Clones assemble! The clonal complexity of blood during ontogeny and disease

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Abstract

Hematopoietic stem and progenitor cells (HSPCs) govern the daily expansion and turnover of billions of specialized blood cells. Given their clinical utility, much effort has been made towards understanding the dynamics of hematopoietic production from this pool of stem cells. An understanding of HSC clonal dynamics during blood ontogeny could yield important insights into hematopoietic regulation, especially during aging and repeated exposure to hematopoietic stress; insults that may predispose individuals to the development of hematopoietic disease. Here, we review the current state of research regarding the clonal complexity of the hematopoietic system during embryogenesis, adulthood and hematologic disease.

Introduction

In humans, a pool of hematopoietic stem and progenitor cells (HSPCs) governs the daily expansion and turnover of billions of specialized blood cells. HSPCs are hierarchical in structure, with a pool of hematopoietic stem cells (HSCs) with self-renewal capacity and advanced multipotency sitting at the top that give rise to more committed and transient progenitors[1,2]. HSCs are classically defined by their ability to give rise to all lineages of the blood after transplantation (HSCT)[1,2] and are routinely used for therapeutic purposes to treat hematologic maladies. Given the unique potency of HSPCs and HSCs and their utility in the clinic, much effort has been made towards understanding the dynamics of hematopoietic production from this pool of stem cells. An understanding of HSC clonal dynamics during blood ontogeny could yield important insights into hematopoietic regulation, especially during aging and repeated exposure to hematopoietic stress; insults that may predispose individuals to the development of hematopoietic disease. Here, we review the current state of research regarding the clonal complexity of the hematopoietic system during embryogenesis, adulthood and hematologic disease.

Clonal complexity of the blood during early ontogeny

Over the last six decades, the embryonic origins of hematopoiesis have been extensively studied with an eye towards developing a deep understanding of when, where and how blood precursors emerge during development[3,4]. The hope is that this knowledge can be leveraged to inform efforts to engineer clinical useful blood cells from differentiating pluripotent stem cells. Until recently, the actual number of cells emerging during early ontogeny to establish life-long blood production has been unclear. Here, we will focus on

reviewing recent progress towards answering this question and assessing how these numbers change over time (Table 1).

The emergence of HSC during embryogenesis

Several recent reviews have nicely summarized current models of hematopoietic development and HSC emergence during embryogenesis[3-6]. Here, we will focus most of our discussion on work in mice and zebrafish, two well-characterized models of vertebrate hematopoiesis[3-5]. Briefly, transplantable HSCs are first detected in murine embryos at embryonic day 10.5 (E10.5)[7-10]. In zebrafish, *runx1+* cells are detected in the dorsal aorta by 24 hours post-fertilization (hpf)[11-14] and CD34 expression (reflective of HSC emergence) is detected in the ventral endothelium of the aorta at week 5 of human gestation[15,16]. Briefly, the current favored model in vertebrates is that hemogenic (or blood-forming) endothelium gives rise to HSCs via an endothelial to hematopoietic transition (EHT) during development[17-19]. EHT is characterized by the budding of hematopoietic precursors and progenitors into the lumen of the major arteries, where they form intra-arterial clusters (IACs) in mice[19,20] and migrate into the cardinal vein in zebrafish[12]. In mice, IACs mostly localize to the ventral wall of the dorsal aorta and vitelline and umbilical arteries[8,15,20-22]. IACs are thought to harbor nascent hematopoietic populations because embryos devoid of *Runx1* expression lack IACs[17,18]. In mammals, HSCs migrate to the fetal liver (FL), where they are mostly found by E12.5[7,23]. In zebrafish, they migrate to the caudal hematopoietic tissue (CHT, thought to be equivalent to the murine FL)[5,11,24]. FL and CHT are considered niches supportive of HSC expansion[23,25]. HSCs complete their journey by moving into the bone marrow (BM) in mammals or the kidney marrow (the

equivalent to the mammalian BM) in zebrafish, where they mostly remain during adult life to support adult hematopoiesis[3-5]. Defining HSC dynamics during embryogenesis can illuminate precise developmental stages and tissues that affect HSC emergence, expansion or quiescence, which can direct an investigator's focus in the hunt for signals that might be exploited to recapitulate HSC specification or expansion *in vitro*. For example, the appearance of transplantable HSCs in the murine mid-gestation embryo reflects a critical time when HSCs are emerging from the hemogenic endothelium[3]. Thus, one might expect a focused study of this developmental stage to identify cues that promote the efficient *in vitro* generation of HSCs[26-29]. Indeed, the derivation of HSCs from endothelium or embryonic stem cells has recently been reported, albeit the process remains highly inefficient[30,31].

Further identification of critical developmental cues and milestones should pave the way for efficient derivation of HSCs *in vitro*. In the next section, we will describe approaches that have been employed to study the development of the adult HSC pool during early ontogeny.

