

# From haematopoietic stem cells to complex differentiation landscapes

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The development of mature blood cells from haematopoietic stem cells has long served as a model for stem-cell research, with the haematopoietic differentiation tree being widely used as a model for the maintenance of hierarchically organized tissues. Recent results and new technologies have challenged the demarcations between stem and progenitor cell populations, the timing of cell-fate choices and the contribution of stem and multipotent progenitor cells to the maintenance of steady-state blood production. These evolving views of haematopoiesis have broad implications for our understanding of the functions of adult stem cells, as well as the development of new therapies for malignant and non-malignant haematopoietic diseases.

When Ernst Haeckel first used the word stem cell ('Stammzelle') in 1868, as a Darwinist he used it to refer to the primordial unicellular organism from which all multicellular life descended<sup>1</sup>. This stem cell therefore sat at the root of a branching family tree, incidentally called a stem tree in German ('Stammbaum', meaning a tree that shows where things stem from). Shortly thereafter, Haeckel's biogenetic law, in which ontogeny recapitulates phylogeny, prompted him to use the stem-cell term to describe the fertilized egg. Histopathologists subsequently applied this stem-cell concept to normal and leukaemic haematopoiesis, putting forward the concept of a common progenitor of red and white blood cells<sup>2</sup> as well as a common precursor of myeloid and lymphoid leukaemic cells<sup>3</sup>. From the very beginning, the stem-cell concept has thus been framed into a tree-like model, in which multipotent stem cells give rise to their progeny through an ordered series of branching steps.

The first *in vivo* assay for stem-cell function was based on the rescue of lethal irradiation by bone marrow transplantation<sup>4</sup>, followed by the first estimation of stem-cell numbers by counting haematopoietic colonies in the spleens of transplanted mice. This not only provided an estimate of the frequency of spleen colony-forming units at 1 in 10,000 bone marrow cells<sup>5</sup>, but also delivered the first definitive proof for *in vivo* multipotent progenitor cell function based on tracking cytogenetic abnormalities within individual spleen colony-forming units<sup>6</sup>. Fluorescence-activated cell sorting subsequently facilitated the purification of transplantable haematopoietic stem cells (HSCs), with a landmark 1988 publication<sup>7</sup> that demonstrated the use of positive and negative selection. HSCs have historically been defined on the basis of two essential properties: self-renewal and multipotency. Operationally, this is tested via transplantation experiments. By contrast, progenitors are defined by the absence of extended self-renewal and a restricted lineage differentiation capacity (most often bi- or unilineage), so that they are usually lost within the first 2–3 weeks after transplantation<sup>8</sup>.

Around the year 2000, the characterization of progenitor populations downstream of HSCs resulted in a model of the haematopoietic differentiation tree that is still shown in many textbooks today (Fig. 1a). In this model, the first branch point segregates lymphoid potential from all other lineages (myeloid, erythroid and megakaryocytic), followed by several further branching steps on either side of the tree progressing from multi- to bi- and finally to unipotent progenitor cells. The subsequent introduction of other surface markers suggested several modifications of this classical tree, including lymphoid and myeloid fates

remaining associated until further down the tree<sup>8–10</sup>, early megakaryocyte branching<sup>11,12</sup> as well as subdivision of the multipotent progenitor compartment into distinct subpopulations<sup>13,14</sup> (Fig. 1b). Moreover, the picture is further complicated because the HSC pool itself is functionally and molecularly heterogeneous<sup>11,12,15–20</sup>. These studies are most advanced in the mouse system, in which we now have what may seem a bewildering number of different structures for the haematopoietic tree. Although it is likely that all these structures capture true aspects of HSC differentiation, collectively they would be difficult to squeeze into a single, rigid branching tree. New ways of not only thinking about, but also graphically representing the process of HSC differentiation are thus required. In this Review, we illustrate how new technologies are challenging the classical view of the haematopoietic hierarchy as a highly compartmentalised and stable structure. The emerging picture is one of a collection of heterogeneous populations organized hierarchically, with gradual progression from one to the next, and which remains highly flexible to meet the changing needs of blood demand.

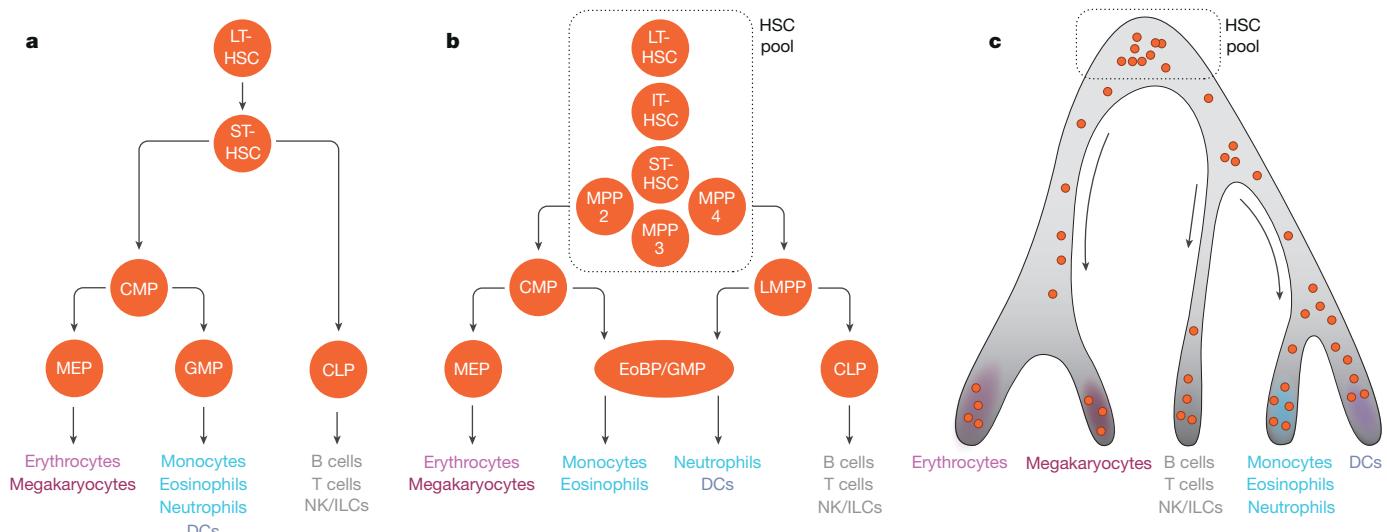
## Stem cell and progenitor boundaries

With self-renewal and multipotency at the heart of what defines an HSC, much research has been invested into understanding the underlying cellular and molecular processes.

