Purpose of review
Hematopoietic stem cell (HSC) transplantation has yielded tremendous information on experimental properties of HSCs. Yet, it remains unclear whether transplantation reflects the physiology of hematopoiesis. A limitation is the difficulty in accessing HSC functions without isolation, in-vitro manipulation and readout for potential. New genetic fate mapping and clonal marking techniques now shed light on hematopoiesis under physiological conditions.

Recent findings
Transposon-based genetic marks were introduced across the entire hematopoietic system to follow the clonal dynamics of these tags over time. A polyclonal source downstream from stem cells was found responsible for the production of at least granulocytes. In independent experiments, HSCs were genetically marked in adult mice, and the kinetics of label emergence throughout the system was followed over time. These experiments uncovered that during physiological steady-state hematopoiesis large numbers of HSCs yield differentiated progeny. Individual HSCs were active only rarely, indicating their very slow periodicity of differentiation rather than quiescence.

Summary
Noninvasive genetic experiments in mice have identified a major role of stem and progenitor cells downstream from HSCs as drivers of adult hematopoiesis, and revealed that post-transplantation hematopoiesis differs quantitatively from normal steady-state hematopoiesis.

Keywords
clonal marking, fate mapping, mathematical modeling, stem cell transplantation, unperturbed hematopoiesis

INTRODUCTION
Compared to solid tissues that are maintained by stem cells such as the gut or the skin, the hematopoietic system is particularly accessible. This is because of the fact that the mostly migratory cells can readily be retrieved from the blood and from lymphoid organs. A detailed phenotypic map of cells, cell populations, tissues, and organs under resting, immune-responding, or pathological conditions is now available. Multicolor phenotyping by flow cytometry has evolved into mass cytometry that allows the detection of tens of markers simultaneously on the cell surface and inside of single cells. Unlike in flow cytometry, the cells are destroyed in the process and cannot further be examined functionally. mRNA sequencing and DNA mutation analyses in single cells have become powerful tools. Collectively, the immune system can now be deconvoluted to the resolution of single cells, and at large scales, that is, for thousands of individual cells [1–4].

In vitro, precursor–product relationships starting from single cells have been examined since the development of hematopoietic colony assays. This area has been much refined by high-resolution tracking of single cells undergoing colony formation, and by simultaneous observation of gene expression associated with, or driving lineage commitment [5–7]. These assays read out the possible potential, and not necessarily the potential that is
KEY POINTS

- Transplantation has been the mainstay of research on HSC and progenitor functions in vivo. To what degree post-transplantation hematopoiesis reflected the physiology of hematopoiesis has been unknown.
- Noninvasive experimental systems have now uncovered major differences comparing normal (polyclonal with low individual HSC contribution) and post-transplantation (oligoclonal with few dominant HSC clones) hematopoiesis.
- During unperturbed steady-state hematopoiesis at least 30% of HSCs (or 5000 HSCs per mouse) are active over time.
- Hundred-fold myeloid over lymphoid bias at the MPP stage has been shown by HSC fate mapping.

realized in vivo. Cells may be exposed to ‘supraphysiological’ conditions, often aiming at maximal cloning efficiencies or burst sizes, which can result in developmental skewing, or even impose a potential on cells. For example, ectopic expression of Notch-ligands, Delta-like-1 or 4 on stromal cells can persuade undecided progenitors toward a T-cell lineage program [8]. Notch signals are essential for T-cell development but forcing progenitors down the T-cell path in vitro does not necessarily identify those cells that would normally take this route [9,10].

Because in-vitro readouts may or may not reveal the physiology of hematopoiesis, transplantation of stem and progenitor cells into myeloablated recipients has been the mainstay of in-vivo hematopoiesis research [11]. Transplantation is a robust assay, the conditions are well established, it works experimentally in animals and clinically in humans, and the reconstituted blood and immune systems are functional long term. Successful reconstitution from a single hematopoietic stem cell ([HSC), in a fraction of the mice] [12–17,18**,19**,20*], proves within this system two postulated key properties of the HSC true:

1. Self-renewal: One HSC needs to reconstitute thousands of cells in the HSC compartment, which can only be achieved by massive proliferation without obvious loss of HSC identity.
2. Multipotency: One HSC generates mature progeny for essentially all hematopoietic lineages, demonstrating, by definition, multipotency of transplanted HSC in vivo.