Methodologies to study clonal complexity during embryogenesis

Methodologies that employ HSC transplantation

Classically, two main approaches have been taken to study the sites and dynamics of HSCs during embryogenesis: **1)** Transplantation of freshly isolated embryonic tissues[7,10,23,32] and **2)** transplantation of *ex vivo* cultured embryonic tissues[33-37]. Most studies that estimate numbers of HSCs during embryogenesis have utilized limiting dilution transplantation into

conditioned adult recipients[3,4,38,39,10,23,32,7-9,40]. However, it is important to note that larger numbers of functional HSCs are detected when conditioned neonates are used as recipients, likely because newly specified HSCs engage more efficiently with a developmentally immature BM niche relative to an adult BM niche[40-42]. Importantly, nascent HSCs not yet developmentally mature enough to successfully engraft the bone marrow niche and/or replenish a fully ablated hematopoietic system are not detected in these types of transplantation studies, even though they may ultimately mature into a cell that contributes to life-long blood production. Hence, any estimate of the initial clonal complexity of the HSC pool obtained by transplant are biased towards numbers of mature HSCs and likely undercount the total number of true HSCs produced during early ontogeny.

To overcome these limitations, Dr. Medvinsky‘s laboratory developed an elegant culture system that allows for the *ex vivo* maturation of E8.5-E11.5 HSC-precursors into transplantable HSCs[33-36,43,44]. Candidate HSC-precursors FACS sorted from dissociated tissues and reaggregated with or without OP9 stromal cells have been shown to specify transplantable HSCs *de novo* using this system[27-30,37]. This has allowed for **1)** a delineation of a maturation hierarchy of HSC-precursors and **2)** quantification of HSC-precursor numbers between E9.5-E10.5 when combined with limiting dilution transplantation studies[34]. Still, technical caveats associated with this strategy that may preclude precise estimates of HSC-precursor numbers and their dynamics must be considered. These include: the disruption of embryonic development and exposure of embryonic tissues to high [O_2], stress associated with enzymatic and physical tissue dissociation and exposure of cells to non-physiological stroma (*i.e.* OP9 stromal cells) and concentrations of growth

factors and cytokines. In short, any system based on *ex vivo* culture suffers from unanswerable concerns regarding the preservation of native development. Thus, although this system is an excellent *ex vivo* surrogate from which many important biological insights have been derived[33-36,43,44], estimates regarding HSC-precursor numbers and their dynamics are likely skewed. Thus, the need for systems to study unperturbed hematopoiesis.

Methodologies to study unperturbed hematopoiesis

To avoid transplantation and stresses that could affect native embryonic hematopoiesis, investigators have recently employed *in vivo* labeling of HSC precursors by means of multi-color-fluorescent systems similar to those employed to study neuronal circuits[37,45,46,47]. We took advantage of an inducible murine *Confetti* allele[37,46], while the Zon laboratory utilized the *Zebrabow* system[45], to track clonal diversity of the early hematopoietic system. Both systems allow for the labeling of pools of HSC-precursors with multiple distinct fluorophores. The Cre-recombinase (CRE)-responsive *Confetti* allele allows for inducible labeling of blood precursors at distinct developmental stages by crossing with tissue-specific CRE alleles (*e.g.* mesodermal precursors can be labeled using *Flk1-Cre*)[37]. Alternatively, unbiased labeling of blood precursors at discrete developmental stages can be achieved using ubiquitously expressed *ERT2-CRE* alleles (*e.g. Ubiquitin-ERT2-Cre*)[37]. The *Zebrabow* system allows one to induce labeling of a pool of cells with about 40 distinguishable fluorescent colors, while the random recombination of the *Confetti* allele renders only four colors[45]. Thus, in a liquid and polyclonal tissue like blood, the *Confetti* allele does not enable tracking of individual clones over time. Rather, using

mouse-to-mouse variance (MtMV) in the final distribution of *Confetti*-induced labeling, the gross clonal complexity of the blood can be assessed over time[37]. To estimate the number of blood precursors in zebrafish, drl:creERT2, which is active in early hematopoiesis, was used to induce *Zebrabow*-based color barcoding of HSCs and their progeny[45]. A hsp70l LASER-inducible method, previously employed to track microglial precursors, was also used to specifically label individual HSC precursors[45,48]. These models minimize disruption of embryonic hematopoiesis, score the number of HSCs present at a specific stages that realize their potential to contribute to adult blood and also capture the cumulative output of all blood progenitors to adult hematopoiesis. It would be very interesting to employ genetic-barcoding-technologies, like the Sleeping-Beauty system[49], PolyLox[50] or CRISPR-based molecular recording or scarring[51,52] to analyze clonal dynamics in embryonic hematopoiesis. The advantage of these technologies would also be their ability to track the contribution of individual embryonic clones to the different blood lineages during adult hematopoiesis.

An evolving model of embryonic clonal complexity: new questions and future challenges

Classic studies solely based on transplantation suggested that at E10.5 and E11.5 of murine development, <1 and 1-2 transplantable HSCs are detected, respectively [7-9]. Around E11, HSCs and HSC precursors migrate from the AGM and major arteries into the FL, where by E12.5 most of the transplantable activity is localized[7,23]. At this stage, about 60 HSCs are detected in the FL[7,10,23,32]. The FL has long been considered a niche supportive of dramatic HSC expansion because the number of transplantable FL HSCs expands to about

1000 by E14.5[7-9,10,23,32]. Although HSCs start their migration to the bone marrow (BM) at E15.5, very few transplantable HSCs are detectable in the BM even as late as E18.5 (≈5-10 HSC in the long bones)[3,4,38,39,53,54]. As HSCs migrate into the fetal BM, their numbers increase until a plateau is reached between days 21-27 post-birth (P21-P27), which is when HSCs are thought to acquire their characteristic adult quiescent state[38]. At this time point, the entire mouse contains about 20,000 HSCs (as the total number of BM cells is $\approx 2 \times 10^8$ and the frequency of HSC among them is 1 /10,000[55-60]).