## Defining the HSC state

At the cellular level, switching off self-renewal coincides with the turning on of lineage programs. It thus seemed plausible that this would also be true at the molecular level, and the concept of multilineage priming was proposed early on as a possible underlying molecular mechanism by which HSCs maintain multipotentiality<sup>21</sup> (Box 1). However, the advent of genomic technologies<sup>14,22–25</sup> coupled with mouse genetic studies has demonstrated that the HSC transcriptional programme is defined by a collection of unique metabolic and cellular properties, which are not intuitively linked directly with multipotency. Approximately 70% of all expression changes between HSCs and early progenitors occur independently of lineage choice<sup>24</sup>, with a similar dichotomy also at the levels of methylation<sup>26–29</sup> and chromatin accessibility<sup>30</sup>. Correspondingly, HSCs reside in a quiescent<sup>19,20,31,32</sup>, autophagy-dependent<sup>33,34</sup> and glycolytic<sup>35,36</sup> state marked by low mitochondrial activity<sup>37,38</sup> and tightly controlled levels of protein synthesis, below those of most other haematopoietic cell types<sup>39</sup>. Stem-cell-specific stress response and quality-control mechanisms allow the preservation of the integrity of the HSC compartment

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**Figure 1 | Timeline of hierarchical models of haematopoiesis.**

**a**, Visualization based on cutting-edge research around the year 2000: HSCs are represented as a homogeneous population, downstream of which the first lineage bifurcation separates the myeloid and lymphoid branches via the common myeloid progenitor (CLP) and common lymphoid progenitor (CLP) populations. DCs, dendritic cells; EoBP, eosinophil–basophil progenitor; GMP, granulocyte–monocyte progenitors; LT, long-term; ILCs, innate lymphoid cells; MEP, megakaryocyte–erythrocyte progenitors; NK, natural killer cells; ST, short-term. **b**, During the years

2005–2015, this visualization incorporates new findings: the HSC pool is now accepted to be more heterogeneous both in terms of self-renewal (vertical axis) and differentiation properties (horizontal axis), the myeloid and lymphoid branches remain associated further down in the hierarchy via the lymphoid-primed multipotential progenitor (LMPP) population, the GMP compartment is shown to be fairly heterogeneous<sup>142</sup>. **c**, From 2016 onwards, single-cell transcriptomic snapshots indicate a continuum of differentiation. Each red dot represents a single cell and its localization along a differentiation trajectory.

after exposure to DNA<sup>40,41</sup> and protein damage<sup>42,43</sup> or metabolic stress<sup>33,34</sup>. By contrast, progenitors are highly proliferative and metabolically active cells that are dependent on oxidative metabolism and mitochondrial function.

Importantly, so-called HSC-specific characteristics are not absolute: HSCs occasionally divide during homeostasis and get activated in response to stress, and therefore transiently pass through a proliferative state. It is also worth noting that early lymphoid progenitors (mouse lymphoid-primed multipotential progenitors<sup>9</sup> or human myeloid-lymphoid progenitors<sup>8</sup>) share common transcription networks with HSCs<sup>24</sup>, which in the former push towards B cell differentiation<sup>24</sup> while in the latter inhibit self-renewal<sup>44,45</sup>. Although the induction of lineage-specific transcriptional programs may occur largely independently of the loss of stem-cell characteristics, regulators such as RUNX1 can be involved in both<sup>46</sup>, suggesting that much remains to be learned about how lineage decisions are coordinated with changes of cellular state.

### HSC self-renewal and cell cycle interplay

Whether the variability in HSC outputs is an intrinsic system property, a reflection of stochastic behaviour, environmental influences, technically related variability or a combination of all of these, has been and still is a subject of debate. Nonetheless, in the past 10–15 years, this heterogeneity in behaviour has been notably formalized, and it is now accepted that HSCs are heterogeneous in terms of durability of engraftment upon transplantation, cell cycle properties and differentiation (Table 1).

HSCs first of all differ in the degree to which they self-renew, equating to the number of symmetric divisions that an HSC can make over its lifetime. Operationally, the field now widely accepts that, both in mouse and human, HSCs that repopulate in transplantation assays for more than 16 weeks in a primary transplantation and at least in a second round of transplantation are considered to be long-term HSCs<sup>17,47–50</sup>. If cells can produce all differentiated cell types and engraft transiently in primary (and in some cases secondary) transplants, they are referred to as intermediate HSCs<sup>16</sup>, short-term HSCs or multipotent progenitors (MPPs)<sup>13,14,47</sup>, depending on the length and robustness of the graft produced.

Heterogeneity in self-renewal capacity seems to be directly correlated to the time HSCs spend in quiescence. Label-retaining studies have demonstrated that the most dormant HSCs (which retain the label over

months at the steady-state) display the most robust and longest repopulation capacity<sup>19,20,51–53</sup>. Two cell cycle parameters are inversely correlated with repopulation capacity: the frequency of division (length of interval between divisions), but also the time a single HSC takes to exit quiescence *in vitro*<sup>16,54</sup>. Of note, the level of pre-existent CDK6 mRNA and CDK6 protein in quiescent HSCs directly determines the kinetics of quiescence exit<sup>54</sup>, and may thus serve as a marker of the quiescent state<sup>32,54</sup> (less CDK6 corresponds to higher dormancy). Dormancy is also associated with high levels of vitamin A metabolism via retinoic acid signalling<sup>32</sup>, high levels of p57<sup>32,55</sup>, low levels of protein synthesis<sup>32</sup>, low MYC activity<sup>32,56</sup>, and may exist as a continuum of quiescent states between the most dormant HSCs and their activated counterparts<sup>32</sup>. Although dormant HSCs forced into activation by stress signals can return to dormancy<sup>19,51</sup>, Bernitz *et al.* estimated that four divisions in adulthood are sufficient for irreversible loss of self-renewal<sup>53</sup>.