Genetic barcoding prior to transplantation is a sophisticated approach to track individually tagged cells and their progeny in mice transplanted with bulk populations of stem cells [21–23]. After transplantation, high-throughput sequencing of DNA barcoding tags has demonstrated self-renewal and multipotency, and revealed that only few, that is, in the order of tens, HSCs contribute to hematopoiesis [21,22].

Self-renewal and multipotency are fascinating but experimentally challenging to demonstrate for normal hematopoiesis. HSC transplantation represents an artificial situation, and little has been known about the degree of self-renewal and multipotency in the normal bone marrow. In-vivo lineage tracing has now provided evidence for self-renewal (maintenance of few labeled HSCs throughout the lifespan of the mouse) and multipotency (full lineage spectrum generated from few HSCs in vivo, although not at clonal level). HSC proliferation [24–26] as such is no indicator of differentiation or self-renewal because proliferation can feed either process. Finally, the relative roles of HSCs vs. downstream stem and progenitor cells for the maintenance of steady-state hematopoiesis, or the differentiation flow through the system remained enigmatic.

Collectively, soon there will be complete structural information of all cellular components of the immune system at very high resolution. Yet, as an outlook, noninvasive experimental methods have to be developed to address key open questions on the operation of hematopoiesis under physiological conditions. Here, we will review recent studies on unperturbed hematopoiesis. It now appears that, while the hematopoietic system can be reconstituted by bone marrow transplantation, HSC engraftment after transplantation does not recapitulate the system as it originally operated in the donor. Their enormous adaptability to demand and huge regenerative capacity allow HSCs to perform under both conditions, albeit with differing division of labor between stem cells.

SINGLE-CELL PHENOTYPING APPROACHES A HOLISTIC SCALE

Phenotypic analysis leads to the description of cells based on parameters that include expression of cell surface markers, intracellular proteins, protein modifications, RNA expression, epigenetic marks, and sequencing of genomic mutations. Phenotyping of living cells plays a key role for the purification of rare cells, including HSCs [18**,19**,27, 28,29*,30], for prospective analysis in adoptive transfer experiments. Owing to technological advances, phenotyping approaches unprecedented depth and resolution. Mass cytometry enables the simultaneous identification of in the order of 40
phenotypic antigens (including, for example, intracellular signaling cues such as protein phosphorylation) at the single cell level [31,32]. In conjunction with large-scale single-cell RNA sequencing, proteomics and epigenetic analyses, these new tools enable deep (detailed biochemical or genetic ‘status’ of a cell) and broad (degree of heterogeneity within populations) biological information on stem cells and progenitors [33–37]. These experiments provide a deconvoluted (yet not easily comprehensible) view on complex cell ensembles [32,34,38]. Recent reviews have covered these developments [1,3,4,39,40].

Most single-cell approaches require the retrieval of cell suspensions from biological samples, and artifacts in the wake of cell isolation, sorting and further manipulations cannot fully be excluded. In this regard, the combination of mass spectrometry with immunohistochemistry is not only offering new avenues in pathology but may also bring some of these new technologies closer (or back) to the tissues [39,41]. Still also here, fixation and other procedures may not be always neutral [2].

Ultimately, an important question is whether this holistic phenotypic approach leads to a detailed ‘structural’ map of the immune system (a highly valuable resource), or whether also developmental routes can be recapitulated, or even be predicted, from these data. Several groups have developed mathematical models to extract developmental trajectories (akin to a movie) from a (standing) picture based on data obtained from large bodies of single cells [31,37,42,43]. To what extent these new approaches will help to unravel developmental pathways in previously uncharted territory remains to be determined. It appears, currently, that the obtained trajectories are either fitted to, or tested against known pathways. This is of course important for validation, yet it does not prove that navigation will be safe with no land in sight. It seems likely that the ‘running machine’ is not readily deducible from stills of its parts. Consider a progenitor cell in which a set of transcription factors turns on erythropoietin receptor mRNA transcription; is this cell always destined to become a committed erythrocyte progenitor, or can it turn off the erythropoietin receptor gene and choose an alternative fate? In-vivo fate mapping shows that Cre recombinase drivers inserted into lineage-specific gene loci can indeed separate branches in vivo; however, this separation is not absolute (e.g., in IL7rCre mice, some myeloid cells are marked, implying that not every cell expressing the IL7r during its ontogeny will become a lymphocyte) [44]. In summary, fate-mapping experiments are likely necessary to complement the phenotypic picture functionally.