The exact cellular mechanisms (specification, symmetric versus asymmetric cell division, apoptosis etc…) leading to these numbers at each developmental stage are not well understood. EHT is widely accepted as the mechanism via which HSCs first emerge in the AGM[3,4]. In this model, cell division accounts for the ≈100-fold expansion in HSC numbers between E11.5 and E15.5 in the FL, supporting the widely accepted view that the FL constitutes an expansion niche that promotes extensive HSC self-renewal[7-9,10,23,32]. Recently, employing explant-reaggregate-cultures paired with limiting dilution transplantation, Medvinsky's laboratory showed that the number of HSC precursors (pre-HSC) in the AGM region matures and expands between E9.5 to E11.5 to a total of about 60 pre-HSC, matching the total number of HSCs present in the FL at E12.5[34]. This suggests that this early burst of transplantable HSC activity in the FL is due to maturation rather than cell division. This further implies that the FL supports, at most, a 16-fold expansion in HSCs between E12.5-E15.5. Recent studies by ourselves and others, as detailed below, have challenged even these more modest expansion numbers, as a larger complexity in the

number of early blood forming clones was observed during the AGM stage of hemogenic specification (≈600)[37,45].

In zebrafish (physically much smaller than a mouse), the number of HSCs was originally studied *in vivo* by imaging of *Tg(cd41:eGFP)* and *Tg(Runx1+23:eGFP)* reporter embryos[11,24]. These studies revealed ≈2 HSCs at 48 hpf in the ventral dorsal aorta region (VDA, equivalent to the AGM region in mouse) and ≈5-10 HSCs at 80hpf in the CHT[11,24]. *Zebrabow*-based clonal fate mapping studies showed a more complex initial origin of the hematopoietic system. 21 pre-HSC clones were detected at 24 hpf, which is prior to HSC emergence, 28 HSC were scored at the peak of HSC budding from the endothelium and 34 HSC clones were seen by 72hpf[45]. The discrepancy in the numbers of HSCs detected by *Tg(cd41:eGFP)* and *Tg(Runx1+23:eGFP)* and the new clonal fate mapping strategies likely results from transgene variegation[11,24,45].

Similarly, our own studies suggest more complex clonal origins of the adult blood system in the mouse. We detected ≈600 mesodermal precursors (E7.5), ≈600 endothelial precursors (E8.5-E10.25) and ≈600 early HSCs (E11.5-E12.5) contributing to adult hematopoiesis using *Flk-1*-Cre, *VE-cadherin*-Cre and *Vav1*-Cre to induce *Confetti* labeling during these specific windows of HSC ontogeny[37]. Because *Vav1*-Cre saturates its labeling of hematopoietic cells by E12.5, it fails to capture any expansion of HSCs that may occur during later stages of FL hematopoiesis. Thus, these data suggest that up until E12.5, very little expansion occurs in the numbers of clones that contribute to life-long hematopoiesis. However, we recently used *Ubiquitin*-ERT2-Cre to induce *Confetti* labeling between E12.5

and E14.5 (M. Ganuza, unpublished data). In these studies, about 1800 clones were estimated to contribute to life-long hematopoiesis during this window. These numbers grossly match the number of HSCs detected by transplantation at E15.5 (≈1,000)[3,4,10]. These data further suggest that the FL HSC pool only expands about 3-fold between E12.5 and E15.5. Although these results challenge the classic model in which the FL supports a dramatic expansion of HSCs fated to contribute to adult blood, they are consistent with a model in which the FL is a site of HSC maturation. In this scenario, nascent HSCs migrate to the FL, where they mature into transplantable HSCs. This is also consistent with Medvinsky's findings that by E11.5 the number of HSC precursors in the AGM matches the number of transplantable E12.5 FL HSCs[34]. However, phenotypic FL-HSCs are clearly actively cycling[4,32,61-63]. To reconcile the apparent absence of a dramatic expansion during mid-gestation in the number of cells that contribute to adult blood and the cycling FL HSC compartment, we imagine two non-mutually exclusive developmental scenarios: **1)** the cell divisions of phenotypic FL-HSCs may be largely asymmetric, which would maintain a relatively constant pool of HSCs with life-long potential in the FL. Supporting this, cultured single mouse and human FL HSCs produce daughter cells which display an uneven distribution of proliferative potential and cell cycle properties, indicating a high functional heterogeneity amongst daughter cells[64,65]. Based on this work, Brummendorf and colleagues proposed the presence of a cell intrinsic control of stem cell fate[64]. **2)** It is also possible that only a sub-set of FL HSCs are capable of migrating and establishing themselves in the fetal BM-niche. This process might be at least in part cell-intrinsic, given the functional heterogeneity of the FL-HSC pool[64,65]. Non-cell autonomous mechanisms may also be in play, such as limited BM-niche space, which could collectively constitute a

developmental bottleneck that precludes some FL HSCs from realizing their potential to contribute to life-long hematopoiesis. It is interesting that during this phase the expression levels of CXC chemokine ligand 12 (CXCL12) are cell cycle dependent, with increased levels during S/G2/M phases, concomitant with an engraftment defect[38].

Further studies employing non-invasive methods will be required to fully describe the dynamics of HSCs during E12.5-P7 when HSCs reside in the FL. This will be essential to definitively establish if the generally accepted role of the FL during HSC ontogeny should be revised. Is the FL truly a niche in which HSCs capable of life-long hematopoiesis dramatically expand? Is it possible that the neonatal bone marrow supports more life-long HSC expansion than the FL? Definitive answers to these questions are essential to choosing the right developmental stage to search for novel signals that might support dramatic expansion of HSCs *ex vivo*.