Several label-retaining assays have been developed to allow the isolation of HSCs based on their division history<sup>19,20,32,51–53,57</sup>. A major limitation of all label-retaining studies so far is that the rate of symmetric divisions is inferred or indirectly measured at the bulk level. However, direct experimental measurement of asymmetric versus symmetric label-diluting divisions will be required to understand the dynamics at play within the HSC compartment. New integrative tools, most likely at the single-cell level, will have to be developed to address this challenge. Nonetheless, it is already apparent that distinct cell cycle properties within the HSC pool are intimately linked to HSC function. Coupled with a wide range in division frequencies (depending on the HSC subset, from once a month to twice a year in mouse<sup>19,51,53,58</sup>), this means that there will be substantial variation in the contribution of distinct HSC subsets to blood formation.

### Heterogeneity in HSC lineage output

The capacity to give rise to all differentiated blood cell types is a fundamental aspect of what constitutes an HSC. It is, however, now accepted that there are distinct differentiation behaviours within the HSC and MPP<sup>13,14</sup> compartments (Table 1, Fig. 1b). Using limiting-dilution analysis and single-cell transplantation, the Müller–Sieburg and Eaves groups described HSCs that differ in their relative myeloid and lymphoid

## BOX 1

## Mechanistic underpinnings of cell-fate choice

Cell-fate decisions entail a choice between alternative gene expression programs, commonly executed by transcriptional and epigenetic regulators in response to extracellular signals. Transcription factors bind to specific sequence motifs within promoter, enhancer and silencer regions. Cell-type-specific expression is achieved through combinatorial transcription factor interactions, which form key building blocks of wider regulatory networks. Transcription factor complexes recruit epigenetic regulators to modulate the activation status of a gene locus, which can be transmitted to subsequent cell generations as ‘epigenetic memory’. Importantly, a progenitor cell can become primed by opening up regulatory elements associated with genes that drive differentiation down a specific mature lineage<sup>144</sup>.

Multipotent cells are thought to exhibit multilineage priming, which entails simultaneous, low-level activation of expression programs for alternative lineages. Lineage choice then constitutes one program ‘winning out’ while the alternative program is extinguished. Cross-antagonism between pairs of lineage-determining transcription factors represents an attractive mechanistic model, which initially focused on the erythroid regulator GATA1 and the myeloid regulator PU.1. More recent single-cell time-lapse imaging, however, questioned whether an erythroid/myeloid fate choice is indeed driven primarily by cross-antagonism between GATA1 and PU.1<sup>145</sup>. Evidence for lineage priming and its resolution by cross-antagonism has been reported at single-cell resolution for other transcription factor pairs, such as the neutrophil or macrophage fate choice that is controlled by GFI1 and IRF8<sup>89</sup>.

Both instructive and stochastic models have been proposed as mechanisms that trigger the upregulation of one lineage program over another. Stochastic here generally refers to random, unequal distribution of molecules after cell division. Instructive models posit that low-level expression of a cytokine receptor is sufficient for a cell to be responsive to external signals, as shown for several myeloid cytokines<sup>146</sup>. Autoregulation and feed-forward loops also have important roles in cell-fate choice decisions. For example, positive autoregulation of GATA1 stabilizes erythroid fate<sup>147</sup>, positive autoregulation of PU.1 stabilizes myeloid identity<sup>148</sup>, and the highly connected triad of GATA2, TAL1 (also known as SCL) and FLI1 is thought to stabilize the stem/progenitor state<sup>149,150</sup>. In feed-forward loops, an upstream regulator induces its target directly as well as through an intermediate regulator<sup>151</sup>. Feed-forward motifs can filter out transient signals, and when coupled with autoregulation, can generate forward momentum.

suggests that the overall picture may be even more complex, with distinct metabolic needs<sup>66</sup> and clonal expansion capacity of HSCs. There are examples in which HSCs with high clonal expansion capacity generate subsets with lower output<sup>67</sup>, but also cases in which HSCs with very modest clonal expansion in the first transplantation, generate the most robust grafts upon serial transplantation<sup>68,69</sup>.

### Rethinking blood lineage relationships

Several recent studies at the single-cell level have questioned the routes by which lineage differentiation occurs.

### Single-cell assays to study potential

Cell-fate decisions (Box 1) are executed at the level of individual single cells. To understand their regulation, it is therefore imperative that both the biological assays testing their cellular function as well as the biochemical assays examining their molecular profiles are performed at single-cell resolution. Although most advanced in the mouse, *in vivo* transplantation assays have been and remain fundamental for our understanding of HSC biology, as the only assay that can test for HSC self-renewal. Because of the suboptimal support of human cells from the mouse microenvironment, xenotransplants cannot robustly read-out all possible differentiation routes, particularly not at the single-cell level. The last 10 years have therefore seen a collective effort in defining the lineage potential of single human progenitor cells by using highly defined *in vitro* models that can support the differentiation of most mature blood cell types. Work from the Dick and Vyas groups, together with studies in mice from the Jacobsen group, suggested that the first restriction in lineage potential does not segregate lymphoid and myeloid potential as postulated by the common myeloid and lymphoid progenitor models (Fig. 1a), but that these potentials remain coupled in the lymphoid-primed multipotential progenitor<sup>9,10</sup> and myeloid-lymphoid progenitor<sup>8</sup> compartments (Fig. 1b). Many other subpopulations, most with either bi- or unilineage capacity, have since been found in both the lympho-myeloid branch<sup>70</sup> and the myelo-erythroid-megakaryocytic branch<sup>71–75</sup>. Altogether, it seems that few single cells read-out as multipotential within the progenitor compartment. There are, however, some caveats with such interpretations, because strong instructive signals provided by the *in vitro* cultures, or potentially high stress levels that HSCs are exposed to during single-cell transplants may promote unilineage output. Moreover, a given cell may be bipotential based on its molecular state, but if it makes a lineage choice before dividing, it will read-out as unipotent in functional assays. The evidence so far suggests that lineage choice occurs earlier than previously thought, and as recently shown for dendritic cells<sup>76</sup>, probably as early as within the phenotypic HSC populations.

