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The single-cell kinetics of hematopoietic stem cell differentiation after transplantation-the steady state and the effect of erythropoietin: the steady state and the effect of erythropoietin

Almut Eisele

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Almut Eisele. The single-cell kinetics of hematopoietic stem cell differentiation after transplantation-the steady state and the effect of erythropoietin: the steady state and the effect of erythropoietin. Human health and pathology. Université Paris sciences et lettres, 2019. English. NNT : 2019PSLET040 . tel-03028817

HAL Id: tel-03028817

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THÈSE DE DOCTORAT
DE L'UNIVERSITÉ PSL

Préparée à l'Institut Curie

**La cinétique de différenciation des cellules souches
hématopoïétiques uniques après transplantation—l'état
d'équilibre et l'effet de la cytokine érythropoïétine**

Soutenue par

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Le 8 Novembre 2019

Ecole doctorale n° 474

**Frontières de l'Innovation en
Recherche et Education**

Spécialité

**Biologie cellulaire et biologie
du développement**

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Chapter I: General introduction

Hematopoietic stem cells (HSC) reside at the apex of one of the most complex and dynamic cellular systems of our body. HSCs ensure the daily production of erythroid cells responsible for oxygen delivery to our organs, and also of all myeloid and lymphoid immune cells involved in the defense against invading pathogens and malignancies. The precise regulation of HSC differentiation to these different lineages is thereby absolutely crucial for a healthy life, and must tightly be adapted to the changing physiological needs of the body. Hormones and cytokines, as erythropoietin (EPO), are important players in this process and already widely used in the clinics to normalize blood composition during different pathologies. Also, after HSC transplantation cytokines are employed to ensure an efficient engraftment and fast reconstitution of the hematopoietic system, especially in the erythroid lineage. Improving our understanding of HSC differentiation after transplantation and cytokine action on HSCs is necessary to guarantee the safety of such clinical applications.

This thesis aims at describing the early HSC engraftment kinetics after transplantation at the single cell level and the effect of erythropoietin (EPO) on this process. In this chapter, I will give an overview of the current status of HSC research, and the role of different lineage tracing systems, especially cellular barcoding, for the field. Furthermore, I will summarize the current knowledge on HSC heterogeneity, and recent findings on the action of cytokines on HSCs with a focus on EPO.

1.1 HSC definition and HSC differentiation models

What is an HSC?

The hematopoietic system makes up around 90% of our bodies cells, is greatly dynamic, and composed of a particularly large number of different cell types with various functions, localizations, and lifespans (Sender, Fuchs, & Milo, 2016). Nevertheless, only one type of stem cell, the hematopoietic stem cells (HSCs), mainly localized in the bone marrow, guarantees its maintenance, through symmetric and asymmetric self-renewal divisions. In mice HSCs can today be defined by two means. Firstly and historically, the HSC activity can retrospectively be determined based on a transplantation assay. A cell with the ability to give long-term, multilineage, hematopoietic output after primary and/or secondary transplantation is traditionally considered to be an HSC. Not fully standardized is the definitions of long-term, multilineage reconstitution. Often long-term reconstitution refers to a period of above 4 months, multilineage reconstitution to the production of at least one myeloid and one lymphoid subset, and reconstitution in general to a donor chimerism of above 1% after transplantation. A second way to define HSCs today is based on the expression of surface markers. This immunophenotypic definition allows the isolation of HSCs for prospective experiments. HSCs are sorted as lineage (Lin) negative (commonly CD19, CD3, CD4, CD8, Ly6G, NK1.1) Sca-1 positive, c-kit positive (together LSK), CD150 positive cells, which are Flt3, CD34, and/or CD48 negative. Other HSC markers, which are less ubiquitously used are for example Tie2 (Arai et al., 2004), Esam1 (Ooi et al., 2009), CD201 (Balazs, Fabian, Esmon, & Mulligan, 2006), Krt18 (Chapple et al., 2018a), Fgd5 (Gazit et al., 2014), Pdzklp1 (Cabezas-Wallscheid et al., 2014), Hoxb5 (J. Y. Chen et al., 2016), CD49b (Wagers & Weissman, 2006), CD244, CD229 (Oguro, Ding, & Morrison, 2013), Evi1 (Kataoka et al., 2011). Furthermore, the immunophenotypic isolation of HSCs is sometimes complemented by the staining for other stem cell characteristics, as slow cycling (assessed through dye efflux (Wilson et al., 2008)), or low mitochondrial activity (assessed through rhodamine-123 staining (C. L. Li & Johnson, 1995)). In humans, the definition of HSCs and the description of HSC specific surface markers is less detailed to date. CD34 is the major marker used to isolate the hematopoietic stem and progenitor population (HSPC) (Civin et al., 1984). Other markers used to distinguish human HSPC are CD38, CD90, CD45RA, CD49f, CD71, CD41, CD10, CD7, and CD135 (Doulatov, Notta, Laurenti, & Dick, 2012) (Scala & Aiuti, 2019). The definition of HSCs can have a profound impact on the way and resolution at which hematopoiesis is envisaged.

HSC differentiation models evolve- from tree, to kinetic, to continuum

Different models of HSC differentiation exist. These are often based on mouse data and have subsequently been applied to the human setting for which less data exists. Since many years prevailing (as far as to be called “classical”) is a model which describes HSC differentiation as a hierarchical process governed by HSC asymmetric divisions. In this model HSCs give rise to more developmentally restricted (“committed”) progenitor cells with less self-renewal capacity, which respectively repeat this behavior, till unipotent progenitors are generated. The entire process is often represented as a tree (the “hematopoietic tree”), of which each branch represents a committed progenitor type (Figure 1 A). The order of lineage restrictions, and the number of branching points in the classical HSC differentiation model is still debated in the murine setting, and even more so for its human counterpart. In older publications the first lineage split during murine hematopoiesis was proposed to occur between the lymphoid and the remaining lineages. The HSC was here thought to generate a common myeloid-erythroid progenitor (CMEP) and a common lymphoid progenitor (CLP) (Abramson, Miller, & Phillips, 1977) (Bradley & Metcalf, 1966). In more recent publications the idea of a first lineage commitment to the lymphoid lineage has been challenged. A “myeloid-based model” of hematopoiesis developed in 2001 proposed for example a first split between a CMEP and a CLP with myeloid potential (CMLP) (Katsura & Kawamoto, 2001). In another common recent adaptation of the “classical model”, the first lineage split does not occur at the level of the HSC at all. Rather, the HSC gives rise to still multipotent but, less self-renewing multipotent progenitor cells (MPP) and only at the level of these MPPs the first lineage split occurs through the production of CLPs and a common myeloid progenitor (CMP). But also, this order of events is debated. Notably the MPP subset has been divided in several other subsets: the MPP1, the myeloid biased MPP2 and MPP3 subsets, and a lymphoid biased MPP4, or LMPP subset ((Cabezas-Wallscheid et al., 2014) (Pietras et al., 2015b)) (Adolfsson et al., 2005). Also in the human setting, the “hematopoietic tree” becomes more detailed with distinction of HSC subsets and MPP based on self-renewal potential (Majeti, Park, & Weissman, 2007) (Notta et al., 2011). For many of the murine myeloid progenitors human counterparts have been identified. To date still only one immature lymphoid progenitor is established in humans (Doulatov et al., 2012). Altogether the classical HSC differentiation model is still evolving.

A drastically different model of HSC differentiation only applied to the murine setting to date, is the sequential determination (“SD”) model, which was first introduced in 1985 (G Brown, Bunce, & Guy, 1985), and revised in recent years (Brown, Hughes, Michell, Rolink, & Ceredig, 2007) (Ceredig, Rolink, & Brown, 2009) (Figure 1 C). Here, in contrast to the “classical” model, the lineage potential of hematopoietic progenitors is not a fixed characteristic, but changing over time. According to this model HSC acquire the potential to produce each lineage individually and sequentially, in a genetically

predetermined order and speed, switching from the myeloid to the lymphoid lineages (more in detail from megakaryocytes (Mk) to erythroid cells (E), to neutrophils (Gr or Neu), to monocytes (Mo or Mono), to B, to natural killer (NK) cell, and finally to T cells). Extrinsic signals are thought to determine whether HSCs really produce a given cell type when having the potential to do so. Besides, the “SD model” proposes that each HSC can go through the sequence of potential changes repeatedly. This point was only recently strengthened with the introduction of a cyclic representation of the “SD model”. Although never as popular as the “classical model”, also several more recent publications are in line with the “SD model” (Lai & Kondo, 2006) (Brown et al., 2007) (Ceredig et al., 2009).

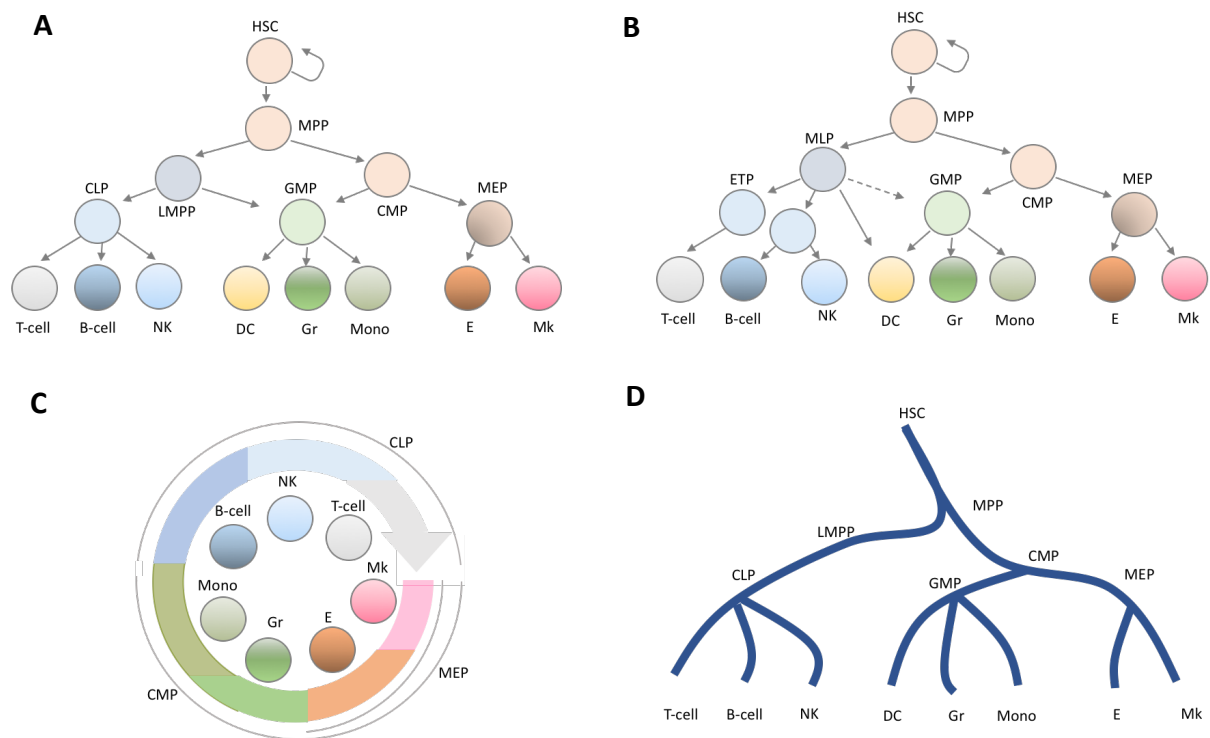


Figure 1: Overview of three different models of HSC differentiation. (A-D) (A-B) the classical model of murine (A) and human (B) hematopoiesis. (C) The sequential determination (“SD”) model (D) The “flow model. Inspired by (Brown et al., 2007) (Kawamoto, Ikawa, Masuda, Wada, & Katsura, 2010) (Ye & Graf, 2007) (Doulatov et al., 2012). Abbreviations as in text and dendritic cell (DC), granulocyte-macrophage progenitor (GMP), megakaryocyte-erythroid progenitor (MEP).

A third model of HSC differentiation as a “flow” has emerged more recently (Figure 1 D) both for the murine and human setting. Its main characteristic is to doubt the binary decisions present in the “classical model”, but also in the “SD model”. The increasing range of progenitor subtypes identified in mouse and human was one driver for the generation of this model (Ye & Graf, 2007). Another was

the increasing availability of single cell RNA sequencing (scRNAseq) data of HSC and other hematopoietic progenitor subsets in both settings (Kowalczyk et al., 2015) (Haas et al., 2015) (Nestorowa et al., 2016) (J. Yang et al., 2017) (Velten et al., 2017) (Giladi et al., 2018) (Tusi et al., 2018) (Pellin et al., 2019) (Karamitros et al., 2018). Many of these scRNAseq studies revealed a spectrum of lineage specific gene expression rather than clear (binary) separations in progenitor subsets. Velten et al. for example concluded from this that uni lineage-restricted cells might derive directly from a “continuum of low-primed undifferentiated hematopoietic stem and progenitor cells” (CLOUD-HSPCs) in human (Velten et al., 2017). In how much gene expression data really predicts potential or actual fate of HSC and progenitors remains to be resolved. Only lineage tracing methods can unambiguously answer these questions and remain the prime technique to set-up, validate or challenge any model of HSC differentiation.

1.2 Lineage tracing techniques in the study of HSC differentiation

An HSC is defined as a cell with the ability to give long-term, multilineage, hematopoietic output after primary and/or secondary transplantation. Lineage tracing is thereby at the very core of HSC research methodology. Today, following several important technical advancements, a broad range of different lineage tracing techniques is available, allowing HSC lineage tracing at different resolution (bulk vs. single cell), and with different throughput, in the clinically-relevant transplantation setting, but also in situ (the steady state without transplantation), in human, mouse and other model organisms (Figure 2).

Lineage tracing after transplantation goes single cell

For many years, lineage tracing in HSC research was restricted to the transplantation setting and notably bulk cell transplantation performed in mice, and also successfully implemented in the clinics as treatment in humans (Till & McCulloch, 1961). Limiting dilution assays (LDA), the transplantation of very low cell numbers to estimate HSC numbers in a transplant, were developed in mice and brought the resolution of HSC lineage tracing closer to the single-cell level (C. J. Eaves, 2015). In the 2000s, single cell transplantation, the prime assay to follow HSC on a clonal level became possible in mice when the immunophenotypically description of HSC was developed enough to isolate HSC based on surface marker expression (Connie J Eaves, 2015) (X). The single cell transplantation technique has however some drawbacks. The technique requires a large number of animals for high throughput. Besides, cells transplanted alone can encounter particular stresses which might influence their behavior.

Single cell transplantation thereby rather assesses HSC potential (under stress) and does not readily reflect clonal behavior during bulk transplantation, or HSC behavior in steady state in situ.

Closer to the clinical transplantation setting, viral integration site (VIS) analysis was the first technique to give an idea of HSC single cell fates during bulk transplantation at higher throughput. Some difficulties have been encountered with VIS detection, as low sensitivity and detection biases, but several improvements have been developed, and the technique is still used in mice and primates (Bystrykh, Verovskaya, Zwart, Broekhuis, & de Haan, 2012) (Sanggu Kim et al., 2014). Interestingly, VIS analysis is also performed today in some first gene therapy trials in human, for example in Wiskott-Aldrich syndrome patients (Aiuti et al., 2013) (Biasco et al., 2016) (Scala et al., 2018).

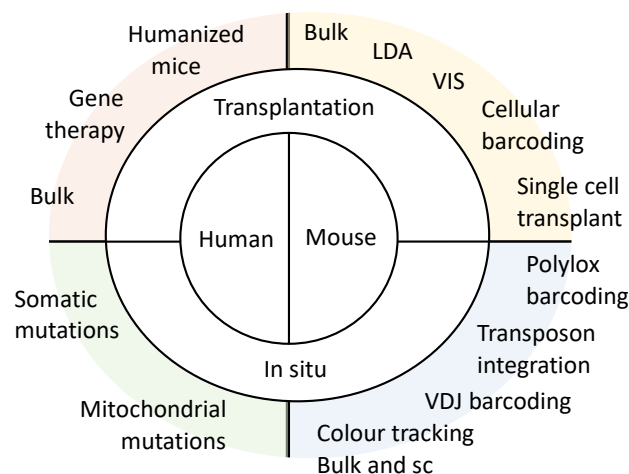


Figure 2: Overview of the different lineage tracing methodologies applied to follow the fate of HSCs. (LDA= limited dilution assay, VIS= viral integration site).