Normal Aging

Transplantation models reveal HSC potency declines with age

The first efforts to identify the hematopoietic output of individual adult clones *in vivo* relied on labeling HSCs or HSPCs *in vitro* followed by transplantation[66-68]. HSCs labeled by retrovirus-mediated gene transfer were transplanted and recipient blood analyzed via Southern blot for the contribution of individual clones[68,69]. These studies identified "classes" of stem cells that could repopulate all hematopoietic lineages as well as stem cells appeared biased towards specific lineages[69]. Given that a majority of recipients were reconstituted by a small number of clones long-term post-transplant[68,69], it was proposed

that any specific lineage-restricted differentiation and/or heightened turnover of clones were due to mechanisms acting on the total pool of clones, rather than the activity of discrete "classes" of stem cells[68]. More sensitive methods emerged for detecting virally tagged stem cells, including solid-phase primer extension with ligation-mediated PCR[70], cellular barcoding[71-76] and vector integration site (VIS) analysis[77]. Using viral genetic barcoding coupled with high-throughput sequencing, Lu *et al.* observed that murine HSCs contributed minimally to mature blood cells, while committed progenitors tracked closely to their expected progeny[72]. Naik *et al.* used barcoding to propose a "graded commitment" model of murine hematopoiesis. In this model, most HSCs provide progenitors for all mature cell types, but the progenitors themselves—with a focus on lymphoid-primed multipotent progenitors (LMMPs)—display heterogeneity in their differentiation output[7]. Cellular barcoding has been used to visualize distinct differences in the clonal output of transplanted young and old murine HSCs. For instance, by assessing peripheral blood and HSC production in recipients, the old HSC pool appeared to be comprised of many clones with 'small' output (also defective in lymphoid potential) relative to the young HSC pool, which although appearing to contain fewer clones, these clones were typified by a 'large' output[74]. However, similar studies assessing the effects of aging on hematopoiesis in macaques did not detect lymphoid deficiency from aged HSPCs[78]. Using VIS analysis in a primate model of transplantation, Kim *et al.* observed clonal succession of HSCs, with the most persistent clones displaying heterogeneity in lineage output[77]. A more recent primate study that used a barcoding strategy presented a model in which HSPCs display initial and persistent heterogeneity in lineage output[76].

A part from viral tags, other methods for *in vivo* tracking of the clonal output of transplanted HSC clones have been applied to animal models[79,80]. Mathematical modeling and computer simulations based on murine transplantation data suggested that each individual HSC clone—as well as daughter HSC—has an intrinsically determined lifespan, which varies greatly between clones[79]. By observing the glucose 6-phosphate dehydrogenase (G6PD) phenotype post-transplant in female Safari cats coupled with computer modeling, Abkowitz *et al.* determined that clonal dominance of transplanted HSCs appears to be random and claimed that stochastic differentiation of clones may be responsible for the range of clonal hematopoietic patterns seen in other animal models[80]. In total, this work presents a landscape in which the most persistent HSC clones—possibly intrinsically defined—are the main drivers of heterogeneic lineage output via progenitor production, and the pool of potent clones declines with age. However, it is important to note that transplantation-based conclusions may be misleading due to random clonal dominance of transplanted HSCs.

In vivo *approaches suggest new models*

While numerous insights into the clonal behaviors of HSCs have come from transplantation studies, it is well-documented that transplantation can impose significant stress on HSCs (reviewed extensively in[81]). Therefore, studies of clonal hematopoiesis utilizing non-invasive *in vivo*-labeling methods can be informative for the study of steady-state hematopoiesis. Using a dox-inducible hyperactive Sleeping Beauty (HSB) transposase, Sun *et al.* labeled individual HSPCs during steady-state and tracked the clonal dynamics of native hematopoiesis[49]. They determined that, unlike transplantation

in which HSCs are the long-term contributors to hematopoiesis, native hematopoiesis is controlled by thousands of long-lived progenitor clones that are successively recruited throughout the murine lifespan[49]. Indeed, subsequent studies with the HSB system found that the pool of multipotent progenitor clones is hierarchically organized into uni- and oligolineage clones, with the lineage fate of HSCs during native hematopoiesis primarily megakaryocytic[82]. By genetically labelling Tie2$^+$ HSCs in murine bone marrow, it was similarly observed that adult hematopoiesis is primarily sustained by less primitive "short-term" HSCs and rarely receives input from more primitive HSCs[15]. However, other studies that also utilized non-invasive labeling methods support a more active role for HSCs in native hematopoiesis[83-86]. Induced labeling in *Pdzk1ip1*$^+$ adult murine HSCs—a serially transplantable HSC population less prone to mobilization—suggested that these cells give rise to other HSCs, as well as progenitors and mature blood cells and were the main drivers of native hematopoiesis[83,85,86]. Using the Polylox barcoding system in adult murine Tie2$^+$ HSPCs, Pei *et al.* reported that myeloid-erythroid and lymphocyte development appear to be bifurcated upstream, supporting a model in which HSCs and their progeny maintain a tiered hierarchy[17]. Multiple additional studies using *in vivo* labelling of HSCs have also suggested that adult HSCs actively contribute to steady-state hematopoiesis[85], and that they display a reduced ability to differentiate as they age[86]. Thus, there is currently no clear consensus on the contribution of HSCs to native hematopoiesis—at least in murine models.

