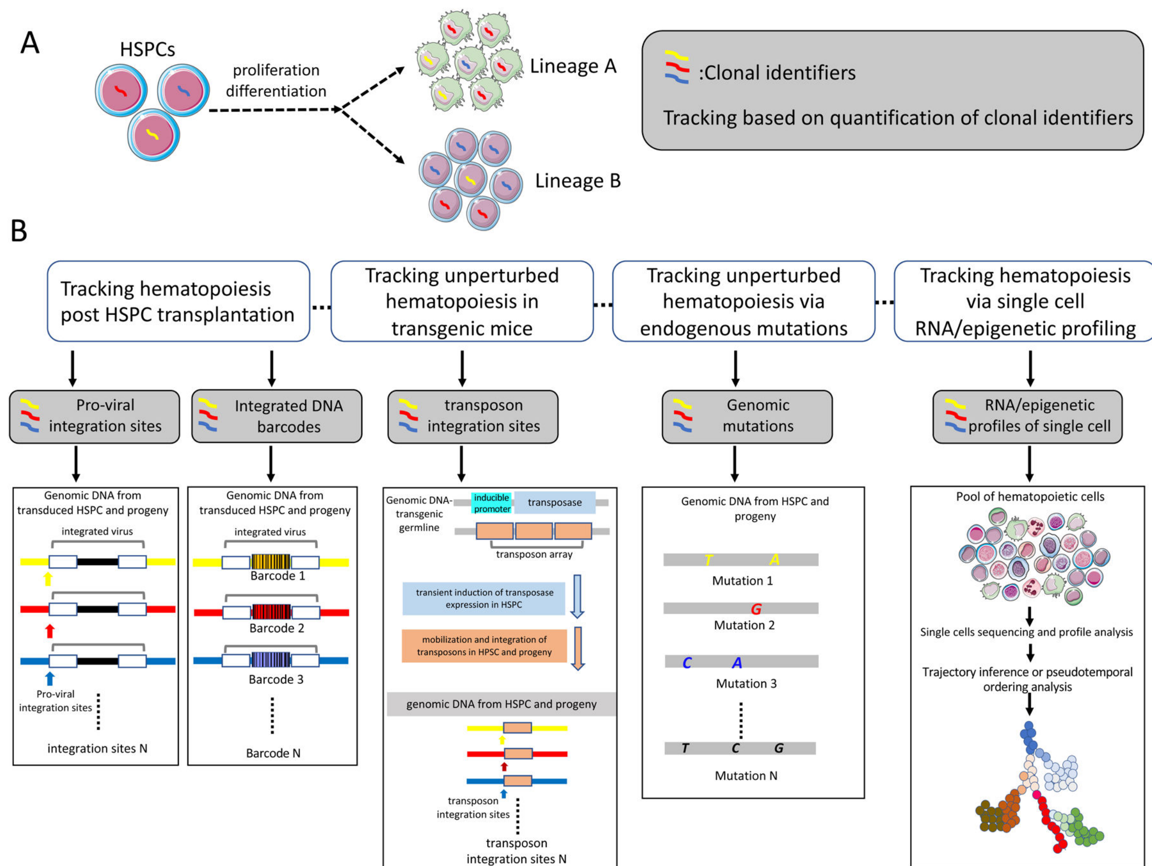

Figure 1.

Clonal Tracking of Hematopoietic Cells.

A) Clonal tracking in hematopoiesis is based on the recovery and quantification of clonal identifiers in different cell types.

B) Clonal identifiers rely on the ability to interrogate high diversity integrated tags. In prospective approaches, the diversity originates from either insertion sites (in gene addition or transposon tagging models) versus exogenously introduced 'barcodes'. In retrospective approaches, clones are distinguished on the basis of somatic mutations. Single cell transcriptome or epigenome analyses permit inference of differentiation potentials.