**TRANSPLANTATION: A WONDERFUL ASSAY BUT NOT THE WHOLE STORY**

Transplantation of HSCs and progenitors has compelling experimental advantages: it is feasible, and the conditions (e.g., dose of radiation; radioprotective cell doses; and engraftment kinetics) are established. Transplantation can separate durable engraftment (long-term reconstitution; by convention referring to HSC activity for at least 4 months) from transient reconstitution (for about 6 weeks and often important for radioprotection). HSC exhaustion can be tested by serial transplantation. Transplantation can reveal competitive advantages or disadvantages when test and competitor cells are coinjected, it can yield functional information on mutant stem and progenitor cells from knockout mice, it can facilitate the analysis of lethal mutants from which at least fetal liver cells can be isolated, and it can be crucial to distinguish hematopoietic intrinsic vs. extrinsic (systemic) phenotypes.

The race for the world record in enrichment and purity in HSCs has been based on repopulating frequencies starting from single or few cells (or graded numbers of cells in limiting dilution experiments). This was the guiding principle in the original descriptions of phenotypically enriched HSC populations [13,14,16,45–47]. It is interesting to observe the reported engraftment frequencies over time. One report claimed near absolute engraftment [48] whereas others reported limitations, for instance because of seeding inefficiencies (e.g., cell loss in the lungs on intravenous transfer or imperfect niche homing) [49]. Repopulating HSCs (also termed long-term [LT] HSCs) are highly enriched in a rare subset (about 0.006%) of cells in bone marrow that have a lineage (Lin)−Kit+Sca1−CD150−CD48− phenotype; sometimes further markers (e.g., CD34−, CD41−, CD41−, and Flt3−) are included. However, some combinations appear redundant because, when tightly gated, some markers virtually exclude cells bearing other markers (e.g., CD34 and CD150 are mutually exclusive).

HSC transcriptomes have been analyzed in the search for new HSC markers and to deduce information on HSC biology [18**,28,30,33,50]. HSCs have successfully been identified in vivo using reporter mice for Hoxb4 [51], and Flgs [29*]. HSC reporter mice have also been used for the in-situ localization of the stem cell niche in the bone marrow [18**,30]. In α-cateninGFP knock-in mice, HSC could be enriched by using only this marker from a frequency of 1/37 000 (unfractionated bone marrow) to 1/7 (α-cateninGFP+ cells) [18**]. Conversely, depletion of α-cateninGFP+ cells from bone marrow reduced the repopulating frequency almost 100-fold over total bone marrow. The highest HSC...
repopulating efficiency (1/3) was achieved by combining \(\alpha\)-catulin\(\text{GFP}\) cells with the Lin\(^-\) Sca1\(^+\)CD150\(^-\)CD48\(^-\) phenotype [18\(^*\)]. One report used Hoxb5 expression to further dissect the HSC compartment [30]. In Hoxb5-triple-mCherry reporter mice, about 20% of phenotypically defined HSCs (Lin\(^^-\) Sca1\(^+\)CD150\(^+\)CD48\(^+\)Hoxb5\(^+\)Flt3\(^+\) cells) were mCherry\(^+\), and only this fraction was highly enriched for long-term repopulating HSCs (at a frequency of 1/2 compared with 1/16 for mCherry\(^-\) cells). It appears difficult, however, to reconcile low frequencies in the Hoxb5\(^-\) population with frequencies in the range of 20–40% that others measured previously for the entire phenotypically defined HSC population [13,14,16,17,18\(^*\)].

The procedures underlying transplantation are not standardized. Details of cell purification and cell sorting (e.g., setting of lineage and other gates in the flow cytometer), choice of recipient strains, conditions of myeloablation, efficiency of cell injections, as well as mouse keeping conditions may matter, effectively precluding direct comparisons of repopulating frequencies. Still, HSC populations can be purified to harbor repopulating frequencies of up to 50% [14,18\(^*\),30,52]. Finally, based on transplantation there is evidence for heterogeneity among HSCs based on expression levels of Kit [53] and CD150 [52,54].