An improvement to VIS analysis is the cellular barcoding technique, in which not a viral insertion site, but an artificially introduced DNA fragment, the “barcode”, and its inheritance by daughter cells is traced from HSC to different lineages (see also next section). In combination with next generation sequencing (NGS) barcode detection cellular barcoding allows today the most accurate quantitative assessment of single cell fates at high throughput. It has been applied in mice (Naik et al., 2013) (Perié, Duffy, Kok, de Boer, & Schumacher, 2015) (Gerrits et al., 2010) (Brewer, Chu, Chin, & Lu, 2016a) (Lu, Neff, Quake, & Weissman, 2011) (Lu, Czechowicz, Seita, Jiang, & Weissman, 2019) (Aranyosy, Thielecke, Glauche, Fehse, & Cornils, 2017) (Evgenia Verovskaya, Broekhuis, et al., 2014) (E. Verovskaya et al., 2013) (Cornils et al., 2014) (L. V. Nguyen et al., 2014a), in humanized mice or xenografts (Cheung et al., 2013) (Merino et al., 2019) (Lan et al., 2017), and primates (Wu et al., 2014) (Koelle et al., 2017) (K. R. Yu et al., 2018).

In situ lineage tracing techniques are emerging

Techniques for the tracing of HSC lineages in situ, meaning in steady state without transplantation, were developed only recently. In 2012 a first trial was performed to apply VIS analysis to the in situ setting through the intra-femoral injection of viral vectors in mice. Viral integration in HSCs was however not very efficient, and results on the in situ hematopoiesis thereby inconclusive (Zavidij et al., 2012). Few years later, the in-situ HSC tracing really took hold when several groups developed, nearly simultaneously, inducible HSC reporter mice, allowing bulk CreERT2-based HSC lineage tracing in steady state. The reporter systems developed include for adult mice today the Fgd5 reporter (Säwen et al., 2018) (Gazit et al., 2014), the Tie2 reporter (Busch et al., 2015), the Pdzk1ip1 (Map17) reporter (Sawai et al., 2016) (Upadhaya et al., 2018), and the Krt18 reporter (Chapple et al., 2018b).

Furthermore, recently, major advancements were made on the development of techniques to track HSC at the single cell level in situ in mice. Four such systems are available today. The Camargo group developed a lineage tracing system in which the integration of an inducible transposable genetic element is followed through HSC differentiation. This was first published in 2014 (Sun et al., 2014). In a second recent study the quantitative aspect of the system was improved (Rodriguez-Fraticelli et al., 2018). A second in situ single cell lineage tracing system is a polylox system developed by the Rodewald group. Here the random recombination of different loxed reporter genes generates an inheritable “polylox barcode” which can be retrieved by sequencing. The system is quantitative and compared to the transposon integration system of the Camargo group not affected by barcode insertion sites. To date it has however mainly been applied to follow the fate of embryonic HSC (Pei et al., 2017). Similar to the polylox system, the Scadden group, developed the “HUE” strain in which fluorescent protein encoding genes are recombined to provide a range of colors which can be tracked (V. W. C. Yu et al., 2016). Finally, a fourth in situ single-cell lineage tracing system, the barcode mouse (“BCM”) strain, was developed by the Schumacher group. Here, barcodes are generated through the recombination of artificially introduced VDJ sequences (Thesis Jeroen W.J. van Heijst, 2010). A first use of this mouse to follow T-cell fate was published (Gerlach et al., 2013). To date the system has not yet been applied to the adult mouse HSC setting, but first studies are ongoing (unpublished as of June 2019). Lineage tracing of human HSCs at the single cell level in situ is developing too. Over the two last years, three groups published single-cell lineage reconstructions of the human hematopoietic differentiation landscape based on somatic mutations (Osorio et al., 2018) (Lee-Six et al., 2018), and mitochondrial mutations (Ludwig et al., 2019). The resolution and throughput of these reconstructions is to date low, but has certainly great potential. It is the only technique available to trace human healthy hematopoiesis in vivo.

All in all, major advancements have been made in HSC research in the development of different lineage tracing systems for the transplantation and steady-state setting. To date cellular barcoding remains the best source for quantitative information on HSC single cell fates in the clinically relevant transplantation setting.

1.3 Lineage tracing of HSC at the single cell level by cellular barcoding

Cellular barcoding allows quantitative assessment of clonality

Cellular barcoding is a lineage tracing technique which relies on the introduction of an artificially created DNA fragment, the “barcode”, in the genome of a cell through viral transfection. When the barcoded cell divides, its “barcode” is inherited by all daughter cells and their subsequent progeny. The analysis of the barcode identity of the progeny cells by sequencing thereby reveals from which originally barcoded cell they originated. When the progeny cells are separated by specific characteristics (e.g. immunophenotypically), it can thus be inferred if the originally barcoded cell gave rise to cells with these particular characteristics. Besides, as the abundance of the “barcode” reads during detection by sequencing is proportional to the number of cells with this barcode- barcode analysis can also give quantitative information. If different subsets of progeny cells are analyzed separately, it can so be determined not only if the originally barcoded cell gave rise to a certain progeny subset, but also how much of its progeny is present in one cellular subset compared to the other. If the progeny of several barcoded cells is analyzed simultaneously their output patterns can further be compared between each other. Applied to HSC research, the “classical” cellular barcoding experiment will so have the following set-up: an HSC is isolated, barcoded, and transplanted into a recipient mouse. After a certain time, mature donor cells from different lineages are sorted and their barcode identity analyzed. This will give information on: 1) which lineages the originally barcoded HSC produced and 2) how much of each lineage it produced. Through the barcoding and transplantation of hundreds of HSCs in a single mouse, the fate of hundreds of single HSCs can be retrieved, analyzed and compared simultaneously.

Already in the 90s DNA-tags were applied to follow cellular fates (Golden, Fields-Berry, & Cepko, 1995). Recently however, new barcode detection methods substantially improved the quantitative aspect of the technique and increased the possible throughput of barcoding experiments. The cellular barcoding technique found new applications. In 2008 Schepers et al. were the first to use a microarrays to detect cellular barcodes in T cells (Schepers et al., 2008). Shortly thereafter, the switch to barcode detection by sequencing, notably NGS by several groups occurred (Gerrits et al., 2010) (Cornils et al., 2014) (Lu et al., 2011) (E. Verovskaya et al., 2013) (Gerlach et al., 2013). And currently at least seven different cellular barcoding libraries are available (Table 1). Each of these is linked to

different strategies to overcome methodological problems, in the initial barcode library design and virus production, but also in the later application of the barcode library in experiments. The major methodological problems being: to guarantee the introduction of only one barcode per cell and to avoid integration of one barcode in several cells, the discrimination of true barcodes from errors/noise generated during PCR and sequencing, and the avoidance of functional effects by barcode integration.

Cellular barcoding libraries have different designs

The currently available cellular barcoding libraries differ markedly in their complexity, as well as the degree to which library production has been controlled, and the design adapted to avoid methodological problems during the subsequent use of the library (Figure 3) (Table 1 and 2).

We employed here a new barcoding library (LG2.2) consisting of barcodes of random stretches of 20 nucleotides. In general, the design of the barcodes across available libraries varies widely in lengths (12- 98 bp, random), and sequence (Figure 3) (Table 1). Random, semi-random, and semi-random sequences with controlled GC-content are used. A recurrent, and likely the least error-prone, barcode design, is a barcode made of random doublets separated by fixed triplets. This design was first introduced by Gerrits et al. but has also been implemented in some newer libraries (Gerrits et al., 2010) (Thielecke et al., 2017) (Cornils et al., 2014). It is meant to avoid accidental generation of restriction enzyme recognition sites, and sequence homologies which can result in secondary structure formation and hinder barcode amplification and analysis by PCR and sequencing (Gerrits et al., 2010), (Cornils et al., 2014) (Thielecke et al., 2017). In one new library three wobble bases and the Illumina adapter sequences were included in the barcode design to allow Illumina phasing, and to avoid PCR cycles during barcode amplification (which can introduce PCR errors) (Thielecke et al., 2017).

Besides, the barcode sequence itself, the different libraries also vary in the degree to which additional sequences allow for multiplexing of the library and selection of barcoded cells (Figure 3) (Table 1). Most commonly, and also the case for the library we employ, the barcode is linked to the expression of GFP without possibility for multiplexing. In the libraries by Cornils et al and Thielecke et al. other fluorescent reporter proteins are employed too and are also used for the multiplexing of the library, in that different fixed triplet sequences of the barcode are linked to different fluorescent reporters. The library by Lu et al. allows multiplexing through library ID sequences which are part of the cell barcode (Lu et al., 2011), (Wu et al., 2014). In the library by Bhang et al (not applied to HSCs) puromycin is used for selection of barcoded cells (H. C. Bhang et al., 2015).

We employ here a barcode library which's barcodes are expressed together with GFP through a CMV promoter. This allows the detection of barcodes from RNA (see Chapter II). Some other libraries are especially designed to avoid barcode expression and any biological effects this could have (Figure

3) (Table 1). Some libraries allow for transgene expression (Cornils et al., 2013). Furthermore, the libraries differ widely in the promoters for barcode and reporter genes, and in the (lenti-)viral vectors used (Figure 3) (Table 1).

During their production, barcoding libraries differ in the presence or not of PCR amplification prior to vector ligation, and the extent of bacterial cloning ((no, partial, or complete) see also (Bystrykh & Belderbos, 2016)) (Figure 3) (Table 1). This is also highly related to library complexity (Figure 3) (Table 1). While most barcode designs, allow for a very large number of possible barcode sequences, the actual size of a barcode library will depend on its production method. For the barcode library we employ here, oligo DNA stretches were ordered and amplified by 10 rounds of PCR before cloning in pRRL-CMV-GFP plasmids and transformation of ElectroMaxStbl4 cells. The 20 bp design of the barcode would allow for over 10^{12} unique barcode sequences. However only approximately 16 000 colonies were picked for amplification and virus production by transfection of HEK293T cells. Likewise, the barcode design by Gerrits et al for example allows for a maximal diversity of above 4 mio barcodes, because bacterial cloning was performed and controlled, the size of the actual library used however is only around 800 barcodes (Gerrits et al., 2010). An even more drastic size reduction exists for the curated, and Hamming-distance controlled “ABC”-library, which is composed of 343 barcodes out of $1,8 \times 10^{19}$ possible ones (Thielecke et al., 2017).

Cellular barcoding experiments can be controlled

Besides in initial library design and production, the available barcode libraries also differ in the strategies used to avoid methodological problems during their application in experiments (Figure 3) (Table 2). To avoid integration of several barcodes in one cell, and of one barcode in several cells, in general the number of cells transduced and the multiplicity of infection are adapted to the actual size of the library (Figure 3) (Table 2). Sometimes further precautions are taken. For the experiments of this thesis, we did adapt the number of cells transduced and the multiplicity of infection (10% transduced cells) to the actual size of the library ($> 10\,000$). Besides we employed one transduction batch for transplantation in several mice, which allows to check back on repeat use of barcodes (Naik et al., 2013). Another strategy developed to control this aspect is a confidence-interval based filtering to remove multiple barcode integration events (L. V. Nguyen et al., 2014a).

Several measures have been taken during PCR, sequencing, and the subsequent analysis to allow to distinguish detection of true barcodes from errors/noise (Figure 3) (Table 2). During PCR it is generally tried to keep PCR cycles low which has been shown to reduce PCR errors best (Thielecke et al., 2017). We employed here, a three-step nested PCR, to amplify the barcode sequence, add the Illumina sequencing primer sequences and a 8bp plate index, and finally the P5 and P7 flow cell

attachment sequences and a 7 bp sample index. Besides, as described before and applied by several groups we split samples in two during PCR. This allows to filter out erroneous or low-confidence barcodes by comparison of the two replicates subsequently (H. C. Bhang et al., 2015) (Gerrits et al., 2010) (Naik et al., 2013). A parameter to control during sequencing is the sequencing depth (Figure 3) (Table 2). To date this has not been well documented. We implemented here a sequencing of 50 reads/ barcoded cell.

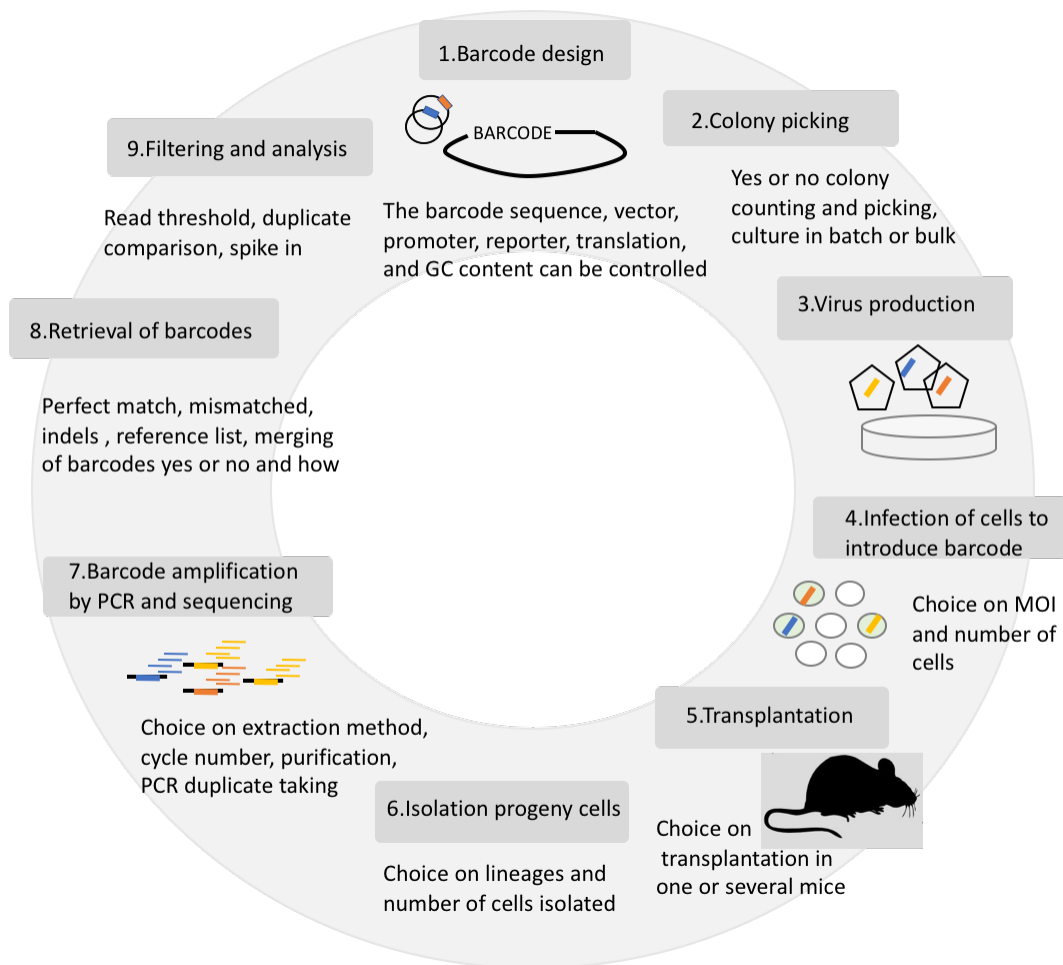


Figure 3: An overview of cellular barcoding library production and application. Many steps are required to perform a cellular barcode experiment. Barcode library design, production, and application, as well as the analysis of cellular barcoding results offer many possibilities to control methodological problems as the introduction of only one barcode per cell and repeat use of a same barcode, the discrimination of true barcodes from errors/noise generated during PCR and sequencing and the avoidance of functional effects by barcode integration.