To our knowledge, Ganuza *et al.* is the only current study to follow the clonal complexity of the hematopoietic system throughout the mammalian lifespan[87]. Here, the *Confetti*

reporter allele (as described in the previous section)[37] was utilized to label hematopoietic clones during embryogenesis and then track global clonal complexity from 2-26 months of age utilizing statistical modeling[87]. Clonal complexity in the peripheral blood (PB) dropped by 30% by 26 months of age. This drop was more dramatic in specific BM populations—including HSCs and multipotent progenitors (MPPs). This is likely a reflection of a reduced pool of HSC clones that have preserved their productive output with age. Interestingly, we also observed an expansion and contraction of clonal pools over the lifespan of individual mice, supporting a model of PB clonal instability and/or clonal succession during native hematopoiesis. Finally, the clonal complexity of HSCs mirrors that of MPPs throughout life, but diverges significantly from downstream HSPCs with aging, suggesting that apex HSCs consistently give rise to MPPs throughout aging, but that downstream progenitors behave more stochastically in terms of their lineage output with age[87].

Human HSC clonality in aging and disease

Attempts have also been made to assess clonal complexity during human hematopoiesis. Initial studies relied on X chromosome inactivation patterns in hematological malignancies and were focused on the clonal origins of leukemia[88,89]. For instance, Fialkow *et al*. assessed G6PD phenotypes in women to determine that chromic myelocytic leukemia CML was clonal in origin: single enzyme phenotypes were found in CML granulocytes but not in nonleukemic granulocytes[89]. More recent studies have relied on hematopoietic output from patients receiving lentiviral gene therapy via HSCT[90,91]. After autologous transplant of gene-corrected $CD34^+$ HSPCs in two X-linked

adrenoleukodystrophy (ALD) patients, integration site analysis showed that hematopoietic output from transplanted cells was polyclonal 24-30 months post-transplant[90]. In another thorough study tracking therapeutically manipulated HSPCs up to four years post-transplant in four Wiskott-Aldrich syndrome patients, it was found that hematopoietic reconstitution was initially sustained by short term HSPCs until ~6-12 months, at which point steady-state hematopoiesis was maintained by HSCs and MPPs[91]. This study also reported that 1,600-4,300 clones were responsible for production of all PB lineage cells, with clonal output higher in the lymphoid compartment compared to the myeloid compartment[91].

As stated earlier, transplantation is stressful for HSPCs[81], and hematopoietic clonal output during steady-state versus transplantation in animal models is highly debated[49,50,83,84]. Therefore, a thorough understanding of hematopoietic dynamics in humans should incorporate assays that are non-invasive and performed in healthy individuals. Catlin *et al*. analyzed the drift of the X-chromosome phenotype (via X-chromosome inactivation patterns) in >1,000 healthy females aged 18-100 years of age and determined that human HSCs replicate once every 40 weeks. Total numbers of HSCs appear to increase from birth until adolescence and plateau during adulthood[92]. By measuring telomere length distributions in granulocytes and lymphocytes from 365 healthy individuals aged 0-85 years, a similar increase in HSC clones in early life with a plateau during adulthood was observed, with a skewing in HSPC potential towards the myeloid lineage with age[93].

In a recent landmark study, Lee-Six *et al*. linked the clonal output of BM HSPCs to mature PB lineage cells in a healthy adult by assessing somatic mutations via whole-genome sequencing[94]. They confirmed that steady-state hematopoiesis in humans results from many clones that originate during embryonic development[49,50,84] and that 50,000-200,000 clones contribute to native hematopoiesis in adulthood[94] (similar dynamics as seen in mice by Ganuza *et al*.[87]). The authors also report the existence of hematopoietic clones with persistent myeloid and B-lymphocyte output throughout life, whereas the clonal dynamics of T-lymphocytes were less clear[94].

Together, these studies support a model of native hematopoiesis in humans in which clonal complexity (*i.e.* total number of clones) peaks during early adulthood and HSCs contribute consistently to the blood throughout life. Given the apparent plateau in total HSC clones during adulthood, the existence of age-associated hematopoietic maladies are not surprising and may reflect "renegade" clones overwhelming the aging blood system.

Effects of aging and disease on clonal complexity

CHIP and aging

Studies of X chromosome inactivation also provided the first suggestions that normal aging is associated with the expansion of HSC clones that harbor initiating driver mutations. Studies performed by the Gilliland laboratory in 1996 detected skewing of the normally random inactivation of the maternal or paternal X chromosome in blood samples from older women (> 3:1 in 40% of women over age 60), but a normal 1:1 ratio in newborn girls [95]. Nearly a decade later, next generation sequencing of