In transplantation experiments, the lineage output has been analyzed at various time points for at least lymphocytes (mostly T and B cells) and myeloid cells (mostly granulocytes). The notion that a myeloid bias is a hallmark of aging hematopoiesis is largely based on the transplantation of young and old HSCs. Moreover, transplanted HSCs have fallen into distinct lineage and kinetic repopulation patterns, suggesting functional heterogeneity with the total HSC compartment [16,17,20\(^*\),52,54,55]. It is unknown whether such HSC subsets exist in situ.

Genetic barcoding of HSCs and progenitors in vitro, followed by transplantation, has been used to track the clonal output of tagged cells in vivo [21–23]. The identification of DNA barcodes in sorted cell populations can uncover the types of lineages and the relative amounts of cells that have been generated in vivo from those tagged HSCs that successfully engrafted. These experiments demonstrated that the number of contributing HSCs (i.e., the number of unique barcodes) is very low (in the order of tens). By extrapolation, this suggested that only few HSCs might contribute also to normal hematopoiesis.

Recently, a barcoding strategy has also been applied to obtain a high-resolution view of the myeloid-erythroid progenitor compartments [56], which is in agreement with a parallel study based on single-cell RNA expression data [37]. This marked heterogeneity within common myeloid progenitors is in keeping with the identification of committed erythroid progenitors within the common myeloid progenitor compartment [57].

From this brief update on transplant hematopoiesis, it is evident that much of the current understanding of the system is based on studies of the fate and functions of HSCs following adoptive cell transfer and engraftment. The flipside is that our knowledge of hematopoiesis under nonperturbed conditions in the bone marrow is limited.

**NONINVASIVE STUDIES OF HEMATOPOIESIS**

In-vivo tracing methods have been developed to ‘visualize’ hematopoietic development from HSCs under native conditions (see [58] for underlying considerations). Inducible lineage tracing of cells emerging from HSCs was reported in mice expressing tamoxifen-dependent versions of Cre recombinase from the Scl locus [27], or from the Runx1 locus [59]. Key for HSC fate mapping is whether or not Cre expression can be as specifically as possible restricted to long-term HSCs in vivo [19\(^*\)]. Other suitable candidate loci include the aforementioned genes Hoxb4 [51], Fgfd5 [29\(^*\)], \(\alpha\)-catulin [18\(^*\)], and Hoxb5 [30]. Busch et al. [19\(^*\)] generated a knock-in mouse expressing from the Tie2 locus Cre recombinase flanked on both ends by a modified estrogen receptor domain; this fusion protein, termed MerCreMer (MCM), is not leaky in the absence of tamoxifen [60]. In Tie2MCM mice, a small fraction (in the order of 1%) of Lin\(^^-\) Sca1\(^+\)CD150\(^-\)CD48\(^-\) HSCs could be induced to express the inheritable fluorescent marker YFP [19\(^*\),61]. In this system, kinetic cell tracing experiments in a large cohort of mice, combined with limiting dilution analysis and mathematical modeling, revealed several unexpected quantitative properties of hematopoiesis from stem cells in the bone marrow [19\(^*\),58]:

1. The number of HSCs that contribute to normal hematopoiesis is in the order of 5000 cells (or more, given that this was a lower estimate) per mouse. Considering that mice have in the order of 17,000 HSCs, this implies that at least 30% of all HSCs participate over time in hematopoiesis.
2. Estimates on the flux of differentiation through the system (between phenotypically-defined stem and progenitor compartments) could be obtained. The by far slowest differentiation step was found between Kit\(^^-\) Sca1\(^+\)CD150\(^+\)CD48\(^-\) phenotype HSCs and short-term
(Kit^+Sca1^+CD150^-CD48^-) phenotype HSCs, which takes almost 1 year. This implies that those HSCs, most apical in the pyramid, are only rarely involved in the daily generation of new blood and immune cells. Steady-state hematopoiesis is thus expected to be largely independent of the activity of HSCs for extended periods of time, which may have implications for time between bone marrow injury and bone marrow failure or hypoplasia.