Table 1: An overview of cellular barcoding libraries I.

Library	Possible diversity	Actual size	Barcode setup	Expression	Express selection marker	vector	multiplexing	References
Lu et al 2011	$1,8 \times 10^{16}$	> 80 000	27 bp random cellular barcode	no	GFP	lentiviral	18 library /IDs of 6 bp	Brewer et al 2016, Nguyen et al 2018, Lu et al 2019
Wu et al 2014			35bp (2x) or 30bp (1x) random barcode as in (Lu et al 2011)	no	GFP	lentiviral	library ID of 6 bp	Koelle et al 2017
Bhang et al 2015 "ClonTracer"	1×10^9	73×10^6	15 repeats of A or T-G or C	no	puromycin	lentiviral	no	
Cornils et al 2014 RGB-barcode library (BC16)	4×10^9	5×10^5	8 pairs of random nucleotides separated by fixed triplets		GFP, mCherry, Venus, Cerulean	lentiviral	Order of fixed triplets matched to reporter	
Thielecke et al 2017 ABC-library (BC32)	$1,8 \times 10^{19}$	343	32 random nucleotides separated by fixed triplets	both	GFP, BFP, Venus, Tsapphire	alpha-retroviral and lentiviral	Order of fixed triplets matched to reporter	Aranysosy et al 2017
Gerrits et al 2010	4×10^6	800	6 sets of random nucleotides separated by fixed triplets	yes	GFP	retroviral		Belderbos et al 2017, Verovskaya et al 2013, Verovskaya et al 2014,
Nguyen et al, 2014	4×10^6	2×10^5	as in (Gerrits et al 2010)	yes	GFP	lentiviral		Cheung et al 2013, Nguyen et al 2015, Lan et al 2017
Naik et al 2013	2×10^4	2500	98 bp barcode ((N)8-(SW)5)5-N8 as in (Scheepers et al 2008)	yes	GFP	lentiviral		Perié et al 2015, Merino et al 2019, Lin et al 2018
This thesis		>10 000	20 bp random nucleotides	yes	GFP or Thy1,1	lentiviral		
Ludwig et al 2019			30 bp random nucleotides	no	mNeonGreen	lentiviral		

Table 2: An overview of cellular barcoding libraries II.

Library	one barcode per cell	PCR+sequencing	Identification of barcodes	Merging barcodes	read threshold
Lu et al 2011	Calculated to have 95% of cells with single barcode (cells 500-1500, MOI of 50%)		mismatches and indels up to 2 bp in barcode. Perfect match for library ID	no	algorithm-generated background threshold
Wu et al 2014	MOI 25	28 PCR cycles	mismatches and indels up to 2 bp in barcode. Perfect match for library ID	no	algorithm-generated background threshold
Bhang et al 2015 "ClonTracer"	MOI for 10%	PCR in duplicate, Phred quality score of at least 10 for all base pairs and average 30	Perfect match to W5X15 pattern, and constant sequence	Merging of barcodes based on hamming distance and relative abundance (HD 1 and 1/8, HD 2 and 1/40)	at least 2 reads
Cornils et al 2014 RGB-barcode library			Perfect match in all 22 nonrandom positions		at least 10 reads
Thielecke et al 2017 ABC-library (BC32)		a single PCR or min 20 cycles, Phred score average 30	Match to fixed triplets with one mismatch allowed	Use a Hamming distance 1/4 of total barcode length max to merge barcodes on readcounts	
Gerrits et al 2010		PCR in duplicate	Perfect match to sample tag and primer sequence	The lower frequency barcode is removed in barcode pairs differing by a single nucleotide	barcodes with frequencies under 0.5% of total within the sample are removed. Samples with < 1000 reads are excluded.
Nguyen et al 2014	Cl-based filtering to remove multiple barcode integration events, Calculated to have >90% of cells with singel	40 PCR cycles, minimum base Phred of 20	Allow 3 mismatches in constant region	Single, double, and triple mismatches are summed sequentially	Spiked in controls to set threshold
Naik et al 2013	MOI for 5-10%, barcoding low number of cells, One transduction batch for several	PCR in duplicate	Perfect match to barcode reference list	no	at least 10 000 reads per sample, filtering on presence, readcount difference, and correlation between
This thesis	MOI for 10%, barcoding low number of cells, One transduction batch for several	PCR in duplicate, 45 PCR cycles	Perfect match to barcode reference list	no	at least 5000 reads per sample, filtering on presence and correlation between replicates, noise removal to match
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A large number of strategies to filter barcode sequencing results exists and aims at discriminating true barcodes from noise (Figure 3) (Table 2). Different groups employed a minimal Phred score for each base (L. V. Nguyen et al., 2014b), or the average of all bases making up the barcode sequence (Thielecke et al., 2017). Once quality filtered, the actual barcode sequences are retrieved from the sequencing data by a match to the barcode pattern. We did so without allowing mismatches or merging barcodes, as described before and employed by several groups (Naik et al., 2013) (Gerrits et al., 2010). Other groups do allow mismatches of few bases (Thielecke et al., 2017), or mismatches and indels (Lu et al., 2011). Also, different strategies have been used to merge barcodes which differ in only few nucleotides, and result likely from PCR or sequencing errors (are “descendants” of each other) (Figure 3) (Table 2). Sometimes the lower abundant of two similar barcodes is crudely omitted (Gerrits et al., 2010). Other groups add the reads of the lower abundant similar barcode to the reads of the most abundant similar barcode (likely the true barcode from which “descendants” were derived) (H. C. Bhang et al., 2015) (Thielecke et al., 2017) (L. V. Nguyen et al., 2014b). Different thresholds to establish similarity have been used in this process (Thielecke et al., 2017) (L. V. Nguyen et al., 2014b). We employed here furthermore a perfect match to a barcode reference list established through sequencing of the library before its use (this also implies omitting reads of “descendants”) (Naik et al., 2013).

Finally, different read number thresholds have been implemented to remove sequencing noise. We implemented here a read threshold which equalizes the sharing of barcode between mice in a same sequencing run to the average barcode sharing of a same transduction batch between sequencing runs. Other groups employed fixed read thresholds (Naik et al., 2013), read thresholds established by an algorithm (Lu et al., 2011), or adapted to the total read sum of each sample (Gerrits et al., 2010).

Cellular barcoding allows for the high throughput quantitative assessment of clonality after transplantation. A large number of different barcoding libraries is available today. These are linked to different strategies on how to avoid methodological problems as the introduction of a unique barcode in each cell and the discrimination of true barcodes from errors/noise generated during PCR and sequencing. Recent studies dedicated to methodological challenges will further improve the reliability of cellular barcoding experiments (Deakin et al., 2014)(Beltman et al., 2016)(Thielecke et al., 2017)(Blundell & Levy, 2014)(Bystrykh et al., 2012)(L. Zhao, Liu, Levy, & Wu, 2018) (Naik, Schumacher, & Perić, 2014). A direct comparison of the different barcode filtering strategies will help in this process.

Functional effects by barcode integration will stay the most difficult aspect to control. For the bulk of barcoded cells the percentage in different lineages can be compared (Naik et al., 2014). The effect of barcode integration on every single cell is however difficult to assess as long as the integration site is not controlled. Vector choice and avoiding translation of barcodes can help. Also arrayed cellular barcoding can increase control over the effects of barcode expression (Grosselin, Sii-Felice, Payen,

Chretien, Tronik-Le Roux, et al., 2013). Nevertheless, it is important to state that for most of the available barcoding libraries, the quantitative aspect and sensitivity have been experimentally validated. The use of cellular barcoding for the lineage tracing of single cells has already led to interesting results which complement the results from other lineage tracing methods. Notably, HSC have been described as lineage-“biased” in their differentiation.

1.4 Lineage tracing reveals heterogeneity in HSC output- HSCs are “biased”

Traditionally, hematopoiesis models assumed that every individual HSC would be multipotent and produce the same amount of each lineage. In recent years however, different studies tracing the differentiation of single HSCs revealed a high heterogeneity, or “biases”, in their lineage output. Meaning that some HSC, while being multipotent, produce relatively more cells of one lineage compared to another. Some studies challenged the current HSC definitions altogether by seeing within the immunophenotypically defined HSC population, not only cells with lineage “biases”, but even lineage restrictions. The heterogeneity of HSC has profound implications for our understanding of hematopoiesis in steady state, under homeostatic challenges, during pathologies, and for clinical applications.

Many ways to define HSC “biases”-how biased is the bias?

Dykstra et al. were some of the first to set criteria for the definition of HSC lineage “biases” (Dykstra et al., 2007). In this single-cell transplantation study, HSCs were assigned to four different categories (α , β , γ , δ) based on their relative donor percentages in the myeloid (granulocyte and monocyte) and the lymphoid lineage (B and T-cells). The α -HSC corresponded to “myeloid biased” HSCs with a myeloid to lymphoid ratio $((G+M)/(T+B))$ of above 2. Beta-HSC represented “balanced” HSC with a ratio of 0.25-2. Cells with ratios <0.25 were further divided in the “lymphoid biased” γ - and δ -HSC categories, taking changes over time into account. Cells which at 16 weeks after transplantation still contributed to both the myeloid and lymphoid lineage with a $\geq 1\%$ chimerism level were assigned to the γ -HSC category. Cells for which the relative lymphoid output increased till 16 weeks (myeloid $<1\%$ chimerism at 16 weeks), were classified as δ -HSCs (Dykstra et al., 2007). The HSC bias definition by Dykstra et al. was subsequently applied in several subsequent single-cell transplantation studies, sometimes even when other lineages (as the erythroid lineage) were analyzed as well (Morita, Ema, & Nakauchi, 2010) (Oguro et al., 2013).

Many other HSC “bias” definitions have been developed later on. Often when considering a myeloid/lymphoid ratio as in Dykstra et al. only three HSC types, “myeloid-biased”, a “lymphoid-biased” and “balanced” HSC have been distinguished. This was for example the case in a VIS study by

Sanggu Kim et al (1:3, and 3:1 ratios defined biases) (Sanggu Kim et al., 2014). Also a recent cellular barcoding study by Lu et al. uses this three categories although with a different threshold. Lineage biased clones are here defined as the ones that produce more than 2,4 times the relative copy numbers in the other lineages (Lu et al., 2019). When other lineages are taken into account sometimes more general rules have been developed as a ≥ 10 fold fractional representation in one lineage (Wu et al., 2014). In a recent study by Sanjuan-Pla et al. a very high ratio of $>50\%$ relative lineage output was used to define bias (Sanjuan-Pla et al., 2013). As in the study by Dykstra et al., a recent study by Carrelha et al. used different thresholds for different lineages based on kinetic considerations. Here a sustained threefold higher lineage output compared to remaining lineages was defined as bias. For the lymphoid bias however, only platelets and myeloid cells, and not necessarily erythroid cells, were considered “owing to their slower decline after HSC exhaustion” (Carrelha et al., 2018). Sometimes the term HSC-bias has been used without clear definition (V. W. C. Yu et al., 2016).

Besides, lineage biases, also lineage restrictions of HSCs have been described. Meaning that cells within the HSC gate are not all multipotent (they do not produce all mature hematopoietic cell lineages), but still have a (substantial) capacity for self-renewal. Sometimes such lineage restriction have been clearly separated from lineage bias (Carrelha et al., 2018) or (Brewer et al., 2016a). Sometimes HSC lineage restrictions have been included in HSC lineage bias categories (Lu et al., 2019).

HSC “bias” definitions have to consider several aspects to be accurate

Different aspects can influence the accuracy of HSC lineage bias definitions (Figure 4). Firstly, the sampling of cells to assess lineage production can play an important role. Hematopoietic cells are widely distributed over the body and the sample taken to assess HSC-lineage contribution might not be representative of its contribution to the lineage in all organs. In some studies, this aspect has been considered, and measures have been taken to minimize sampling error. In a recent study by Lu et al. peripheral blood cells were for example obtained through animal perfusion and analyzed in their entirety. Besides, bone marrow cells were gained from all limb bones by crushing (Lu et al., 2019). Sampling is also related to the detection sensitivity of the lineage tracing technique employed.

A second aspect important for the accuracy of HSC-bias definitions is the consideration of differentiation kinetics. The lifespan of hematopoietic cells can vary widely. Murine erythroid cells live around 40 days, platelets around 5 days, while lymphoid cells as B and T can live for many years till a life-time (O’Connell et al., 2015). The studies by Dykstra et al and Carrelha et al took this into account to some extent (Dykstra et al., 2007) (Carrelha et al., 2018). Besides the different life-span of hematopoietic cells, production kinetics can influence bias assessment. The recently developed inducible HSC reporter mice, allowing bulk CreERT2-based HSC lineage tracing in steady state, often focused on this aspect. Consistently, all could report on the faster production of platelets, than of

erythroid cells, than of myeloid, and lymphoid cells in steady state (Upadhaya et al., 2018) (Sawai et al., 2016) (Säwen et al., 2018) (Gazit et al., 2014) (Chapple et al., 2018a) (Busch et al., 2015). Similar, though slower, production kinetics were described on the bulk level after transplantation (Säwen et al., 2018) (Boyer et al., 2019) (Forsberg, Serwold, Kogan, Weissman, & Passegué, 2006) (Pietras et al., 2015a). In situ production kinetics on the single cell level in situ have not yet been described, and also after transplantation only few data is available (Oguro et al., 2013).

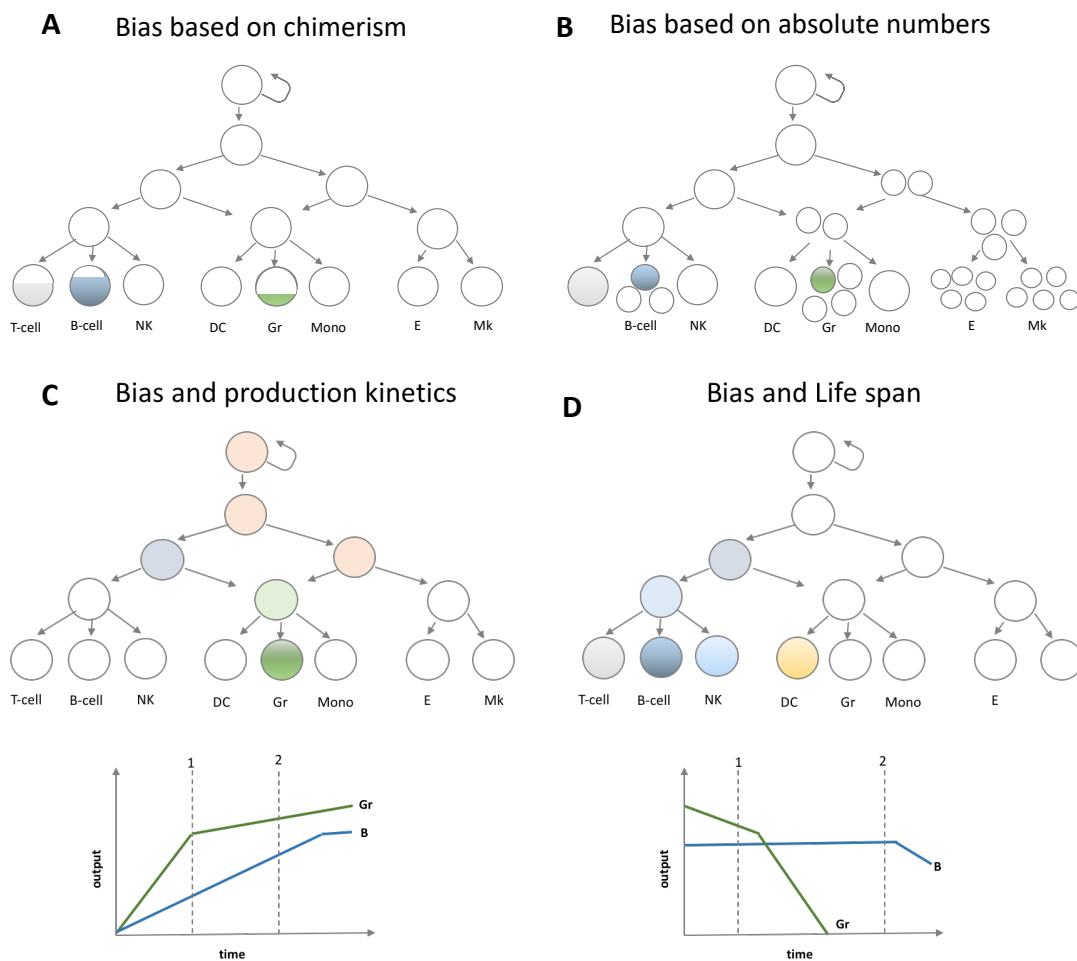


Figure 4: Overview of different aspects influencing HSC lineage bias. (A-D) Bias can be defined based on chimerism (A) or absolute cell numbers (B). The production kinetics (C) and the life span (D) of different lineages can be considered. For (C-D) dashed lines represent different analysis timepoints after the production of lineages from an HSC. The choice of this timepoint will have a big influence on the detected “bias” of the HSC. For (D) the analysis timepoint 1 will give a myeloid-biased profile, while analysis timepoint 2 will give a lymphoid-restricted profile.