### Single-cell transcriptional landscapes

Single-cell expression analysis of RNA was first reported over 25 years ago<sup>77</sup>, but remained low throughput in the number of genes and cells until microfluidic approaches were introduced. These quickly prompted studies that reported the expression of dozens of genes in hundreds of single HSCs and haematopoietic stem and progenitor cells (HSPCs)<sup>78</sup>, and provided new insights into core regulatory circuits<sup>79</sup>, new progenitor populations<sup>72,73</sup>, cellular hierarchies in normal and transformed haematopoiesis<sup>80</sup>, dissociation between self-renewal potential and activation of lineage programs<sup>81</sup>, and the molecular overlap between HSC populations purified with four different cell-sorting strategies<sup>82</sup>. It is not practical to assay more than 200 genes per single cell with PCR-based methods, and these genes need to be predefined, thus limiting the scope for new discoveries. A real breakthrough was therefore provided by technical innovations, which now make it possible to perform transcriptome-wide RNA sequencing (RNA-seq) in thousands of single cells.

Following a landmark paper reporting the transcriptomes for more than 2,600 mouse single myelo-erythroid progenitor cells<sup>83</sup>, a subsequent report of 1,600 transcriptomes ranging from true long-term HSCs to progenitors of all major lineages focused on generic stem-cell functions such as metabolic and cell cycle status<sup>84</sup>. Several algorithms have been

output<sup>15,59</sup>. More recently, the Jacobsen and Nakauchi groups identified HSCs that predominantly differentiate towards megakaryocytes and platelets (platelet-biased)<sup>11,12</sup>. Subsequent transplantation experiments have highlighted that single HSCs execute only a limited repertoire of lineage fates patterns, and the only long-term unilineage read-out observed in this study was that of platelets<sup>60</sup>. There are limitations to single-cell transplantation, particularly as very low contributions to certain lineages may be missed. Moreover, a cell with unilineage read-out may have the potential to give rise to other lineages in a durable fashion in other conditions. HSCs with platelet-biased output in unperturbed haematopoiesis acquired broader potential after transplantation<sup>61</sup>, and similarly HSCs with platelet-biased output after transplantation could produce myeloid and lymphoid cells *in vitro*<sup>60</sup>. Finally, both platelet-biased HSCs and long-lived platelet progenitors<sup>11,62,63</sup> may coexist and be difficult to distinguish.

Importantly, HSC heterogeneity is not simply stochastic, as it can be propagated by serial transplantation<sup>15,18,64,65</sup>, indicating intrinsic programming, the molecular basis for which remains unclear. As discussed later, these findings have important conceptual implications, because they question at what cellular stage lineage choices occur. Recent evidence

**Table 1 | Phenotypic or functionally defined HSC subsets**

Species	Name	Cell-surface phenotype	Self-renewal	Cell cycle properties	Differentiation	References
Mouse	LT-HSC	Lin <sup>-</sup> Sca1 <sup>+</sup> cKit <sup>+</sup> CD34 <sup>-</sup> CD150 <sup>+</sup> CD135 <sup>-</sup> CD48 <sup>-</sup> ± EPCR <sup>+</sup> ± Rho <sup>lo</sup>	High			17, 47, 48, 141
	IT-HSC	Lin <sup>-</sup> Sca1 <sup>+</sup> cKit <sup>+</sup> CD34 <sup>lo</sup> CD135 <sup>-</sup> Rho <sup>o</sup> CD49b <sup>hi</sup>	Intermediate	Short G <sub>0</sub> exit		16
	ST-HSC/MPP1	Lin <sup>-</sup> Sca1 <sup>+</sup> cKit <sup>+</sup> CD135 <sup>-</sup> CD150 <sup>-</sup> CD48 <sup>-</sup>	Low			
	α	NA	High	NA	Ly-deficient	15, 108
	β	NA	High	NA	Balanced	
	γ	NA	Intermediate	NA	My-deficient	
	δ	NA	Low	NA	My-deficient	
	MPP2	Lin <sup>-</sup> Sca1 <sup>+</sup> cKit <sup>+</sup> CD135 <sup>-</sup> CD150 <sup>+</sup> CD48 <sup>+</sup>	Low	Similar	Ly-deficient	13, 14
	MPP3	Lin <sup>-</sup> Sca1 <sup>+</sup> cKit <sup>+</sup> CD135 <sup>-</sup> CD150 <sup>-</sup> CD48 <sup>+</sup>	Low		Balanced	
	MPP4	Lin <sup>-</sup> Sca1 <sup>+</sup> cKit <sup>+</sup> CD135 <sup>+</sup> CD150 <sup>-</sup> CD48 <sup>+</sup>	Low		Ly-biased	
Human	d-HSC	NA	Higher	Dormant		19, 20, 51–53
	a-HSC	NA	Lower	Activated		
	LT-HSC	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> CD45RA <sup>-</sup> CD49f <sup>+</sup> CD90 <sup>+</sup> ± Rho <sup>lo</sup>	High	Long G <sub>0</sub> exit		50, 54
	ST-HSC/MPP	Lin <sup>-</sup> CD34 <sup>+</sup> CD38 <sup>-</sup> CD45RA <sup>-</sup> CD49f <sup>+</sup> CD90 <sup>+</sup>	Low	Short G <sub>0</sub> exit		
CD34 <sup>+</sup> LT-HSC	CD34 <sup>+</sup> CD38 <sup>-</sup> CD93 <sup>hi</sup>		High	Highly quiescent		68