(3) An individual HSC is giving rise to differentiated progeny (cell divisions leading to differentiation) approximately once per 110 days. The rate of proliferation (one division per 110 cells and day, or approximately 1% of HSC per day) is lower than actually measured proliferation rates in HSCs based on the literature [26], which are in the order of a few percent. This difference should reflect those cell divisions that compensate for cell loss.

(4) The observation of many mice over long periods of time with low HSC labeling frequencies indicates very slow periodicity of HSCs (averaged over the labeled HSC population) and no obvious signs of random or stochastic HSC activation. The relative homogeneity of the output from few labeled HSCs argues for one HSC pool with rare individual HSC activity rather than two pools, one active and one dormant, within the labeled cells.

(5) Under steady-state conditions, the contribution of individual HSCs (output) to the hematopoietic system is low. This would imply relatively small clone sizes of mature immune cells, and ensure that many different HSCs contribute to the maintenance of hematopoiesis.

(6) Hematopoiesis in adult mice (not only aged mice) is characterized by a very strong myeloid bias, i.e. the flow from HSCs into myeloid lineages is several 100-fold larger compared with the lymphoid lineages. Because, in general, myeloid cells are shorter-lived than lymphoid cells, these different production rates could be explained by the higher demand for de novo myelopoiesis compared with lymphopoiesis.

(7) 5-FU-induced leukopenia enhances the flux from HSCs.

(8) In-situ labeled HSCs, when grafted using conventional bone marrow transplantation, fail to reestablish their original representation (i.e., clonal composition) in the host bone marrow. Differences in HSC contributions over time under steady state and post-transplantation are depicted in Fig. 1. Quantitative hallmarks that distinguish steady-state and post-transplant hematopoiesis are summarized in Table 1. Genetic barcoding is a powerful tool to study precursor product and inter-lineage relationships at very high, possibly clonal resolution. The

FIGURE 1. Schematic depiction of HSC diversity and contribution to hematopoiesis tested in transplantation or by fate mapping. (a) Transfer of HSC leads to a transient multiclonal contribution in the first weeks after injection. The majority of HSC clones is lost within the first 4 months, and long-term engraftment is achieved by only a few dominating HSC clones. Individual clones are symbolized by colored lines. (b) Tamoxifen induces Cre/loxP-mediated recombination of the Rosa26 reporter locus, resulting in YFP-expression in Tie2^+ HSCs and their progeny. This reveals polyclonal participation of many HSCs in hematopoiesis, with each individual HSC contributing only rarely (on average about once every 110 days, with perhaps some variation in individual timing) [19**]. Individual clones are indicated by colored lines, and waves indicate periods of activity. HSC, hematopoietic stem cell; Mer, mutated estrogen receptor site; MerCreMer, Cre recombinase fused to two Mer sites.
Table 1. Quantitative and qualitative hallmarks of hematopoietic stem cells, and experimental advantages and limitations (shown on top) vs. experimental categories discussed in this review (shown on left).

| Number of contributing HSC | Progeny/clone size | Self-renewal | Multipotency | Advantages | Limitations |
|----------------------------|--------------------|--------------|--------------|------------|-------------|
| Transplantation of HSC     | Number of long-term contributing HSCs low | Expansion of few HSCs | High | Directly measurable for single cells | Measures stem-cell behavior in vivo but only in the context of transplantation |
|                           |                    | HSC exhaustion after 4–5 serial rounds of transplantation | Evidence for HSC heterogeneity | Direct comparison of stem-cell properties by competitive transplantation | Shows whether defects are intrinsic or extrinsic of hematopoietic system |
|                           |                    | Size of individual clones large | | | Irradiation affects stem-cell niche |
| In-situ fate mapping       | High | Lower than in transplantation | Law | Yes, for labeled cell pool. At the clonal level unknown; requires ‘endogeneous barcoding’ | Physiological context; insight into unperturbed HSC functions; led to data on frequencies of active HSCs, flux through the system, and on ageing |
| (inducible genetic marks in HSC in vivo) | Low individual contribution | Clone sizes strictly regulated to avoid dominant clones/ prevent leukemia | | | Locus has to be HSC specific, no background in the absence of induction is essential |
| Transplantation of barcoded HSC | See above | See above | See above | See above | Marking and analysis of HSC on clonal level |
| | | | | | High resolution of HSC output and lineage relationships under conditions of transplantation |
| | | | | | Integration sites may not be neutral |
| | | | | | Only low number of transferred clones contribute long term |
| | | | | | Representative for all transferred HSCs? |
| | | | | | Labelled HSC subset might not be representative for the whole population |