Finally, a third aspect to consider for an accurate HSC-bias definition are the absolute numbers of hematopoietic cells. When the ratio of donor chimerism percentages in different lineages is used to set the thresholds for HSC-biases it is often not completely acknowledged that a same chimerism can represent greatly differing cell numbers. A study by Boyer et al, recently studied this aspect. The vast number of erythroid cells made them conclude that the erythroid differentiation is the default HSC behavior (Boyer et al., 2019).

HSC “biases” after transplantation and in steady state

The reports on HSC biases after transplantation in different studies do show some agreement. When restricted progenitors were explicitly analyzed after single cell transplantation Mk-, MkE-, and MkEM- restrictions in lineage output could repeatedly be detected (Yamamoto et al., 2013a) (Carrelha et al., 2018). Also, several cellular barcoding studies seem to detect M-restricted HSC clones (Lu et al., 2011) (Lu et al., 2019) (Brewer, Chu, Chin, & Lu, 2016b) (Wu et al., 2014) (E. Verovskaya et al., 2013) (Evgenia Verovskaya, Broekhuis, et al., 2014) (Koelle et al., 2017) (this can often only be deduced from plots). Erythroid output was only assessed once (Perié et al., 2015) and megakaryocyte output was not assessed in HSC cellular barcoding studies to date. This means M-restriction could include MkEM-restricted HSC as detected in the single cell transplant studies. Cellular barcoding studies often also seem to detect B-, BT-, and T-restricted HSC (Wu et al., 2014) (Lu et al., 2011) (Brewer et al., 2016a) (Evgenia Verovskaya, Broekhuis, et al., 2014). The single cell transplantation studies however do not confirm lymphoid-restricted lineage output patterns. In Yamamoto et al, B-restricted output was not seen (Yamamoto et al., 2013a). In Carrelha et al. B, BM-, and BE-restricted output was not detected, unless preceded by transient multilineage reconstitution (Carrelha et al., 2018). And also in cellular barcoding studies the number of B-restricted HSC is sometimes stated to be low (Koelle et al., 2017). In others however it encompasses a considerable fraction of HSCs (Brewer et al., 2016a).

Biases in HSC lineage output reported are very divers. Studies are difficult to compare once the differently defined bias categories are used for analysis. Some studies give however the “raw data” so that the extent of a bias, and the importance of differently-biased HSC to mature lineage output can be compared. Sometimes this is possible for different timepoints after transplantation. Most commonly analyzed, and thereby the easiest to compare are here myeloid, B, and T lineages. One cellular barcoding study reported a high percentage of lymphoid (B, T, BT) biased HSC (Brewer et al., 2016b). The number of lymphoid-biased HSC in other studies is in general however rather lower than the number of myeloid- and balanced-HSCs (Lu et al., 2019) (Lu et al., 2011) (Evgenia Verovskaya, Broekhuis, et al., 2014) (Koelle et al., 2017)(Wu et al., 2014) (Carrelha et al., 2018) (Oguro et al., 2013). The importance of each category to the total lineage output has been reported differently. In the VIS study by Kim et al. and also in a cellular barcoding study in macaques by Sanggu Kim et al., the greatest proportion of cells

was produced by rather balanced-HSCs at > 5 months after transplantation (Koelle et al., 2017) (Sanggu Kim et al., 2014). In a study by Lu et al., dominant and non-dominant clones exhibited similar proportions of lineage bias and balance when transplanted after irradiation. When transplanted after ACK2-treatment however, dominant clones did exhibit lineage biases, whereas nondominant clones exhibited lineage balance (Lu et al., 2019).

Indeed, several studies reported that the transplantation conditions can influence the type and number of biased HSCs. Both the studies by Carrelha et al. and by Lu et al. reported that lineage bias was affected by the type of competitor cells transplanted. In the presence of wild type cells, HSC showed higher lymphoid output (Carrelha et al., 2018). Similarly, in Lu et al. increasing the number of helper cells resulted in a higher percentage of lymphoid-biased HSC clones (Lu et al., 2019). Also, Brewer et al. observed less myeloid-producing HSC at higher competitor cell to HSC ratios (Brewer et al., 2016b). Lu et al. also compared the influence of different transplantation conditioning regimens on HSC biases. In this study HSC were biased when transplanted after irradiation and ACK2-treatment. Half-lethal irradiation did produce significantly fewer lymphoid-biased clones and unconditioned transplantation even resulted in Gr to B balanced HSC output. This raises the question if HSC-biases seen after transplantation are also seen in situ.

The techniques allowing lineage tracing at the single cell level in vivo are only emerging and few data on HSC-biases in situ is available yet. Biases seem to be present also in situ. Mainly three studies can be considered. The first one is the study of Yu et al using the “HUE” strain in which fluorescent protein encoding genes are recombined to provide a range of colors which can be tracked to follow HSC differentiation (V. W. C. Yu et al., 2016). Here HSC-biases could be identified and one lymphoid-and myeloid-biased HSC clone was compared. The study does however not give clear statistics on the occurrence and importance of HSC-biases. The second study which can give information on in situ HSC-biases is Pei et al. who used “polylox barcodes” generated by the random recombination of different loxed reporter genes to follow embryonic and adult HSC fate (Pei et al., 2017). “Polylox barcodes” were found in several different lineage combinations. Both multilineage, oligo-lineage, and uni-lineage. From the heatmaps given, HSC multilineage output seem most important, but some EM-biased clones were abundant too. The third study which allows to assess HSC biases in steady state is Rodriguez-Fraticelli et al. who used transposon integration to track HSC differentiation (Rodriguez-Fraticelli et al., 2018). Following the induction of transposon integration, Mk-restricted and Mk-biases could be observed. Besides, EM-, LM, and LEM-restrictions, but not MKE-restricted transposon integrations were detected (Rodriguez-Fraticelli et al., 2018). Even more than in other studies, it is here however unclear how much HSC, and how much progenitor bias was monitored. This also relates again to the question of how much sampling and kinetics influence biases observed.

Is the HSC “bias”-really an HSC “bias”?

Biases in HSC output do not necessarily have to originate at the HSC level if one considers HSC differentiation as a multi-step process. The proliferation of a more committed progenitor subset could result in the observation of an HSC bias too. In other words one can ask, if heterogeneity in HSCs is necessary for peripheral blood clone size heterogeneity or if a neutral model can explain clone size differences (Xu, Kim, Chen, & Chou, 2018). Is the HSC bias really an inherent, stable phenotype of HSC clones? Several aspects are in line with a real “HSC bias”. Some data suggests kinetic phenomena and proliferation of more committed progenitors could play an important role too. In the next sections I will discuss HSC marker expression, the relation of HSC and mature cell clonality, and secondary transplantations with respect to this question.

Speaking for an inherent HSC bias is the fact that markers for the isolation of biased-HSCs have been described. These are for example CD229 (Carrelha et al., 2018)(Oguro et al., 2013), CD86 (Shimazu et al., 2012), CD41 (Gekas & Graf, 2013), BMP-activation (Crisan et al., 2015a), Vwf (Pinho et al., 2018)(Carrelha et al., 2018) (Sanjuan-Pla et al., 2013), Mfn2 (Luchsinger, de Almeida, Corrigan, Mumau, & Snoeck, 2016), Msi2 (Park et al., 2014), Hdc (Xiaowei Chen et al., 2017), CD61 (Mann et al., 2018), and LIGHT (Xufeng Chen et al., 2018), Per2 (J. Wang et al., 2016). Also, the LSK side population (SP) gating of HSCs based on Hoechst 33342 staining has been described to allow discrimination of differently biased HSCs. Myeloid-, and lymphoid-biased HSCs were described to lay in the lower and upper LSK SP gates respectively (Challen, Boles, Chambers, & Goodell, 2010).

The relationship of HSC and other committed progenitor clones, with mature cells based on which lineage biases are defined suggests kinetic phenomena could play a role in “bias” detection. In general, the percentage of HSC clones reported to be found back in mature cells is moderate after bulk transplantation. Repeatedly a number of HSC clones detected through cellular barcodes or VIS analysis have not been found back in mature hematopoietic cells (E. Verovskaya et al., 2013) (Evgenia Verovskaya, Broekhuis, et al., 2014) (Biasco et al., 2016) (Aiuti et al., 2013) (Lu et al., 2019); implying that not all HSC are active after bulk transplantation, but that some can be quiescent, senescent, or self-renewing without differentiation. The clonality of other progenitor compartments has in general been reported to be similar to mature cells (Lu et al., 2011) (Lu et al., 2019) (Biasco et al., 2016), meaning all clones differentiate (to some extent). Some studies allowed to relate the presence and abundance of clones in HSC and other progenitor compartments to lineage biases in the mature subsets. In these studies, both lymphoid and myeloid biased clones could be found back in HSCs which implies that both do have some self-renewal capacity. The percentage of balanced, myeloid-, and lymphoid-biased clones which is present in the HSC compartment was though not the same. In Sanggu Kim et al., the majority of the balanced (57-96%) and myeloid-biased clones (38-80%) was found back in the HSPC pool. In contrast only 17-50% of lymphoid-biased clones were found back (Sanggu Kim et al., 2014). Likewise,

in Aiuti et al. the percentage of lymphoid VIS found back in bone marrow was much lower than for VIS found in myeloid cells (Aiuti et al., 2013). Also, in Carrelha et al., lymphoid-biased and lymphoid-restricted reconstitution, which was always preceded by multilineage reconstitution, was not associated with long-term reconstitution of the HSC compartment. This could be in line with some lymphoid-biases being caused by the long lifespan of lymphoid cells and not reflecting the lymphoid bias of a currently active HSC. Alternatively, lymphoid-biased HSC might self-renew much less than myeloid-biased HSC and be present under the detection limit. To assess the change of biases over time can help to discriminate these two scenarios.

In the most thorough study monitoring biases over time these were found to be “extremely” stable (Koelle et al., 2017). This would again imply an inherent HSC bias. The comparison of bias stability upon repeated transplantation, is however rather contradicting this idea. Cells lying in the immunophenotypically defined HSC gate do not all lead to reconstitution upon secondary transplantation. This is the case for 86% of the myeloid-restricted progenitors identified by Yamamoto et al after single cell transplantation (lymphoid-restriction was not identified in this study) (Yamamoto et al., 2013b). When this was compared between differently biased HSCs it was repeatedly the lymphoid-biased HSCs which showed less reconstitution capacity upon secondary transplantation. In Dykstra et al. 90% of myeloid biased HSC were able to reconstitute upon secondary and third transplantation. None of the lymphoid-biased HSCs could (Dykstra et al., 2007). Likewise, In Grosselin et al., 5/13 of myeloid-biased but only 3/13 lymphoid-biased clones differentiated upon secondary transplantation. All of this could still be caused by a much lower self-renewal of lymphoid-biased HSC than myeloid-biased HSC; lymphoid-biased HSC might not be transplanted. A real hint towards kinetic phenomena underlying biases comes from the comparison of biases before and after secondary transplantation. Repeatedly > 50% of clones did keep their general lineage bias upon secondary transplantation (Dykstra et al., 2007) (Grosselin, Sii-Felice, Payen, Chretien, Roux, et al., 2013)(Carrelha et al., 2018). However, when a switch in output pattern was observed, this was always a switch from a platelet, to a myeloid, to a multilineage, to a lymphoid bias. In Grosselin et al. and Dykstra et al. clones switched for example sequentially from the α to the β , γ , and δ category. In Carrelha et al., where Mk- restricted HSC were transplanted, these either maintained high levels of Mk-restricted secondary reconstitution, or produced some low levels of erythroid, myeloid or even lymphoid cells (Carrelha et al., 2018). Also, in Verovskaya et al the number of lymphoid-restricted clones after secondary transplantation was higher (13/36) than after primary transplantation (6/43). Although here one single clone switched from lymphoid to myeloid cell production (Evgenia Verovskaya, Broekhuis, et al., 2014). In Morita et al., some cells displayed barely detectable myeloid engraftment in primary-recipient mice, but progressive and multilineage reconstitution in secondary recipient mice (the corresponding HSC were termed “latent”) (Morita et al., 2010). The few data available comparing in

situ lineage biases to biases after transplantation describe both a change and stability of bias. In Rodriguez-Fraticelli et al., Mk-restricted HSC become multilineage-outcome HSC after transplantation (Rodriguez-Fraticelli et al., 2018). In Yu et al. stability of lineage biases in situ and after transplantation is seen, but only few clones are assessed and no statistics are given (V. W. C. Yu et al., 2016).

In recent years a high heterogeneity in the lineage output of HSC has been described. Notably Mk-, MKE-, MkEM-restricted, and lymphoid- and myeloid-biased HSC have been described after transplantation and also in vivo. Markers for lymphoid and myeloid-biased HSCs have been described implying bias to be a stable characteristic. However, individual HSC can also change bias and this has been described to follow a constant flow from erythroid, to myeloid and to lymphoid bias. Kinetic phenomena as production kinetics and life span of different hematopoietic lineages might therefore be one cause for the detection of lineage biases. The heterogeneity of HSC has profound implications for our understanding of hematopoiesis in steady state, under homeostatic challenges, during pathologies, and for clinical applications. Also our understanding of cytokine action on hematopoietic cells has been influenced by the discovery of HSC heterogeneity.

1.5 The effect of cytokines on HSC differentiation

HSCs guarantee the production of a particularly large number of different cell types with each having a crucial and specific role for the functioning of our body. When physiological needs change, following invasion of pathogens, malignancies, or mere physical activity, also the requirement for the production of each of these different hematopoietic cell types changes. The fast and adequate adaptation of the hematopoietic differentiation is needed to match the current demand. Hormones and cytokines, as erythropoietin, are important players in this process and can act on cells throughout the hematopoietic tree, including on HSCs.