polymorphonuclear cells and buccal epithelial cells from elderly (> 65 years) women identified somatic *TET2* mutations in samples that also had clonal expansion by X-inactivation. *TET2* mutations were found in 5.6% of 180 subjects with age associated X-inactivation skewing, but were not found in 105 elderly subjects without X-inactivation skewing or in 96 subjects younger than 65 that had X-inactivation skewing[96]. A few years later, two large studies leveraged target capture and whole exome sequencing to evaluate for the presence of 65 – 160 leukemia-associated mutations in the blood of 12,000 and 17,000 presumably healthy adults[97,98]. Clonal populations of blood cells with leukemia-associated mutations were detected in blood samples at a mutant or variant allele frequency (VAF) as low as 2%. Both studies identified the epigenetic regulators, *DNMT3A*, *TET2*, and *ASXL1* as the most common (66 – 78% of identified mutations) somatic mutations in the blood of study participants. This expansion of hematopoietic stem cell clones with acquired driver mutations in myeloid leukemia-associated genes in individuals that lacked overt hematologic disease was termed clonal hematopoiesis of indeterminate potential, or CHIP[97]. Consistent with the Gilliland studies, the incidence of CHIP was found to increase significantly with age, with an incidence of 2.5 - 3% in individuals under age 40 and 15 – 20% in people over 70[98,99]. A large whole genome sequencing study puts the incidence of clonal hematopoiesis even higher. Zink et. al. identified clonal hematopoiesis in about 50% of individuals >85 years of age[100]. Intriguingly, the majority of individuals with clonal hematopoiesis in this study did not carry mutations in common leukemia-associated genes. Computational modeling of clonal hematopoiesis arising from “neutral drift” operating on a small, aging population of aging HSCs led the authors to conclude that clonal hematopoiesis may be an

'inevitable consequence' of aging. In each of these studies, the presence of a clone with or without a driver mutation was associated with an increased risk of developing a hematologic malignancy.[97,98,100] CHIP is now considered a pre-cancerous state carrying a 0.5 – 1% risk of leukemic conversion per year. CHIP clones with driver mutations were also unexpectedly found to be associated with an increased risk of coronary heart disease and ischemic stroke [97].

Subsequent studies have shown that more sensitive sequencing techniques can identify CHIP mutations in the blood of young, apparently healthy adults.[99,101] Two separate groups have demonstrated that ultra-deep targeted sequencing can reliably detect CHIP clones with a VAF as low as 0.8% in the peripheral blood. In addition to showing an exponential increase in the prevalence of CHIP mutations with age, from 2.5% in the 20 – 29 age group to over 20% in the 60 – 69 age group[99], both studies also identified age restrictions associated with a subset of genes. Mutations in *DNMT3A* and *JAK2* were identified in individuals as young as 22, however, cancer-associated mutations in the RNA splicing factors *SF3B1* and *SRSF2* were only identified in subjects over age 70.[99,101] These findings suggest that there are likely age-associated changes to the bone marrow niche that facilitate the selection and expansion of different mutant HSC clones.

CHIP in the absence of aging

Although CHIP was initially defined as a feature of the aging hematopoietic system, more recent studies have identified additional hematopoietic stressors that can increase the selection and expansion of CHIP clones in "younger" adults. Studies of adults

undergoing treatment with cytotoxic chemotherapy for solid and hematologic malignancies have demonstrated a higher prevalence of CHIP and more mutations per patient compared to the general population[102-104]. Sequencing of 401 bone marrow samples from patients with non-Hodgkin lymphoma that received cytotoxic chemotherapy prior to autologous stem cell transplantation (SCT) identified at least one CHIP-associated mutation in 29.9% of patients[102]. In this study, the prevalence of CHIP was 10% in patients aged 30 to 39 and as high as 40% in patients over 60. As in age-associated CHIP, 66% of patients that developed a secondary myeloid neoplasm had a detectable CHIP clone prior to SCT and patients with CHIP had a significantly inferior overall survival rate (30.4% vs 60.9%) ten years after autologous SCT. This difference was driven primarily by an increased risk of death from ischemic stroke or cardiovascular disease. At least one CHIP mutation was also identified in 25.1% of 8,810 patients with solid tumors, with a similar increase in prevalence in younger patients. Although CHIP was also associated with a statistically significant inferior overall survival in this study, the most common cause of death in these patients was disease progression. This is likely due to the aggressive nature of their primary cancers. Both studies identified a difference in the affected CHIP genes in patients that received chemotherapy, suggesting that selective pressures other than normal aging can influence clonal hematopoiesis. Mutations in the p53 pathway genes, *PPM1D* and *TP53*, were frequently identified in addition to mutations in *DNMT3A*, *TET2*, and *ASXL1* in these patient populations.[102,103]

Therapy-related CHIP

The effect of different cellular stressors on clonal hematopoiesis was further evaluated in a cohort of 119 patients with lymphoma or multiple myeloma[104]. Error-corrected next generation sequencing demonstrated a higher incidence of CHIP in 81 patients that received cytotoxic chemotherapy compared to 38 patients that did not receive chemotherapy. Consistent with the initial mutation developing in an HSC, CHIP mutations were detected in sorted myeloid (monocytes) and lymphoid (T cells) cells. Although the incidence was lower, mutations in *DNMT3A* or *TET2* could be identified in aged-matched "healthy" donors as well as patients with hematologic malignancies, however, CHIP clones with *TP53* or *PPM1D* mutations appeared to have a fitness advantage and were enriched in patients that received cytotoxic chemotherapy. In addition to the replicative stress induced by chemotherapy, autologous SCT also alters the bone marrow microenvironment, which may further select for HSCs that have acquired a competitive advantage. CHIP clones were followed for 6 – 12 months after autologous SCT in 40 of the lymphoma patients. Most CHIP clones engrafted with a VAF similar to the pre-SCT sample, however 33% of *DNMT3A* CHIP clones expanded more than 2-fold after engraftment and 30% of *PPM1D* CHIP clones decreased after SCT. Targeted next generation sequencing of 500 "healthy" related SCT donors age ≥ 55 identified CHIP clones in 16% of donors for allogeneic SCT.[105] Not unexpectedly, *DNMT3A, TET2*, and *ASXL1* were the most frequently mutated genes. Donor-derived CHIP was associated with an increased risk of chronic graft vs host disease and serial sequencing of 22 recipients with donor-derived CHIP revealed clonal expansion (11 recipients) or stable engraftment (10 recipients) of the mutant clones. Notably, the recipient's leukemia relapsed in half of the patients with linear expansion and only 2 recipients with expanding