Hematopoiesis
generation of barcodes in vivo without the use of viral vectors possesses an experimental challenge. Sun and colleagues [62] developed a new mouse model in which they labeled cells with unique genetic barcodes in vivo. The system was driven from the ubiquitously expressed Rosa26 locus, doxycycline-inducible transposon mobilization occurred in approximately 30% of cells, and the labeling included stem or progenitor cells as well as fully differentiated cells. At early time points, the presence of tags in the periphery was unrelated to development, but following chase periods up to 40 weeks, the turnover of label revealed data on the dynamics of native hematopoiesis under steady-state conditions.

Kinetic resolution of peripheral blood samples at 4–6 week intervals revealed highly polyclonal granulopoiesis that originates from successive, distinct sets of progenitors during steady-state granulopoiesis [62]. Because the residence time (time cells spend in a compartment) for multipotent progenitors (MPP) is on average 70 days [19], these results are compatible with the idea that a given set of MPP generates (at least) granulocytes over several weeks before another set takes over. Sun et al. found only very few overlapping barcodes comparing LT-HSC in the bone marrow and mature cells in the periphery, and concluded ‘that LT-HSC have limited lineage output under unperturbed conditions for at least 40 weeks’ [62]. However, the output from HSCs is not zero, but an estimated number of 150 HSCs still contribute per day [19]. The absence of common tags could in theory also be explained by loss of tags from the HSC pool because of stem cell consumption during differentiation (in that case, proliferation would always lead to two differentiating daughter cells that leave the HSC compartment, causing loss of the original barcode from the HSC pool).

This barcoding system has also been used to compare hematopoiesis in situ and post-transplant. Akin to the model depicted in Fig. 1, many clones contributed early and transiently after transplantation but, with time, an oligoclonal pattern arose, providing strong support for the notion that upon transplantation the population of donor HSCs fails to reestablish its original composition, clone sizes or activity rhythm in the host. Finally, this barcoding system also provided hints into lineage relationships. Only 10% of MPP clones showed bipotent development into myeloid and lymphoid cells. Although different lifespans of myeloid and lymphoid lineage could mask bipotency, the experiments indicate that multipotency exists at this progenitor level in vivo.
CONCLUSION

Until recently, information on the functions of HSCs and progenitors was largely based on transplantation experiments. New experimental systems have been developed to explore the functions of normal steady-state hematopoiesis in the bone marrow in mice. These noninvasive experiments have uncovered major differences comparing normal and post-transplantation hematopoiesis; hence both are in vivo but different. Following transplantation, hematopoiesis is initially driven by many transient clones, which fade over time, resulting in hematopoiesis that is maintained by few HSCs (oligocolonal hematopoiesis). By contrast, new studies now show that normally very large proportions of HSCs (at least 30% or about 5000 HSCs per mouse) are active over time. HSCs have a very slow periodicity, for example, long lag times between two incidents of activity with no evidence for distinct dormant vs. active HSC compartments. Tracking of the emergence of the label from HSCs via differentiation into mature cells led to estimates on rates of differentiation flux, rates of differentiation-associated proliferation, and residence times in compartments. In vivo barcoding data gave insights into the clonal dynamics (number and diversity of barcodes distributed over time) of hematopoiesis, and suggest that at least at the MPP stage most in-vivo potential is myeloid rather than bipotent lymphoid plus myeloid. This is in line with the several 100-fold myeloid over lymphoid bias at the MPP stage deduced from HSC fate mapping data.

Collectively, these developments provide a quantitative framework for the dynamics of normal hematopoiesis (whereas refining the structure and relatedness of the lineage pathways under normal conditions will require even more sophisticated tools). The regulation of the HSC output and the hematopoietic flow under stress conditions or during pathology will be key questions in the future. Moreover, massive single-cell analyses will provide an unprecedented depth of the structure of the hematopoietic system. Eventually, these routes of investigation should merge in a detailed functional description of the physiology and pathology of hematopoiesis.

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Conflicts of interest

There are no conflicts of interest.

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