Listed are subtypes of HSCs defined based on combinations of cell-surface markers and/or function. A global interpretation of the literature on HSC biology is complicated by the fact that each study usually uses only one of the classification schemes, so the extent of overlap between HSC subsets remains to be clarified. This list is not exhaustive. EPCR, endothelial protein C receptor; IT, intermediate-term; LT, long-term; Ly, lymphoid; My, myeloid; NA, not assessed; ST, short-term.

developed<sup>85–89</sup> based on the idea that single-cell transcriptomes represent snapshots of single cells as they traverse differentiation landscapes (Fig. 1c). A recent study on human bone marrow haematopoiesis comprehensively sampled the HSPC compartment<sup>90</sup>. Computational predictions suggestive of early lineage restriction were underpinned by *in vitro* single-cell culture assays, which led the authors to propose a model in which acquisition of lineage-specific fates is a continuous process, and unilineage-restricted cells emerge directly from a continuum of low-primed undifferentiated HSPCs, without any major transition through the multi- and bipotent stages. This is supported by other studies<sup>71,83,87</sup>, which highlighted the abundance of unipotent progenitors within compartments that, at the population level, are multipotent.

There are, however, caveats with this new model and its heavy reliance on single-cell RNA-seq (scRNA-seq). A major conundrum here is that combinations of surface markers can readily split the HSPC compartment into functionally distinct subpopulations, including within the space proposed to be a continuum of low-primed cells when analysed by scRNA-seq. One possible explanation is that there is a decoupling between steady-state mRNA and protein expression levels. The counter argument is that multiomics analysis of highly purified bulk HSPC populations has shown good concordance between mRNA and protein for most genes<sup>14</sup>. It is possible also that functional heterogeneity of HSCs is primarily determined at the epigenetic level. Future measurements of chromatin accessibility and histone marks ideally at single-cell resolution may reveal such mechanisms. A third possible explanation is that even though purifying cells based on protein markers followed by functional assays suggests clean splits into distinct cell types with different biological functions, the changes in biological functionality are in reality much more gradual, where there is no binary biological difference between, for example, long-term HSCs and MPPs, but instead any individual cell sits somewhere along a continuous spectrum. A fourth possible explanation is that current scRNA-seq data analysis methods are not effective at distinguishing between closely related cell types, because many of the shared biological processes (such as the cell cycle, metabolism, motility) generate substantial heterogeneity, which may exceed the number of differentially expressed genes between closely related stages of haematopoietic maturation. The degree to which early haematopoiesis is characterized by a continuum versus distinct populations therefore remains a question that requires further investigation.

### New representations of haematopoiesis

An immediate challenge for the field is how all the recent findings can be reconciled into new graphical models that describe the hierarchical organization of haematopoiesis. It seems clear that a tree in which a circle depicts each successive restriction in potential and each circle is connected to a few others by arrows is an over-simplification. First, the circle is intuitively viewed as a homogeneous set of cells with specific characteristics,

a vision incompatible with the large degree of heterogeneity observed experimentally. Second, these trees indicate a restricted set of possible transitions between circles, which probably underestimates the possible differentiation journeys *in vivo*. Because cell-surface markers can highly enrich for particular behaviours (differentiation, self-renewal or proliferative output), a model in which all lineages branch out directly from the HSC compartment also seems unrealistic (Fig. 1c). We thus propose an alternative visualization (Fig. 2a), in which the trajectories of differentiation are mapped over the areas that have long been represented as circles, highlighting both the diversity in possible routes and the prevalence of early lineage choice. In addition, it is important to remember that one HSC will produce a very large number of progeny, which increases exponentially with each division, an element so far ignored in graphical representations of haematopoiesis (including Fig. 2a). Divisional histories are difficult to measure, and are likely to be heterogeneous, but should nevertheless be incorporated in future experimental and computational analyses, which could result in new graphical representations of the blood system (Fig. 2b).

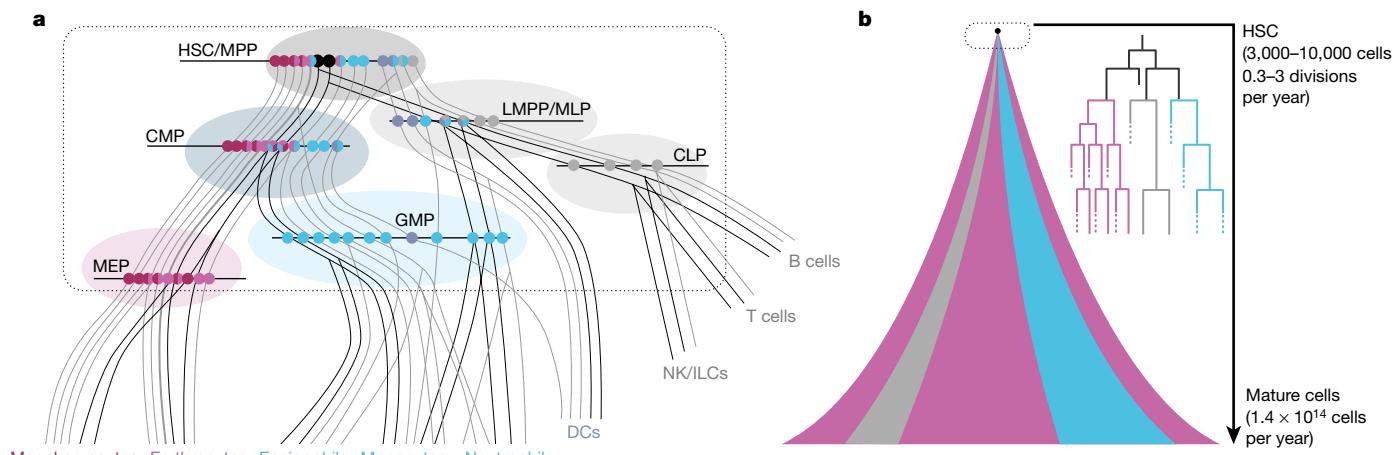
### Making blood at steady state and under stress

In addition to defining the routes of lineage differentiation, another important question is to understand the quantitative contributions of HSCs and progenitors to daily and emergency haematopoiesis.