clones developed donor-cell leukemia notable for clonal evolution and the acquisition of additional mutations in myeloid leukemia genes. In a smaller study of donor-engrafted CHIP in allogeneic SCT recipients, *DNMT3A* mutations were identified in 5 of the 6 SCT recipients with unexplained cytopenias.[106] These studies suggest that the higher incidence of CHIP in younger patients undergoing cancer treatment may be due to the selection of HSC clones with CHIP mutations that confer a growth advantage in response to different cellular stressors.

Inflammatory stress and CHIP

Hematopoietic stress caused by chronic inflammation has also been shown to have distinct effects on clonal dynamics. Aplastic anemia is an acquired autoimmune disorder that results in the immune mediated destruction of bone marrow HSCs. Clonal patterns of X-inactivation have been noted in female patients with aplastic anemia.[107] The risk of developing MDS or AML in aplastic anemia patients treated with immunosuppressive therapy has been shown to be as high as 15% and independent of treatment with growth factors such as G-CSF[108,109]. Targeted deep sequencing of peripheral blood identified CHIP mutations in 36% of a cohort of 439 patients aged 2 – 88 years with moderate to severe aplastic anemia.[110] Although the prevalence of mutations increased with age, mutations in *BCOR* or *BCORL1* and *PIGA* were more common than mutations in the age-associated CHIP genes, *DNMT3A* and *ASXL1*. Mutations in these 5 genes accounted for 77% of identified CHIP mutations in aplastic anemia and the underrepresentation of *TET2*, *JAK2*, and *TP53* suggests a different mechanism of clonal selection occurs in an aplastic bone marrow microenvironment. Serial sampling found *BCOR*, *BCORL1*, and

513 *PIGA* mutant clones decreased or remained stable over time. These mutations were
514 independently associated with a better response to immunosuppressive therapy and
515 higher overall and progression-free survival. In contrast, *DNMT3A* and *ASXL1* mutant
516 clones tended to increase in size over time and were associated with worse outcome.
517 However, clonal dynamics as whole were highly variable and did not consistently predict
518 response to immunosuppressive therapy or long-term survival among individual patients

519

520 Stress hematopoiesis, inflammation, and chronic immune stimulation leading to the
521 selection of HSC clones with CHIP mutations may also explain the increased risk of
522 myeloid malignancies identified in patients with autoimmune diseases. Distinct from
523 lymphoproliferative disorders, secondary leukemias that develop in patients with
524 autoimmune diseases are far more likely to be myeloid rather than lymphoid in nature.
525 Two large population based studies have identified a 1.2 to 1.7 fold higher risk of
526 developing acute myeloid leukemia (AML) and a 1.5 to 2.1 fold higher risk of developing
527 myelodysplastic syndrome (MDS) in patients with autoimmune disease compared to the
528 general population[111,112]. This increase in AML and MDS risk was initially thought to be
529 secondary to the cytotoxic treatments used to treat autoimmune diseases, however
530 numerous studies have since shown that leukemia risk is not associated with specific
531 treatments with the exception of cyclophosphamide, azathioprine, and mitoxantrone.
532 (reviewed extensively in [113]) The Engels study compared 13,486 patients over age 67
533 with myeloid malignancies to 160,086 population-based matched controls and identified
534 several systemic autoimmune diseases, namely rheumatoid arthritis (RA), systemic lupus
535 erythematosus (SLE), polymyalgia rheumatica, autoimmune hemolytic anemia, systemic

vasculitis, ulcerative colitis, and pernicious anemia, that were associated with a significantly increased risk of AML (odds ratio 1.57 to 6.23). RA and pernicious anemia patients also had a significantly increased risk of MDS (odds ratio 1.52 – 2.38)[111].

Smaller studies have shown that the risk of developing a myeloid neoplasm is associated with chronicity and severity of the autoimmune disease[114]. This could be consistent with the expansion of a CHIP clone that acquires additional mutations. Two recent studies have examined the incidence of CHIP in patients with autoimmune disorders. One study of 59 patients with RA (mean age 54.7 years) identified CHIP clones in 17% of participants, with a prevalence of 25% in patients over 70. They note that this is not significantly different from the incidence of CHIP in the general population and found mutations in *DNMT3A*, *TET2*, and *ASXL1* to be the most common[115]. A slightly larger study of 112 patients with antineutrophil cytoplasmic antibodies (ANCA)-associated autoimmune vasculitis aged 18 to 84 identified CHIP in 30.4% of subjects; a much higher prevalence than an age matched cohort[116]. The youngest participant with CHIP was 18 years old, however the median age of patients with CHIP was higher than CHIP negative patients (70.5 compared to 63.0 years). Mutations in *DNMT3A*, *TET2*, and *ASXL1* were found in 63% of CHIP positive participants. Clone size was serially followed, range 3 to 100 months, in 19 participants with available samples. Most clones (12) remained stable and 5 clones increased in size. No differences in peripheral blood counts or cardiovascular disease incidence were noted between CHIP positive and negative subjects.