Cytokines act throughout the hematopoietic tree

For a long time, the enhanced production of specific hematopoietic cell subsets in times of need was mainly considered to result from the induction of proliferation of mature hematopoietic cells, or uni-lineage committed hematopoietic progenitors. A prime example is the proliferation of mature antigen-specific lymphoid cells upon antigen-recognition to guarantee an adaptive immune response after pathogen exposure. Also during innate immune responses this principle was considered to be major. Emergency granulopoiesis, the rapid production of neutrophils after infection, has for example been described to be initiated by the cytokine-mediated induction of proliferation and maturation in committed myeloid progenitor cells (Manz & Boettcher, 2014; J. L. Zhao & Baltimore, 2015). Likewise, erythropoietin was described to induce erythroid cell production in anemic conditions by increasing

survival of committed erythroid progenitors (Richmond, Chohan, & Barber, 2005). Only more recently, the action of cytokines on less committed hematopoietic progenitors gained more/new interest. To allow the production of a specific hematopoietic cell type, cytokines have been envisaged to act here, not by induction of proliferation, but rather through the instruction of lineage, meaning a “biasing” of differentiation. Such effect has for example been shown for M-CSF, and IFN- γ acting on GMP (Rieger, Hoppe, Smejkal, Eitelhuber, & Schroeder, 2009) (De Bruin et al., 2012). More recently, also insulin has been described to induce such bias, namely a lymphoid lineage commitment in MPPs (Xia et al., 2015). All in all, it seems today established that the combination of cytokine-induced proliferation and lineage instruction can regulate hematopoietic differentiation for most hematopoietic progenitors. The action of cytokines on HSC themselves is still more controversial.

Cytokines and HSCs-what, where, and how?

That cytokines can act directly on HSC differentiation is obvious from in vitro culture. Still, the precise action and importance of different cytokine actions on HSCs during fast physiological responses, and especially for the production of specific hematopoietic cell subsets remains unclear. Different cytokines have been shown to regulate proliferation of HSCs in general. Luteinizing hormone (LH), for example, was described to limit HSC expansion of HSC during puberty via the LH-receptor (Peng et al., 2018). Contrary, IFN- α was described to promote exit of HSCs out of a dormant state into proliferation during infections, at least partly via the IFN α -R (Essers et al., 2009). Also for IFN- γ such effect has been suggested (Baldrige, King, Boles, Weksberg, & Goodell, 2010).

Such regulation of proliferation of HSC in general, however can only lead to general increases or decreases of hematopoietic output. For the production of specific hematopoietic cell subsets, two different mechanisms of cytokine actions have been suggested: selective proliferation of “biased” HSC and, as already established for other hematopoietic progenitors, the direct instruction or “biasing” of HSC differentiation. Indeed, for both phenomena some first evidence exists. Two studies from the Challen group suggested cytokine-induced selective proliferation of HSC (Matatall, Shen, Challen, & King, 2014) (Challen et al., 2010). Matatall et al. suggested IFN- γ would selectively promote the differentiation, and thereby depletion, of myeloid-biased HSCs defined based on SP distribution. They tracked down this effect to a 20-fold increased expression of IFN- γ receptor 1 in myeloid-biased HSCs (Matatall et al., 2014). Likewise, Challen et al. described TGF- β as inducer of proliferation for myeloid-biased, but not lymphoid-biased HSCs (Challen et al., 2010). Two other studies suggested cytokine-induced lineage instruction on the HSC level (Mossadegh-Keller et al., 2013) (Grover et al., 2014). In a study by Mossadegh-Keller et al. the effect of M-CSF on HSC differentiation was assessed. It could be established using video imaging that culture of HSCs with M-CSF increased the expression of myeloid genes including the myeloid master transcription factor PU.1. Also in vivo, high systemic levels of M-

CSF directly stimulated M-CSF receptor-dependent PU.1 expression. Furthermore, the transplantation of in vivo M-CSF primed HSC in mice resulted in an increased ratio of GMP to MEP progenitors in spleen after 2 weeks and an increased myeloid to lymphoid cell ratio in peripheral blood after 4 weeks. All of this led the authors to conclude that M-CSF directly induced a myeloid lineage bias in single HSCs. A recent study could however not confirm parts of the study, namely the M-CSF induced expression of the PU.1 in HSC in vitro (Etzrodt et al., 2019). The effect of M-CSF on HSC stays thereby open. The second study suggesting cytokine-induced lineage instructions on the HSC level, studied the effect of EPO on HSCs (Grover et al., 2014) (see also next section). Grover et al. could observe that increased systemic EPO levels, changed the ratio of erythroid to other lineage-producing hematopoietic progenitors. Besides, in vitro exposure of HSC with EPO changed their transcriptome, increasing erythroid-, and decreasing the expression of myeloid-specific genes. Finally, transplantation of in vivo EPO-exposed HSCs in mice resulted in higher donor chimerism in erythroid cells than in myeloid cells in peripheral blood after 4 weeks. The authors concluded, that EPO induced an “erythropoietic superhighway” by skewing differentiation towards the erythroid lineage at every bifurcation in the hematopoietic tree starting from the HSC. As EPO was administered systemically in the study by Grover et al. it is however not clear if the effect of EPO on the differentiation of HSC was really direct, and linked to the transcriptional changes observed after in vitro culture with EPO.

Different mechanisms have been suggested by which cytokines can act on hematopoietic progenitors and HSCs, to increase the production of one specific hematopoietic lineage when needed. First evidence for different mechanisms has been given, but data is to date rather sparse and not fully conclusive. This is also the case for the cytokine EPO. The action of EPO on HSC and other (hematopoietic) cell subsets remains to be reconciled.

1.6 Erythropoietin and HSCs

Erythropoietin-classical view and new applications

Among all the different hematopoietic cells originating from HSCs, one cell type dominates in terms of numbers and turn-over. Of the 90% of our bodies cells comprised of hematopoietic cells, around 84% have been approximated to be erythrocytes (Sender et al., 2016). With an estimated 1% of daily renewal (Koury, 2016), erythropoiesis, the generation of erythrocytes from HSC, is thereby among the most productive hematopoietic processes. In the classical model of hematopoiesis, erythropoiesis proceeds from the MEP, to the generation of the restricted erythroid blast forming unit (BFU-E) and colony forming unit (CFU-E) progenitors, which further differentiate into pro-erythroblasts (ProEB), erythroblasts, and finally reticulocytes. These reticulocytes mature in the blood stream into the terminally differentiated erythrocytes which transport oxygen throughout the body (Koury & Haase,

2015). Considered as the major regulator of erythropoiesis is the cytokine erythropoietin (EPO), a 30 kDa, highly glycosylated protein, mainly expressed by a subset of adult renal peritubular interstitial cells. Although EPO is constantly secreted by these cells (with circadian rhythm) to guarantee the normal physiological erythroid turn-over, its production increases dramatically in hypoxic conditions (up to 1000 fold from the homeostatic serum concentrations of 2,6-18,5 mIU/ml (Mayo clinic) (Grote Beverborg et al., 2015) (Kaushansky, 2006)) to augment erythrocyte production. On a molecular level, this effect has been attributed to an EPO-induced increase in viability of CFU-E and ProEB. Both CFU-E and ProEB express the EPO receptor (EPOR) dimer in complex with other associated proteins as the transferrin receptor 1 and 2 (Tfr1/2), and Scribble at the highest recorded levels (1000 EPOR/cell)(Khalil et al., 2018) (Forejtnikova et al., 2010). Through binding of EPO to the EPOR complex, conformational changes in the receptor are thought to activate a cascade of signaling pathways from Janus kinase-2 over STAT5, Erk, PI3K-Akt, STAT3, which finally lead to an increased expression of anti-apoptotic proteins as Bcl2, and a decreased expression of apoptotic proteins as Fas and Fas ligand (Richmond et al., 2005). Thereby, EPO is believed to inhibit a local negative feedback loop based on Fas and Fas ligand expression of the erythroid progenitors at their site of production (Socolovsky, 2007). The potent action of EPO in increasing erythroid cell production, made recombinant EPO become an important medication. Indeed, recombinant EPO is today one of the most sold biopharmaceuticals in the world (Walsh, 2014) and different EPO formulations are routinely used in the clinics to counteract anemia in many different settings, including chronic renal failure, malignancy, prematurity, HIV infection, post-chemotherapy, post-radiotherapy, post-transplantation, or during surgeries when blood transfusion is disallowed.

Aside the very established function and use of EPO in the regulation of erythropoiesis, other functions and working mechanisms of the cytokine have been acknowledged more recently. EPO has for example been shown to be produced in several other organs, including the skin, liver, brain and bone marrow, often in a less sialated form but at high concentrations, implying a rather paracrine/autocrine action (Brines & Cerami, 2012) (Rankin et al., 2012) (Su et al., 2015) (Nairz, Sonnweber, Schroll, Theurl, & Weiss, 2012). Besides, different receptors for EPO and/or EPO peptides have been identified as mediators for the erythroid, but also neuroprotective, and anti-inflammatory effects. EphB4 has been identified as alternative EPOR involved in tumor growth and progression (Pradeep et al., 2015), but also implicated in erythropoiesis, through its expression on human erythroid progenitors in BM and human umbilical cord blood (Suenobu et al., 2002). CD131, usually part of the IL3, IL5, and GM-CSF receptor, has been described to bind EPO, or EPO-peptides, alone or as part of a heterodimer with the EPOR called tissue-protection receptor (TRP) (Cheung Tung Shing et al., 2018). The TRP was described to mediated anti-inflammatory actions. Finally, CRFL3, which might be involved in the negative regulation of cell cycle progression (F. Yang et al., 2009) was recently identified as alternative EPO

receptor in insects (Hahn et al., 2017). Still to be elucidated remain also the role of two isoforms of the EPOR generated by alternative splicing: a soluble protein that lacks the transmembrane and intracellular domains, and a truncated EPOR that lacks large parts of the intracellular domains.

EPO has an established function in the regulation of erythropoiesis for which it is widely used in the clinics. The additional functions of EPO discovered recently will, and already do, foster the application of EPO in even more clinical settings. To avoid side effects in all of these circumstances it becomes more and more important to understand the effect of EPO on stem cells as the HSC.

Erythropoietin-and HSCs

The induction of erythropoiesis by EPO acting on the committed erythroid progenitors CFU-E and ProEB is well established. Less studied has been the possibility that EPO might be acting on HSC, in an instructive or selective way. Indeed, the main EPO receptor, EPOR, is highest expressed on CFU-E and ProEB suggesting a major action on these cell subsets. This does not mean however, that other hematopoietic cell subsets would be devoid of functional EPOR expression. Several studies described EPOR gene expression in less committed hematopoietic progenitors, including HSCs (Akashi et al., 2000; de Graaf et al., 2016; Forsberg et al., 2006; Mooney et al., 2017; Nakauchi et al., 1999; Shiozawa et al., 2010). These levels might be lower than in CFU-E and ProEB for the bulk HSC population, however on the single cell level sufficient for effective EPO-signaling, given the reduced expression of cell internal threshold setters in HSC (Sarrazin & Sieweke, 2011). Indeed two studies did demonstrate EPOR protein on a low percentage (5-10%) of HSC (Shiozawa et al., 2010) (Suszynska et al., 2014) (Others did not (Singbrant et al., 2011)). Also alternative EPO receptors are expressed on HSC. EphB4 was first cloned in HSC, and has been implicated in erythroid and megakaryocyte HSC differentiation (Z. Wang et al., 2002) (Bennett et al., 1994). The alternative EPO receptor CRFL3 is widely expressed among hematopoietic cells, also on HSCs. Despite all these reports on the expression of EPO receptors on HSC, the effect, and notably the direct effect of EPO on HSC has not often been considered and remains controversial.

Older studies have associated EPO to a differing rate of HSC differentiation or to increased HSC proliferation, without an increased erythroid production in vitro or in vivo (Cheshier, Prohaska, & Weissman, 2007) (Erslev, 1971) (Dubart et al., 1994). Also some recent studies assessing HSC subject to high systemic EPO levels, could only report a changed proliferative state. In Giladi et al. HSC and other hematopoietic progenitors were analyzed by scRNAseq after two days of consecutive EPO treatment. This suggested that EPO disrupts HSC dormancy non-specifically besides the known expansion of erythrocyte progenitor populations (Giladi et al., 2018). Similarly, a scRNAseq study assessing hematopoiesis after acute blood loss (increasing EPO levels) reported an increased rate of proliferating HSCs. HSCs started here to express a number of immune response genes unrelated to

lineage differentiation. However MPP induced express of myeloid, megakaryocytic and erythroid genes (J. Yang et al., 2017). This is similar to the study by Tusi et al. which performed scRNAseq of bone marrow of mice treated with EPO for 48h. They observed a remarkable conservation of the hematopoietic progenitor landscape. As in Giladi et al., the proportion of erythroid progenitors increased, however again uncommitted MPPs altered their gene expression. This included a downregulation of C/EBP β , a transcription factor that biases differentiation away from erythroid/megakaryocytic fate (so increasing erythroid/megakaryocytic potential) (Tusi et al., 2018). Other recent studies assessing HSC subject to high systemic EPO levels did however suggest a changed HSC differentiation or transcriptional profile besides induction of proliferation. A study by Singh et al. analyzed HSC and MPP in EPO transgenic mice (Tg6). Here HSC were significantly enriched in genes directly related, not only to cell cycle, but also to red blood cell production. MPP increased their expression of genes related to the innate immune response and the myeloid lineage (Singh et al., 2018). Furthermore, Shiozawa et al. reported the EPO-induced expression of BMP2 and BMP6 in HSC which have been linked to HSC lineage biases (Shiozawa et al., 2010) (Crisan et al., 2015b). Finally, Grover et al. suggested most clearly the biasing of HSC towards erythroid differentiation. In this study, in vivo and in vitro EPO-exposed HSC led to an increased expression of erythroid genes (increased level and number of HSC expressing erythroid genes) which was accompanied by a decreased myeloid gene expression. Furthermore, for the first time, the transcriptional changes observed in HSC were also linked to real lineage biasing in that in vivo EPO-exposed HSC produced more erythroid and less myeloid cells after transplantation (Grover et al., 2014). As in most other studies it remained however unclear if the effect of EPO on HSC was really direct, via EPOR or alternative EPO receptors. EPO has been described to have major effect on bone marrow cells, being part of the HSC “niche”. EPO has for example repeatedly been involved in bone homeostasis. Promotion of bone formation through EphB4 signaling as well as induction of bone loss upon EPO exposure have been reported (C. Li et al., 2015) (Singbrant et al., 2011). Interestingly this also affected the EPO-induced erythroid response (Singbrant et al., 2011). EPO has been linked to an altered adipocyte homeostasis, and a reduced adipocyte size, both in line with the low body weight, serum insulin, and fat mass of Tg6 EPO transgenic mice (American Society of Hematology, Alvarez, & Noguchi, 2017) (Zhang et al., 2014). EPO action on endothelial cells has been shown to lead to the egress of immature B-cells from the bone marrow (Ito, Hamazaki, Takaori-Kondo, & Minato, 2017) (Singbrant et al., 2011). The direct effects of EPO on the lineage differentiation of single HSC have not been studied yet.

1.7 Aims of my thesis

The quantitative description of the hematopoietic system under steady-state and in response to different challenges has started and will be the substrate for the single-cell characterization of other stem cell system. Recent technical advances in lineage tracing techniques of HSC at the single cell level, as cellular barcoding, are a major driver in this process. This thesis aims to explore the behavior of HSC early after transplantation and the effect of erythropoietin on this process at the single cell level. In Chapter II, we describe the early reconstitution kinetics of HSC in the myeloid, lymphoid, and erythroid lineage after transplantation at the single cell level. We have discovered that distinct HSC clones with different life-spans guarantee the production of erythroid and other hematopoietic lineages in the first weeks after transplantation. In Chapter III we assess the direct effect of EPO on this single-cell differentiation kinetic after transplantation. We found that EPO directly changed HSC clonality and induced HSC self-renewing without differentiation and the dominance of myeloid-erythroid (ME)-biased and myeloid B-cell (MB)-biased HSC. In Chapter IV, I will discuss the findings and implications of my thesis.