### Studying unperturbed haematopoiesis

Although the haematopoietic differentiation tree is widely used as a model for how a hierarchically organized tissue is maintained, it is important to remember that this tree was largely derived from experiments that measure cell potential in colony or transplantation assays, rather than cell fate during steady-state differentiation. However, just because a single cell gives rise to two lineages in a colony assay, this does not prove that the same cell, when left alone in an unperturbed bone marrow environment, would have done the same. To understand the dynamics of blood formation, cells can be individually tagged (for example, by retro- or lentiviral insertions or barcodes) and transplanted into recipient mice to measure the contribution of each clone over time. Progressively more sensitive methods have been used in mice, primates and humans<sup>91–95</sup>, collectively supporting the lineage biases and/or restrictions observed in single-cell transplants. Importantly, only a limited number of HSCs produce the vast majority of the differentiated cells in a transplantation setting, consistent with studies of the divisional history of HSCs<sup>19,20,51–53,57</sup>, which demonstrate that only the very rare most dormant HSC can provide life-long reconstitution after transplantation.

Substantial excitement was raised by new technologies to assess the lineage output of individual stem and progenitor cells in unperturbed haematopoiesis<sup>96</sup>. Doxycycline-induced mobilization of a sleeping beauty transposon in stem and progenitor cells was used to generate



**Figure 2 | Trajectory-based visualizations of the haematopoietic hierarchy.** **a**, Two-dimensional visualization of early haematopoiesis. Continuous lines denote trajectories of differentiation for different types of single cells present in the phenotypic HSC compartment (grey shaded area). Along these trajectories, cells and their progeny pass through progenitor compartments commonly defined by specific combinations of cell surface markers (shaded areas). Horizontal lines represent snapshots of the lineage potential of the cells present in each phenotypic compartment (single-colour circles denote unilineage cells; two-colour circles denote bilineage cells; three-colours circles denote trilineage cells; black circles denote multipotent cells). In most progenitor compartments, the number of unilineage cells outnumbers that of bi- or trilineage cells. The figure illustrates differentiation trajectories reported in the literature so far, but

unique integration events, which serve as barcodes that can be tracked by sequencing. Consequently, the comparison of barcodes in mature lineages after pulse-chase labelling analysis allows reconstruction of single-cell behaviours in native, unperturbed haematopoiesis. In contrast to transplantation approaches, analysis of unperturbed haematopoiesis suggested that (i) MPPs contribute predominantly to the myeloid lineage during the steady state, and (ii) cells functioning as HSCs in transplantation do not have a notable role in steady-state haematopoiesis, which instead seems to be driven almost entirely by cells within the MPP compartment.

An alternative genetic fate-mapping system based on *Cre-loxP*-induced recombination of a transgenic barcode cassette recently achieved temporally controlled barcode induction in single cells, and demonstrated that when HSCs are labelled at the fetal liver stage, their descendants in the adult will mostly contribute to multiple lineages<sup>97</sup>. However, megakaryocytic fate was not analysed, and when the analysis was repeated in adult bone marrow, few barcodes were detected in HSCs as well as mature progeny. Given that each fetal liver HSC divides and therefore give rises to several HSCs in the adult, conclusions about the lineage contribution of individual adult HSCs therefore remained preliminary. Another study from the Camargo group<sup>61</sup> addressed this issue more comprehensively by carrying out a 30-week pulse-chase experiment in adult mice with the sleeping beauty barcode system. 133 barcodes were detected in HSCs and at least one of four mature lineages (megakaryocyte, erythroid, granulocyte or B cell). Interestingly, more than half of these 133 HSC barcodes were present only in megakaryocytes, and only a minority of the remaining barcodes were present in more than one mature lineage. Coupled with analysis at shorter pulse-chase intervals and comprehensive single-cell RNA-seq analysis, this study therefore concluded that during homeostatic unperturbed haematopoiesis, megakaryocytes can arise independently from other lineages, and the phenotypic long-term HSC population as defined by transplantation assays actively contributes to megakaryocyte output.

In the HSC compartment, a transposon tag may often be present in just one or two cells, because HSC clones will rarely amplify during unperturbed haematopoiesis, thus making barcode detection less reliable.

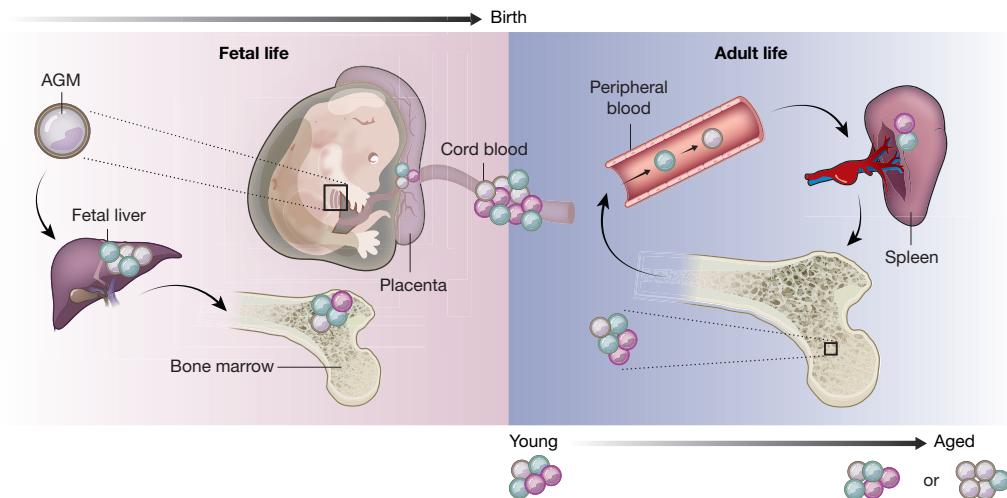
their proportions may not reflect the *in vivo* situation. **b**, Three-dimensional visualization of the progeny of a single HSC. Pink, blue and grey represent the erythroid, myeloid and lymphoid lineages, respectively. Cell history, division and progenitor expansion should all be considered when modelling the differentiation journey of one HSC and all of its progeny. In an adult human, there are an estimated 3,000–10,000 HSCs, which probably divide from only once every 3 months to once every 3 years<sup>58</sup>. Humans produce an estimated  $1.4 \times 10^{14}$  mature blood cells per year<sup>143</sup>. The amplification from a few thousand HSCs is therefore staggering, and must include a strong contribution from a transient-amplifying compartment. Also, because there are many more terminally differentiated erythroid cells than myeloid cells, and even less lymphoid cells, all with different turnover rates, the flux into each compartment must be highly regulated.