559 Taken together, the deep sequencing studies described above have demonstrated that
560 cellular stressors associated with aging, chemotherapy, and inflammation can lead to the
561 clonal selection of HSCs with mutations in myeloid malignancy genes that confer an
562 increased risk of MDS/AML or cardiovascular disease. Chemotherapy and inflammatory
563 bone marrow stress from conditions such as aplastic anemia strongly select for specific
564 mutations (*PPM1D*, *TP53*, *BCOR*, *BCORL1*, *PIGA*) in addition to the genes commonly
565 mutated during aging (*DNMT3A*, *TET2*, and *ASXL1*). Of note, clonal hematopoiesis due
566 to revertant somatic mosaicism, spontaneous correction of a pathogenic allele, has also
567 been identified as a mechanism to partially restore normal hematopoiesis in several
568 hypocellular bone marrow failure syndromes, including aplastic anemia.[117] PCR analysis
569 of *FANCA* exon 29 revealed the absence of a maternal mutation in multiple mature blood
570 cell lineages and HSPCs in a patient with Fanconi Anemia, although the mutation was
571 present in fibroblasts.[118] Single nucleotide polymorphism (SNP)-array based analysis of
572 peripheral blood from 306 patients with aplastic anemia identified copy number-neutral
573 loss of heterozygosity at chromosome 6p (6pLOH), leading to loss of one HLA haplotype
574 and presumably escape from cytotoxic T cell recognition, in 40 patients (13%).[117] SNP-
575 array analysis also identified spontaneous correction of an autosomal dominant
576 pathologic *TERC* mutation in multiple blood lineages, but not in other tissues, in six
577 individuals from four families with dyskeratosis congenita.[119] More recently, targeted
578 deep sequencing identified acquired stop-gain mutations in two patients with *SAMD9L*-
579 mutant familial MDS. These patients had a transient monosomy 7 clone that was replaced
580 by a large somatic clone with paternal 7q uniparental disomy.[120] Whereas normal
581 hematopoiesis and thus incomplete penetrance is one of the main findings in patients with

582 revertant SAMD9L or TERC mutations,[119,120] the patient with Fanconi Anemia also
583 developed clones with leukemia driver mutations in non-revertant cells,[118] and 6pLOH
584 HSCs cannot repopulate the bone marrow of aplastic anemia patients without
585 immunosuppressive therapy, which also targets the inflamed bone marrow
586 microenvironment.[117] These studies again highlight how inflammation can lead to the
587 expansion of HSCs clones with pathologic mutations.

588

589 *Modeling CHIP in animals*

590 Mouse models of CHIP mutations commonly identified in humans have been used to gain
591 further insight into the pathophysiology of CHIP and evaluate potential treatments.
592 Mutations in *DNMT3A*, *TET2*, *ASXL1*, and *JAK2* were commonly identified in four case-
593 control studies of a total of 4276 older individuals (age 60 to 70) with cardiovascular
594 disease compared to 3529 controls)[121]. Two independent groups used *Tet2*-deficient
595 (*Tet2*$^{-/-}$) murine HSCs to study the effect of the second most commonly mutated gene in
596 CHIP on coronary heart disease[121,122]. Fuster *et. al.* transplanted lethally irradiated
597 atherosclerosis prone, low-density lipoprotein receptor-deficient (*Ldlr*$^{-/-}$) mice with 10%
598 *Tet2*$^{-/-}$ CD45.2^{+} HSPCs and 90% wild type (*Tet2*$^{+/+}$) CD45.1^{+} HSPCs and then fed the
599 recipient mice a high-fat/high-cholesterol diet to promote atherosclerosis development.
600 As expected, clonal expansion of *Tet2*$^{-/-}$ HSPCs, indicated by peripheral blood CD45.2
601 chimerism, increased from 28% CD45.2^{+} cells at 4 weeks post-transplant to 56%
602 CD45.2^{+} cells at 12 weeks post-transplant without an effect on blood cell counts. Notably,
603 plaques in the aortic root of *Tet2*$^{-/-}$ HSPC recipients 12 weeks after SCT were 60% larger
604 than the plaques in wild type controls. Analysis of aortic wall immune cells was notable

for clonal expansion of immune cells with 62% CD45.2^{+} macrophages. Jaiswal *et. al.* made similar findings and both groups demonstrated that *Tet2*$^{-/-}$ macrophages aberrantly overexpress proinflammatory cytokines[121,122] Fuster *et. al.* went on to show that an inhibitor of the NLRP3 inflammasome (MCC950) decreased interleukin-1β production by TET2-deficient macrophages and that MCC950 decreased atherosclerotic plaque size in *Tet2*$^{-/-}$ HSPC recipients by ~50% without affecting the expansion of TET2-deficient cells.[122]

Conclusions

HSC clonal dynamics after development vary greatly in response to aging and disease. Targeted sequencing has identified CHIP mutations in several different contexts, however additional studies are needed to define the genes commonly mutated in autoimmune disorders such as SLE, where CHIP clones may affect morbidity and mortality independently of the primary disease, and other conditions associated with chronic systemic inflammation such as type 2 diabetes or sickle cell disease. Targeted sequencing provides data at discrete time points and further work with animal models of CHIP mutations and chronic inflammation will be needed to improve our understanding of HSC clonal dynamics in response to disease and inform how these clones should be monitored and/or treated.

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