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Chapter 2: Early hematopoietic stem cell engraftment kinetics at the single cell level

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2.0 Abstract

The reconstitution of the different hematopoietic lineages after bone marrow transplantation can take several weeks. In this anemic phase patients are especially vulnerable to infections and might require transfusions of red blood cells and platelets. The differentiation of single hematopoietic stem cells (HSCs) to the erythroid (E) lineage after bulk transplantation has not been assessed to date because of the difficulty to trace unnucleated mature red blood cells over time. We overcame this limitation here by detecting cellular barcodes in the erythroid lineage from RNA, and followed the differentiation of hundreds of single HSC in the erythroid, myeloid (M) and B-cell (B) lineage over the first six weeks after transplantation into sub-lethally irradiated mice. By monitoring clonality in mature cells and progenitor populations, we could establish that early high erythroid cell production after transplantation was maintained by distinct short-lived HSC clones engrafting the spleen. In contrast, high myeloid cell production after transplantation was maintained by long-lived balanced multi-outcome HSCs residing in the bone marrow. We hope the detailed description of the engraftment kinetics of single HSC after transplantation in the erythroid, myeloid and B-cell lineage provided, will be a step towards improved transplantation protocols fostering faster erythroid reconstitution and causing less burden for patients. The detection of cellular barcodes from RNA described paves the way for new applications of cellular barcoding in the future, especially its combination with scRNAseq profiling.

2.1 Introduction

The transplantation of HSCs is today widely used in the clinics as treatment for a large number of different pathologies (Scala & Aiuti, 2019). The survival rate after transplantation has increased over the last years but further improvement is a major focus of clinical research (Bhatia, Armenian, & Landier, 2017). One phase which remains particularly critical for the success of a transplantation are the first weeks after transplantation in which the different blood lineages did not normalize yet (Tomblyn et al., 2009). Notably the initial phase of anemia after transplantation often makes hospitalization and treatment of patients necessary. Red blood cells and platelet transfusions might be required (Radia & Pamphilon, 2011). A better understanding of the production kinetics of the different lineages, notably erythroid cells, from HSCs in this phase after transplantation, could foster the development of new transplantation protocols and increase the success of clinical transplantations.

In basic HSC research, the transplantation assay is very prominent, however often rather considered as a tool to define and analyze HSC function retrospectively. The unique resolution, notably the single cell level, at which HSC behavior can be studied in murine transplantation models has therefore not always been used to focus on clinically relevant questions. Notably, HSC differentiation early after transplantation has not been given a lot of attention, also because HSC definition was historically based on long-term engraftment outcome. The bulk production kinetics of different lineages in blood shortly after HSC transplantation are known from the clinic and in the murine model (Boyer et al., 2019) (Forsberg et al., 2006). Indeed the different lineages appear with different kinetics - myeloid and erythroid cells are produced faster than lymphoid cells (appearance in blood around day 5-10 and 20 after transplantation respectively (Boyer et al., 2019)). This sequence is in line with current assessment of bulk HSC production kinetics in situ, although the production kinetics are here overall slower with a major detection of platelet and granulocyte production at week 4 and 8, and the start of B-cell detection at week 12 or beyond (Upadhaya et al., 2018) (Säwen et al., 2018). It remains to be determined how single HSC contribute to the bulk production kinetics of the different lineages, notably to the erythroid lineage.

Over the last years, multiple studies could show that different lineage-restricted, and biased cell subsets, as well as cells with different self-renewal capacity exist within the HSC population currently gated as LSK CD150⁺, Flt3⁻ (and or CD48⁻ and CD34⁻). Notably E-restricted, M-restricted, ME-restricted, and myeloid- and lymphoid-biased HSCs have been described besides multi-outcome short-term (ST) and long-term (LT) HSC (see also Chapter I). After single-cell transplantation, these subsets have been described to have different differentiation kinetics. In a study by Yamamoto et al, EM-restricted cells were for example detected earlier in the EM- lineages (week 2) than E-restricted cells (week 3-4). Besides, ST-HSC were described to produce the MEB lineages at week 2 already, while for

LT-HSC only platelets were produced this early, and the MEB lineages only at week 3-4 (Yamamoto et al., 2013b). In a study by Dykstra et al, myeloid-biased HSC started to generate output only at week 4 after transplantation, while balanced and lymphoid-biased HSC produced myeloid cells before week 4, and lymphoid cells after week 4 (Dykstra et al., 2007).

Some evidence on how these different HSC subsets interact to generate the waves of lineage production observed when transplanted together is coming from a number of cellular barcoding and viral integration site (VIS) studies for different myeloid and lymphoid cell subsets. When transplanting barcoded CD34⁺ cells in rhesus macaques, clones detected in blood granulocytes (Gr), monocytes (Mono), and B- and T-cell at one month after transplantation, were reported not to contribute significantly anymore at month 3 or thereafter (Wu et al., 2014) (Koelle et al., 2017). This would be in line with a clonal succession and the dominance of short-term engrafting clones early after transplantation. In several barcoding studies in mouse, it was seen however, that also early differentiating clones can stay active on the long-term. In Verovskaya et al, clones present at week 12 seemed to be present at week 6 already, although Pearson correlation between the Gr, B-cell, and T-cell lineages was only high from week 12 on (E. Verovskaya et al., 2013). In a study by Brewer et al., 50% of clones detected at one month in Gr were long-term engrafting, and these early differentiating clones, contributed most cells of the Gr, B-cell, and T-cell lineage at 6 months (Brewer et al., 2016b). Also Sun et al. reported that a fraction of clones present in Gr at 6 weeks after transplantation, was still found back at week 11, and 16 (Sun et al., 2014). Finally, in a VIS study by Kim et al. some clones detected at week 2, 3, and 4 after transplantation stayed active for over 3 years (Sanggu Kim et al., 2014). Thereby these studies favor the idea that some clonal succession exists, but also that (some) LT-multi-outcome HSC do differentiate early after transplantation. The clonality in the erythroid lineage, especially relevant for anemia has not been assessed over time after bulk transplantation to date.

Here we established the detection of cellular barcodes in the erythroid lineage from RNA, and followed the differentiation of hundreds of single HSC in the erythroid, myeloid and B-cell lineage over the first six weeks after transplantation into sub-lethally irradiated mice.

2.2 Results

Erythroid, myeloid and B-cells are produced with different kinetics after transplantation

Cellular barcoding was used to study HSC differentiation in the erythroid, myeloid, and B-cell lineage at 2, 4, and 6 weeks after transplantation. HSC (LSK Flt3⁻ CD150⁺) were isolated from tibia, ilium, and femur of Rosa26CreER^{T2}; mTdT/mGFP or CD45.1 donor mice, and labelled with unique genetic barcodes by lentiviral transduction. A new lentiviral barcode library (LG2.2) of over 10 000 barcodes was applied, which's barcodes consist of random stretches of 20 nucleotides which are

integrated in transduced cell in conjunction with GFP, both expressed through the CMV promoter. The transduction protocol, and controls for single integration of barcodes were applied as previously described, which include a low transduction efficiency of 10%, and a low number of transduced cells relative to the capacity of the barcode library (Naik et al., 2013). Transduced HSC were transplanted at 2500 cells/mouse (of which around 250 barcoded HSC) without helper cells in sub-lethally irradiated mice. After 2, 4, and 6 weeks, barcoded (GFP⁺) erythroid (E; Ter119⁺ CD44⁺), myeloid (M; Donor Ter119⁻CD19⁻CD11c⁻CD11b⁺), and B-cells (B; Donor Ter119⁻CD19⁺) (Figure S1) were sorted from the spleen, and their barcode identity was analyzed through nested PCR and NGS detection. At the same timepoints the E, M, and B lineage chimerism was assessed in blood samples (Figure 1 A).

In line with previous studies, we could see differences in the bulk production kinetics of the different lineages after transplantation (Figure 1 B-E). Erythroid cells, which have been shown to be the least affected by irradiation (Boyer et al., 2019), were the most abundant cellular subset in blood and spleen throughout all timepoints. This was followed by the myeloid lineage at week 2. At week 4 and 6, B-cell numbers had recovered, and represented the second most abundant lineage in spleen and blood, although at different relative proportions to the erythroid lineage (around 1:10 in spleen, and 1:100 in blood) (Figure 1 D, E). In terms of chimerism, the myeloid subset was the first to rise at week 2. The erythroid, and B-cell chimerism increased from week 2 to 4 (Figure 1 B, C). As erythroid cells lose their nucleus during terminal differentiation to mature red blood cells, the production of the Tomato protein, used to determine the chimerism, is not sustained and depends on the long-half live of the protein. The erythroid chimerism is therefore likely under-estimated (Figure S1 D).

The clonality of HSC at 2, 4, and 6 weeks after transplantation assessed in spleen

To analyze if the different lineages had been produced by the same HSC, we went on to analyze the presence and overlap of barcodes in the E, M, and B lineage in spleens at week 2, 4, and 6 after transplantation (Figure 2 A-D) (Figure S2). The overall percentage of barcodes found in the three lineages was similar over the three timepoints, meaning that the same percentage of transplanted HSC produced each lineage at 2, 4, and 6 weeks after transplantation (Figure 2 B). To assess if the transplanted HSC had also the same lineage restrictions, we classified the barcodes according to presence and absence in the different lineages into uni- and multi-lineage outcome categories, corresponding to uni- and multi-lineage outcome HSCs (Figure S3). The percentage of HSCs in these different uni- and multi-lineage outcome categories changed over time (Figure 2 C). At all timepoints, the highest percentage of barcodes (40-60%) was present in all three lineages analyzed (MBE multi-lineage outcome category) and contributed the majority of cells (>90%) in all lineages (Figure 2 D). The occurrence of the other outcome categories was rather equally low and constant over the different timepoints. Two exceptions were B-outcome HSC and ME-outcome HSC. At week 2, no B-, BE-, and

MB-outcome HSC were detected. All B-producing HSC were multi-lineage outcome. Besides, a particular high percentage (25%) of ME-outcome HSCs was present at the earliest timepoint, and decreased over time, to encompass a significantly lower percentage of HSCs at week 6 (<5%) (Figure 2 C). Both lack of B-restricted cells, at week 2, and the higher percentage of ME-outcome clones, could be in line with differences in the kinetic of lineage production at the single cell level, as the switch of HSC lineage outcome pattern from ME, to MBE, to MB, and B (possibly after extinction). If such changes are really caused by the same cells can only be verified through a kinetic analysis, which we aimed for next.

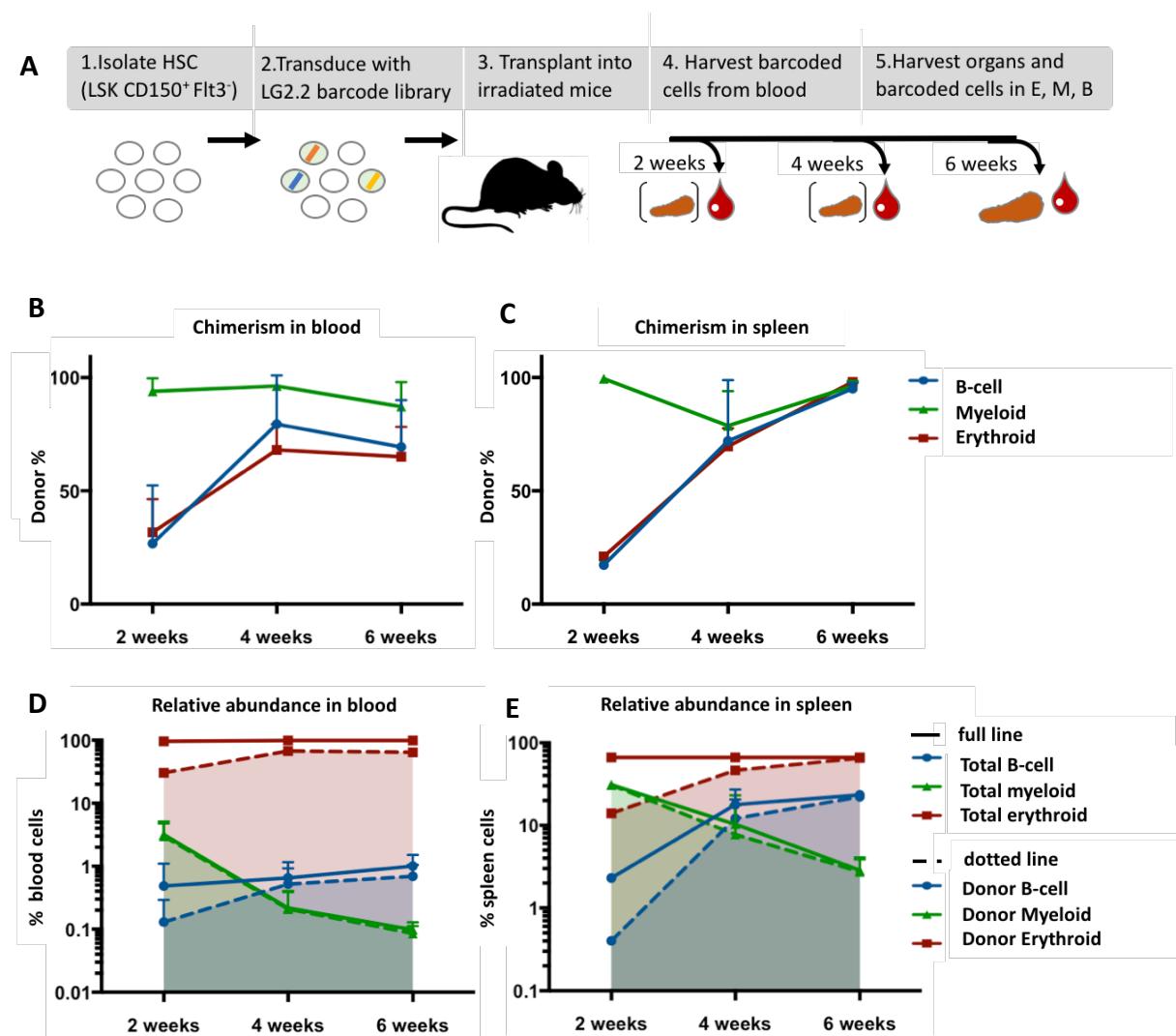


Figure 1: Chimerism and sample composition in spleen and blood shortly after HSC transplantation. (A-E) HSC were sorted from Rosa26CreER^{T2}; mTdTtomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. The relative abundance of the E, M, and B lineage, and chimerism (based on Tom expression) was assessed at week 2, week 4, and week 6 in PB and spleen of sacrificed individual mice. (A) Scheme of experimental set-up. (B-C) The fraction of Tom⁺ cells among the indicated cell types in PB (B) and spleen (C). (D-E) The relative abundance of the indicated cell types in spleen and blood

samples. The overall relative abundance (line) and corrected for donor chimerism (dotted line). Shown throughout are values from individual animals (n=8 week 2, n=7 week 4, n=3 week 6 for blood, n=1 week 2, n=14 week 4, n=4 for spleen, collected over 3 different experiments). For all plots mean and S.D. between mice are depicted.