It is therefore noteworthy that both the Rodewald and Reizis groups<sup>98,99</sup> found that the HSC compartment contributed more to multilineage blood production than what was estimated by the transposon approach. Busch *et al.* also estimated the kinetics with which cells transit through their differentiation trajectories. Interestingly, flux into the lymphoid branch is 180-fold less than in the myeloid branch. Flux into the erythroid lineage was not assessed, but is likely to be even higher than in the myeloid lineage (Fig. 2b). Flux analysis also found substantial self-renewal capacity in the short-term HSC/MPP compartment, consistent with a recent report of long-term normal haematopoiesis in mice in which the HSPC compartment was ablated by 90%<sup>100</sup>.

Future approaches are likely to use barcodes that are expressed under a strong promoter, and therefore can be detected reliably by scRNA-seq. This would afford true single-cell resolution for the analysis of clonal relationships and single-cell transcriptomes, offering the exciting possibility of defining the native hierarchy agnostic of sorting strategies that were developed using transplantation. Another pertinent question is to what extent laboratory mice kept under sterile, pathogen-free conditions are a suitable model for human haematopoiesis, which is constantly challenged by exposure to infectious agents, and has to function over a much longer lifespan. Long-term follow-up of patients who underwent autologous transplantation has already revealed previously unknown functional aspects of human haematopoiesis, such as the number, stability and dynamics of individual HSCs over many years<sup>101</sup>. Background somatic mutations represent unique barcodes that can be exploited to reconstruct clonal lineage relationships<sup>102,103</sup> and may thus represent another approach to investigate the dynamics of single human HSPCs over extended periods of time.

### Haematopoiesis is flexible in space and time

Blood production needs to have the flexibility to adapt to drastic changes of demand, with evidence accumulating that HSC properties and differentiation journeys can adapt. As reviewed elsewhere<sup>104</sup>, haematopoietic development in the embryo is complex, with a series of transient haematopoietic waves across several organs (Fig. 3). Importantly, fetal and adult



**Figure 3 | The composition of the HSPC compartment changes in space and time.** HSPCs are found in many organs in the body across a lifetime. Cells of different colours represent distinct HSPC subsets. It is unclear whether all HSPC subsets and differentiation trajectories are present in the same proportions in each of the organs. Current evidence suggests that

HSCs have fundamentally different regulation and behaviours (reviewed in ref. 105). In mice, there is a switch from a proliferative to a quiescent state between 3 and 4 weeks of age, which coincides with a decrease in self-renewal<sup>106</sup>. In humans, the timing of the fetal-to-adult switch differs. When compared to adult bone marrow, human HSCs from neonatal cord blood have increased proliferative potential (as expected from mouse studies), but their cell cycle properties already resemble the adult configuration<sup>54</sup>. Consistently, telomere length in granulocytes, a surrogate for the division rate of HSCs, rapidly declines during the first year of human life<sup>107</sup>. There is also evidence that terminally differentiated cells are produced differently during fetal and adult haematopoiesis, because many more single progenitor cells from human fetal liver produce two lineages or more than from adult bone marrow<sup>71</sup>. Interestingly, the relative proportion of HSC subsets also change over time with balanced HSC predominating in fetal liver, whereas lymphoid-deficient (also known as myeloid-biased) HSCs accumulate during ageing<sup>108</sup>. The effects of ageing on HSCs and more generally on blood production are numerous and have been reviewed elsewhere<sup>109</sup>. Changes in both the composition of the HSC pool<sup>108,110,111</sup> as well as the molecular circuitry of individual HSCs<sup>34,112–114</sup> can be due to either extrinsic or intrinsic properties, including alterations in the microenvironment, the proliferative history and accumulation of mutations.

The HSC niche is a highly complex ecosystem that sustains HSC function, in particular promoting survival and long-term maintenance of the HSC pool<sup>115</sup>. If and how niche interactions shape the activity of distinct HSC subsets and the differentiation journeys of HSCs remains unclear. Single-cell transplants and clonal tracking analysis have shown that clones display stereotypical behaviour over serial rounds of transplantation, arguing that their characteristic outputs are not extensively niche-dependent<sup>15,59,64,65</sup>. However, it is possible that distinct HSC subsets may have different niche preferences. Furthermore, even though the vast majority of HSC are located in the bone marrow in adults, a small percentage of HSCs are released in the blood with circadian-clock controlled patterns<sup>116</sup>, and HSCs can also be found in the spleen<sup>117</sup> and lungs<sup>118</sup> (Fig. 3). The role of these extramedullary niches and whether they host specific subsets of HSCs or influence their differentiation or clonal expansion capacity will have to be explored at single-cell resolution. Finally, many types of stress directly affect HSC function: DNA damage<sup>40,41</sup>, inflammation<sup>119</sup>, acute or chronic infection<sup>120,121</sup>, psychosocial stress<sup>122</sup>, metabolic stress<sup>33</sup> and obesity<sup>123</sup>. For most of these processes, insights have been gained on the molecular mechanisms that drive the changes in HSC or progenitor cell function. There are also examples that show that

age-related changes result from a combination of shifts in the composition of the HSPC pool, as well as phenotypic changes in particular cell types driven by intrinsic genetic or epigenetic changes and systemic alterations of the microenvironment. AGM, aorta gonad mesonephros.

at the cellular level, not all HSC or progenitor subsets respond equally to these stresses. For example, in emergency myelopoiesis, MPP2 and MPP3 drive enhanced production of GMPs<sup>13,124</sup>, which reorganize themselves spatially and activate a self-renewal network<sup>124</sup>. We are thus only beginning to understand how stress responses can reshape the relative abundance and possibly differentiation trajectories of different HSPC subsets, and less still is known about the cellular and molecular control/feedback mechanisms that maintain and/or re-establish homeostasis.

### Implications for human disease

Our understanding of haematopoiesis is currently undergoing several shifts. First, the demarcations between stem and progenitor cells that were previously considered rather rigid are becoming increasingly blurred. Second, cell fate choices upstream of the classically defined bi/oligopotent progenitor cells may be more prevalent than previously thought. Third, the loss of key stem-cell characteristics may be largely decoupled from the initiation of specific lineage differentiation programmes. Finally, measuring cellular fates *in vivo* without the need for highly disruptive transplantation procedures has highlighted a previously underappreciated importance of short-term HSC/MPPs in unperturbed haematopoiesis. These and other revisions of our understanding of haematopoiesis as a stem-cell developmental system have major implications for the diagnosis, prognosis and treatment of haematological diseases.

Haematology as a clinical discipline has a long track record of being an early adopter of the latest technological developments, which recently included the first successful gene therapy trials<sup>125,126</sup> as well as some of the first comprehensive cancer genome studies<sup>127</sup>. With respect to haematopoietic malignancies, much current research focuses on identifying the cell of origin, which acquired the first somatic mutation within the multistep progression towards a full-blown malignancy. It is widely accepted now that stem and progenitor cells have a major role in the development of myeloid malignancies (chronic myelogenous leukaemia and acute myelogenous leukaemia) and myeloproliferative neoplasms. More recent evidence also implicates HSCs and lymphoid progenitors in the early stages of hairy cell leukaemia<sup>128</sup> and lymphoma<sup>129</sup>. It is beyond the scope of this Review to list the effects of each of the driver mutations on HSC and progenitor cell function, but it is worth noting that each one of them will reshape the balance of differentiation trajectories and generate complex clonal dynamic patterns. Because cellular context influences the potential effect of leukaemogenic mutations, the newly recognized fluidity of cellular states within the HSPC compartment suggests greater disease heterogeneity between patients, because even when two patients

carry identical founder mutations, the likelihood that they arose in identical cellular states is small. Moreover, the malignant transformation is likely to open up new molecular states and trajectories. In acute myelogenous leukaemia, for example, leukaemic stem cells are defined by a chimaeric transcriptional state<sup>10,130</sup>, and single-cell proteomic approaches have demonstrated the existence of distinct differentiation trajectories for malignant cells<sup>131</sup>.

Given the increasing recognition of discrepancies between the clonal behaviour of native unperturbed HSPCs versus transplantation, xenotransplantation of human leukaemic cells into immunocompromised mice will share similar limitations, in which exposing leukaemic cells to a transplantation assay may induce cellular behaviour that would never occur in a human patient. Exciting prospects may be offered here by new models that may permit transplantation without irradiation<sup>132</sup> and the use of ossicles templated with human bone marrow stromal cells<sup>133,134</sup>. It nevertheless seems imperative to invigorate research efforts that directly track disease in human patients. This will require careful design of patient cohorts and new screening technologies, including multiomic single-cell technologies to define the cellular state as well as the mutation burden of individual cells, as demonstrated recently for the gene fusion *BCR-ABL*, found in most patients with chronic myelogenous leukaemia<sup>135</sup>.

Bone marrow transplantation is likely to remain an important curative therapy for many patients with leukaemia, and with progress in the cell and gene therapy field, may find much wider applicability. Because suitable donor material for bone marrow transplantation remains rate limiting, a promising approach is to produce HSCs from other cell types by borrowing regulatory processes known to be important during developmental haematopoiesis, as illustrated by the recent generation of stem cells with long-term engrafting capability from human pluripotent and mouse adult endothelial cells<sup>136,137</sup>. A better understanding of the HSC state and cell fate decision making will provide new possibilities to develop protocols for *in vitro* amplification of HSCs<sup>138–140</sup>, and also facilitate optimization of protocols for robust genome engineering of HSCs with vast potential for treating common diseases ranging from inherited red blood cell to autoimmune disorders. Expansion of clinical applications will greatly benefit from a better understanding of the self-renewal and differentiation potential of individual HSPCs, coupled with robust prediction algorithms from molecular profiling data to evaluate the efficacy of cell therapy products. Haematopoiesis is therefore well positioned to lead the way in the gene and cell therapy arena, and we may not be too far away from a future in which haematopoiesis will be firmly established not just as a stem cell, but also as a therapeutic model.

**Received 30 July; accepted 8 November 2017.**

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**Acknowledgements** We thank D. Kent for critical reading of the manuscript. E.L. is supported by a Sir Henry Dale fellowship from the Wellcome Trust (WT)/Royal Society. Research in the Laurenti and Gottgens laboratories is supported by the WT, CRUK, Bloodwise, MRC, BBSRC, NIH-NIDDK, and core support grants by the WT and MRC to the Wellcome-MRC Cambridge Stem Cell Institute.

**Author Contributions** E.L. and B.G. contributed equally to the writing and editing of the manuscript as well as to figure preparation.

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