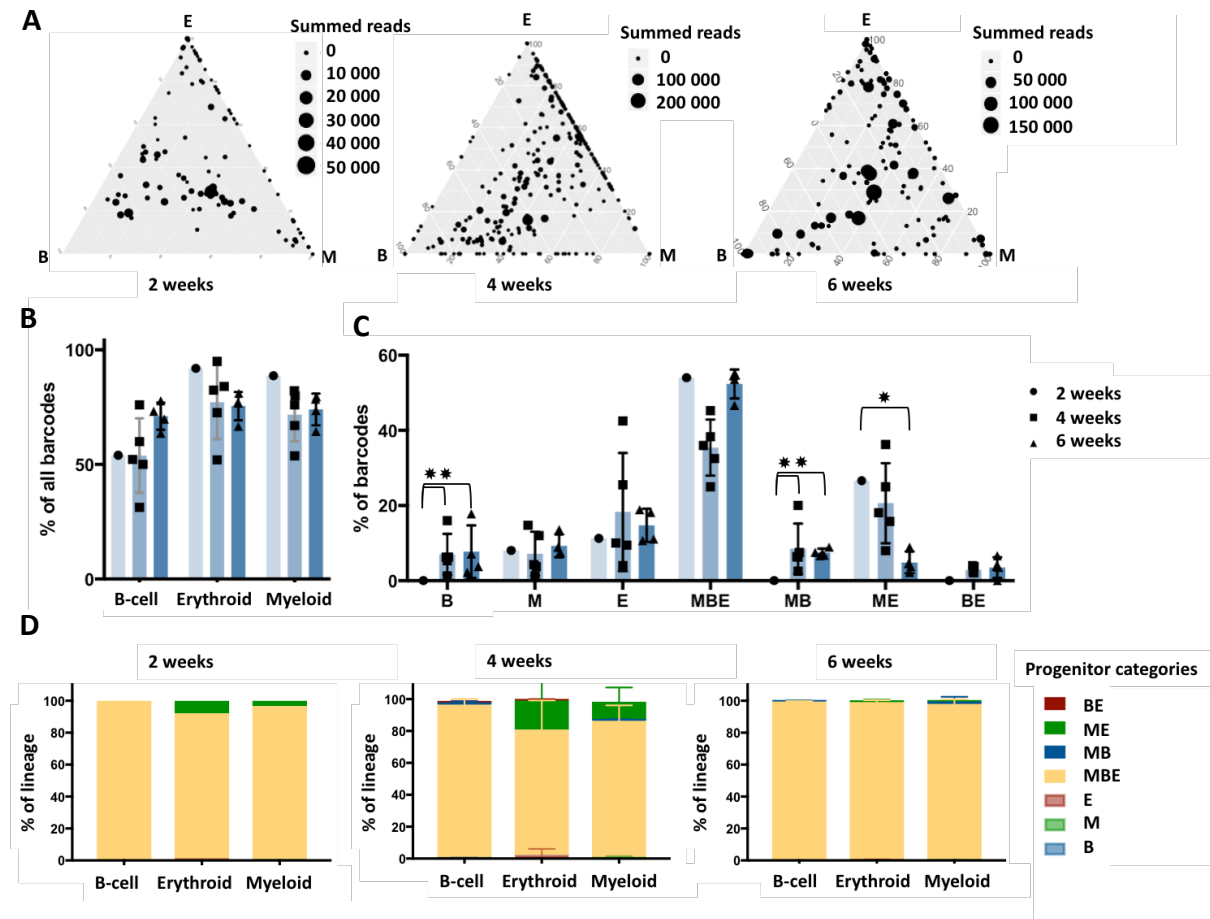


Figure 2: HSC output patterns at 2, 4, and 6 weeks after transplantation as assessed from spleen. (A-D) HSC were sorted from Rosa26CreERT²; mTdtTomato/mGFP mice or CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage in spleen of mice sacrificed at week 2, 4, and 6 post-transplantation. (A) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output at week 2, 4, and 6 after transplantation. (B-C) Percentage of barcodes found back in the indicated lineage (B) and lineage combinations (C) at week 2, 4, and 6 after transplantation. (D) The percentage of each lineage produced by the indicated uni-, or multi-outcome barcodes. Shown are values from several animals (n=1 week 2, n=5 week 4, n=4 week 6 (collected over 4 different experiments)). For all bar graphs mean and S.D. between mice are depicted.

HSC clone activity can be assessed from blood over time

After assessing the barcode profiles in E, M, and B cells at 2, 4, and 6 weeks after HSC transplantation independently, we wanted to assess how these are related e.g. how the output of single HSCs changes over time, early after engraftment. To do so, we set off to take blood samples at 2 and 4 weeks and compare these with spleen samples taken at week 6. To implement this strategy, we wanted to assess two aspects in more detail: 1) the comparison of blood and spleen barcode profiles and 2) the assessment of barcodes in the erythroid lineage from RNA in blood.

The percentage of nucleated erythroblasts is low (<5%) in murine blood (O'Connell et al., 2015) which hampers the analysis of cellular barcodes from DNA. To overcome this limitation, we tested the amplification of barcodes from RNA of erythroid cells in spleen and blood (Figure 3 A-M). The direct comparison of RNA and DNA profiles revealed a variable overlap. In the spleen around 30% of barcodes detected from DNA were also detected from RNA. The reverse overlap percentage was higher. The majority of barcodes (55%) detected from RNA was also detected from DNA (Figure 3 A, E). The barcodes shared between RNA and DNA sample, were notably the highest abundant barcodes in the DNA samples and ranking of barcodes stayed similar between RNA and DNA detection (Figure 3 I). The RNA barcode profile seemed thereby a subsample of the DNA barcodes in spleen. In blood, the barcode profiles of erythroid cells obtained from DNA and RNA were less similar. We could see however, that the RNA-derived barcode profile was more similar to the splenic profile, than the DNA-derived blood barcode profile. It encompassed notably more of the high abundant barcodes in spleen (Figure 3 B). We therefore concluded that the analysis of barcodes from RNA in blood erythroid cells, was valid, although representing a subsampling of spleen erythroid cell barcodes.

The comparison of blood and spleen for the M and B lineage showed a similar picture (Figure 3 C-D, G-H, L-M). The barcodes detected in blood were generally also found back in spleen samples of the corresponding lineage (75-100%). However not all barcodes detected in spleen, and notably the low abundant barcodes, were found back in blood (5-70%). The ranking of the barcodes detected in blood and spleen however matched markedly (Figure 3 C-D, L-M).

We concluded that the detection of barcodes from RNA of erythroid cells, and from DNA of myeloid and B-cells in blood, could be used to assess changes in HSC clonality from blood samples over time. The barcodes detected in blood are however rather a subsample of barcodes in spleen for all three lineages, implying that the low production of each lineage cannot be excluded based on barcode profiles acquired from blood samples.

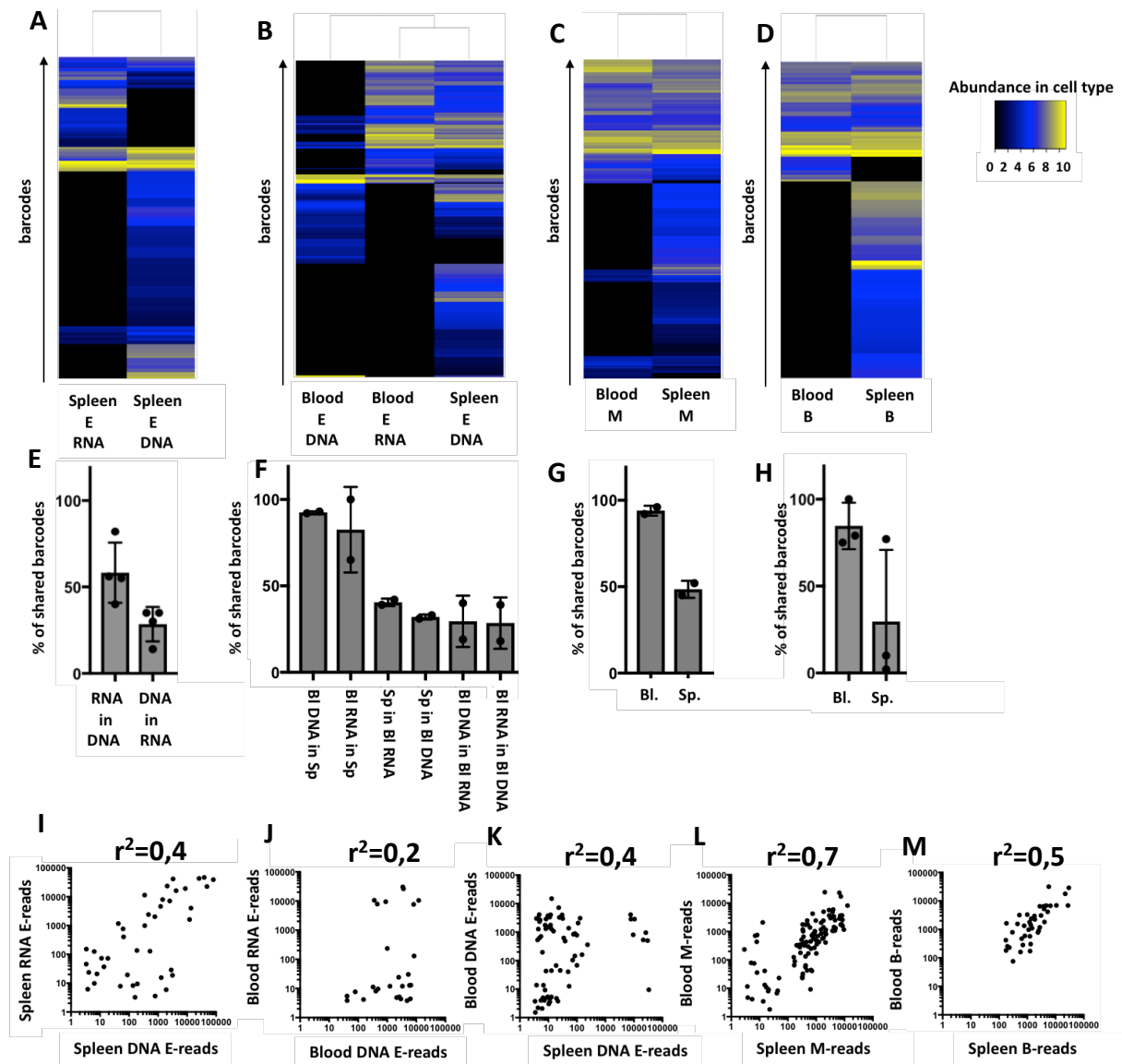


Figure 3: Comparison of DNA and RNA-derived barcode profiles in spleen and blood. (A-M) HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice or CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage in spleen of mice sacrificed at week 2, 4, or 6 post-transplantation from DNA or RNA. (A-D) Heatmaps showing the output of individual barcodes (rows) in different samples as indicated. Data is log transformed and clustered by complete linkage using Euclidean distance. No output is represented in black. (E-H) The percentage of barcodes shared between samples as in (A-D). In all plots Mean and S.D. between mice are depicted. (I-M) the abundance of barcodes shared in different samples as in (A-D). The spearman correlation is indicated. Shown are values from several animals (n=4 for (A, E, I), n=3 for D, H, M, n=2 for remaining plots (data was collected over 2 different experiments)).

Clonal succession and clonal stability co-occur after HSC transplantation

After comparing the barcode profiles of E, M, and B cells in blood and spleen independently, and the amplification of barcodes from RNA in the erythroid lineage, we started a cohort for which blood samples were taken at week 2 and 4 and, the spleen analyzed at week 6, to assess changes of individual barcodes over time. As the B-cell percentage is very low in blood at week 2 after transplantation (Figure 1 D) we only sorted the M, and E lineage at this timepoint. Overall, we could observe lower barcode numbers in blood than in spleen samples (not shown). Of all the barcodes detected at the three timepoints (average of 89), on average only 6 were found back at all timepoints, and 22 were found back at two timepoints, making it possible to assess a change in output over time (Figure 4 A-B). The low number of barcodes recovered throughout the three analysis timepoints, already suggested a clonal succession taking place over time. When considering the summed output in all lineages over the three timepoints, it became apparent that the majority of clones at any timepoint was detected only once. These included some high output clones (Figure 4 C), However, some high output clones were also detected throughout the monitoring window (Figure 4 C), meaning both clonal succession and stability were observed among clones, and among high output clones. Although the number of barcodes in the blood samples was lower than in spleen, the presence of high output clones among clones present at only one timepoint makes it very unlikely that sampling can explain the clonal succession observed.

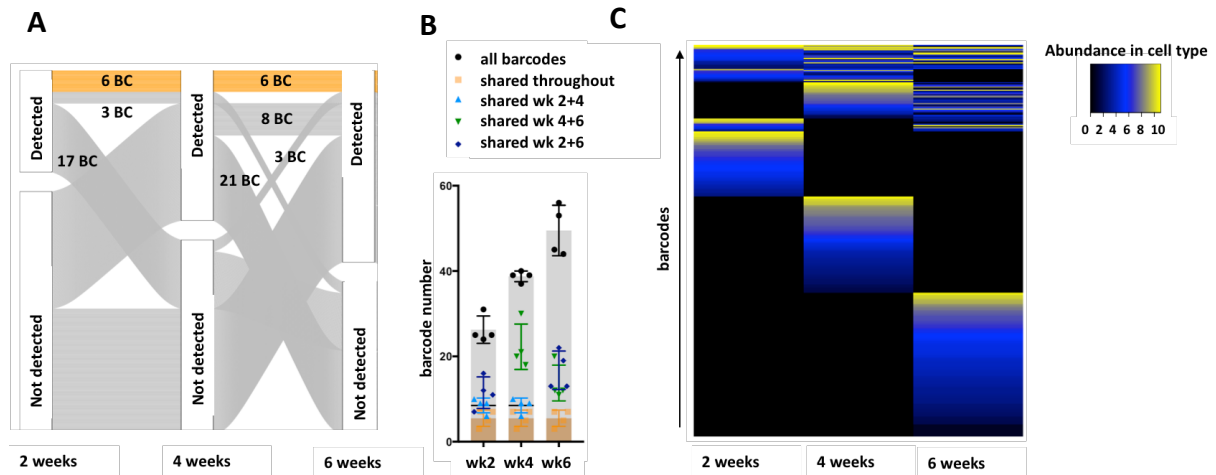


Figure 4: Overview of clone detection over time for individual barcodes. (A-C) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen at week 6 in the same individual mice. (A) Sankey diagram showing detection of barcodes over time for all mice combined. Orange color is used for barcodes detected at all three timepoints. (B) Bar-graph showing variability between mice in detection of barcodes over time. Mean and S.D. between mice are depicted. (C) Heatmaps showing the summed output of individual barcodes (rows) over time for all mice combined. Data is log transformed. No output is represented in black. Shown are values from the same animals throughout (n=4 from one experiment).

Clonal contributions to E, M, and B lineage after HSC transplantation in blood

After assessing the barcode profiles irrespective of lineage contribution at 2, 4, and 6 weeks after HSC transplantation, we wanted to assess how these are related, e.g. if clonal succession and stability are similarly important for each lineage and which lineage changes occur. In a first instance we assessed how the blood barcode profiles at week 2 and 4 compared to the spleen profiles at these timepoints (Figure S4). When analyzing the percentages of different uni- and multi-lineage outcome categories between blood and spleen we could observe a decreased percentage of MBE outcome HSC in blood as compared to spleen (Figure S4). As we detected overall lower number of barcodes in blood, we wanted to assess in how much this profile of blood samples can be mimicked by subsampling of spleen data (Figure S5). Indeed, a subsampling across all three analyzed lineages resulted in uni- and multi-lineage outcome HSC distributions similar to the distribution observed in blood (Figure S5). Therefore, we cannot exclude that uni- and bi-lineage outcome HSC observed in blood are due to sampling. Alternatively, the lower percentage of MBE outcome HSC in blood as compared to spleen could also be caused by different production kinetics and migration of the analyzed cell subsets between blood and spleen.

HSC contribution to different lineages changes over time

After assessing the barcode lineage contributions in blood from the cohort of mice analyzed at week 2, 4 and 6, we went on to assess if clonal succession and stability are similarly important for each lineage and which lineage changes occur. Indeed, switches in output pattern, and from detection to no detection, were not evenly present in the different lineages (Figure 5 A-G). E-restricted clones were for example more likely not to be detected at a subsequent timepoint than clones with other output patterns (Figure 5 A, C and Figure S6). Clones detected as ME-restricted at week 2 were more likely to be producing B (as MB, MBE, or B) at week 4, as M- and E-restricted clones. Indeed, in general, B production was more than M- and E-production preceded by detection. This is in line with a longer production time (or delayed production) for B-cells at the single cell level. Besides, multi-outcome clones were more likely to stay, and to stay multi-outcome (Figure 5 A and Figure S6). In line with this, while from all clones detected throughout none kept its exact output pattern, some were detected in ME, MBE, and MBE over the different timepoints, staying multi-outcome throughout. For the clones detected in two lineages over several timepoints, we did assess the bias over the different timepoints (Figure 5 E-G). Switches in all directions existed. It was however apparent that strongly biased clones had a lower chance to be detected at the next timepoint. So not only multilineage, but also balanced production of the three lineages favored subsequent detection, and detection in B-cells.

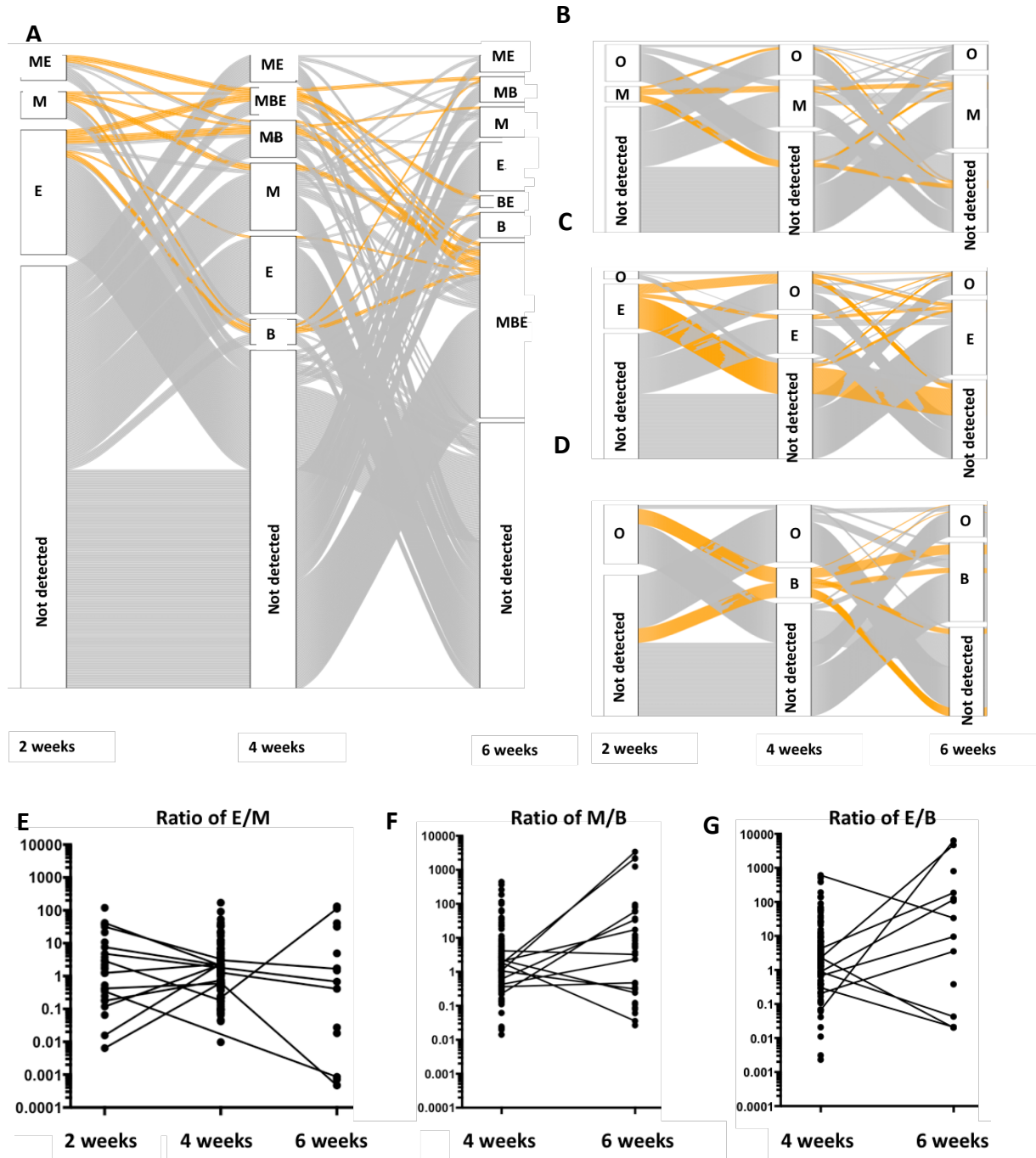
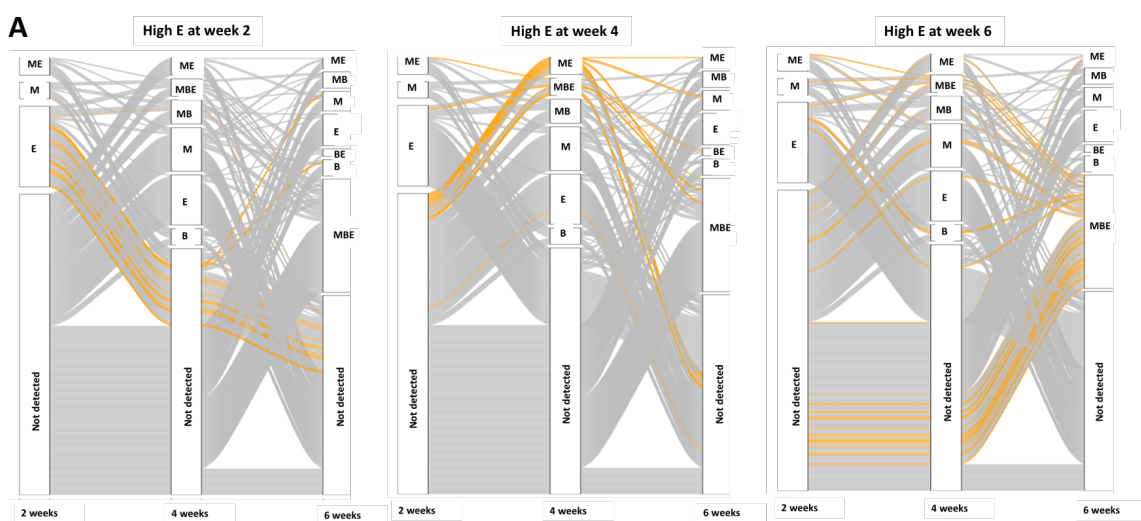


Figure 5: Changes in lineage restriction of individual HSC over time. (A-G) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen 6 in the same individual mice. (A) Sankey diagram showing detection of barcodes in different lineages over time for all mice combined. Orange color is used for barcodes detected at all three timepoints. (B-D) Sankey diagrams as in (A) distinguishing only between detection in the myeloid (B), erythroid (C), or B-cell (D) lineage (highlighted in orange), detection in the respective other lineages (symbol O), and no detection. (E-G) Line graphs depicting the ratio of E/M (E), M/B (F), and E/B (G) output when the respective lineages were detected over time. Shown are values from the same animals throughout (n=4 from one experiment).

High output in the myeloid, but not erythroid lineage characterizes stable clones

After analyzing the switches in output for all detected barcodes, we next thought to focus on lineage switches of the highest output clones; e.g. the clones producing over 1% of a lineage at a specific timepoint (Figure 6 A-B). Interestingly, the high output clones in the erythroid lineage behaved differently than the high output clones in the myeloid and B-cell lineage. At the week 2 timepoint, the high E output clones were detected only in the E-lineage and mostly not detected at subsequent timepoints (Figure 6 A-B). At the week 4 timepoint again a high percentage of erythroid clones was only detected at this timepoint. However, the high E output clones were not found in this category but rather among ME-restricted and multi-outcome MBE clones, and many of these clones were also detected at week 6 (Figure 6 A-B). Thereby, early high erythroid output clones were not stable; distinct clones maintained early high erythroid cell production. In contrast, the high M producing clones at week 2, 4, and 6 were evenly distributed between the different myeloid-outcome categories, and often detected thereafter. All of these clones kept producing myeloid cells, and some stayed among the highest abundant clones from week 2 till the week 6 timepoint (Figure 6 A-B). High myeloid output, contrary to early high erythroid output thereby was correlated to clonal stability. The high B producing cells at week 4 were mostly found in the multi-lineage outcome category (Figure 6 A-B). Besides, in contrast to high E and M producing clones, the majority had already been detected at week 2 (Figure 6 A-B). Which is, as for all clones, in line, with on a clonal level longer production time for B-cells, and speaking against existence of B-restricted HSCs.



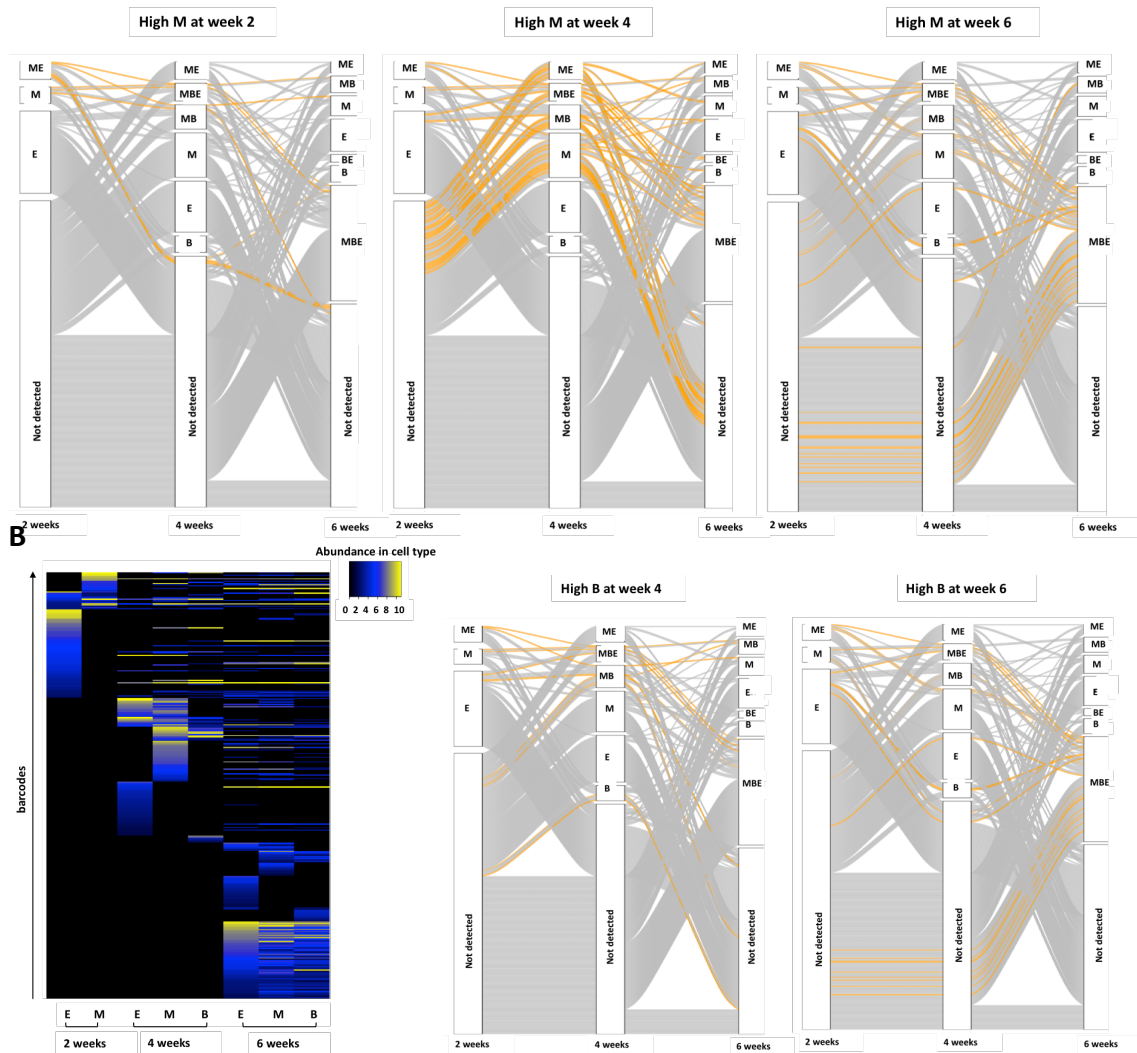


Figure 6: Fate and origin of clones with high abundance. (A-B) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen 6 in the same individual mice. (A) Sankey diagrams showing the fate of clones with high erythroid, myeloid or B-cell output at week 2, 4 or 6 as indicated (respective clones are highlighted in orange). (B) Heatmaps showing the output of individual barcodes (rows) over time for all mice combined. Barcodes are clustered according to their abundance at the week 2 timepoint. Data is log transformed. No output is represented in black. Shown are values from the same animals throughout (n=4 from one experiment).

Which HSC did self-renew, which differentiated, and where are they located?

After we had analyzed the change in mature cell output of HSC at different timepoints, and over time after transplantation, we wanted to assess the presence of these same barcodes in HSC, and other hematopoietic progenitors (Figure 7 A). This was meant to give insights on the relation of mature cell production and self-renewal of HSC shortly after transplantation. At 2 weeks after transplantation, a rather low percentage (19%) of mature cell barcodes was found back in bone HSCs (Figure 7 B). This

increased at week 4 and 6 to over 50%. In contrast most HSC barcodes at any timepoint were found back in the mature cell subsets (above 80%), although with a low decrease over time (from 100% at week 2 to an average of 91% and 82% (S.D 5 and 11) at week 4 and 6 respectively). This would be in line with few transplanted HSC which only self-renew, but some HSC which only differentiate, and self-renew very little. However, to our surprise, the percentage of mature cell barcodes which was found back in HSC in the spleen was close to 50% at week 2 (Figure 7 C). Thus, implying that the early differentiated cells, did self-renew too, but were localized in the spleen, rather than the bone marrow. In line with a possible movement of HSC from spleen to bone, nearly 100% of HSCs present in bone were shared with spleen HSC (and mature cells) (Figure 7 D).

The abundance of HSC and their differentiation capacity in different lineages

After we had established that HSC self-renewal was mostly linked to differentiation, we wanted to assess this relation in more detail. More precisely, we wanted to analyze how abundance in bone HSC (self-renewal) is related to abundance in mature cell subsets (differentiation) in general, and for each lineage in particular, at the different timepoints early after transplantation (Figure 7 E-F and Figure S7). The mean abundance in mature cell subsets was clearly higher for barcodes found back in bone marrow HSC and spleen HSC although the distribution was wide (Figure 7 E-F). This is again in line with self-renewal and differentiation being correlated and also at a quantitative level; A high abundance in HSC through high self-renewal was correlated with a high abundance in mature cells due to high differentiation. HSC clones with low self-renewal, which might have been missed due to a detection limit, did produce less mature cells on average.

When analyzing this relation for the different lineages, we could see that at week 2, the barcoded HSC in bone corresponded rather to multi-lineage outcome HSC which produced more B cells than cells in the E and M lineage (and could be considered as “B-biased”) (Figure 8 A-D and Figure S7). Indeed, also the abundance of barcoded bone HSCs correlated best with the abundance in the B lineage (Figure 8 C), this was not the case for HSC present in the spleen at week 2 (Figure 9 A-C). Here, as for all other timepoints in spleen and bone, the highest percentages of overlap between mature cell and HSC barcodes was found in the outcome categories MBE, ME, and the very rare BE (Figure 8, 9, S7 and S8). Also, within the MBE outcome category, the barcodes found back in HSC produced more cells in the E and M lineage than in the B lineage, resulting in a high correlation of HSC with E and M lineage but not with the B lineage (Figure 8 C). Indeed, especially high abundant HSC had such an “EM-biased” output profile (Figure 8 D).

The high correlation between HSC and E+M, but not B abundance could be explained by different phenomena. One of these is a higher number of amplification steps (higher exponential growth) from the HSC to the mature E and M lineage than to the B lineage. The absence of this phenomenon for bone

HSC at week 2 could be explained by their origin in the spleen. As we described before some evidence suggest a movement from splenic HSC to bone at this early stage. It seems that a specific subset of HSC (namely multi-lineage outcome and rather “B-biased”), and especially HSC with high abundance in spleen were also found back in bone (Figure 9 A-C). It could be that this is related to a specific location of this HSC subset in spleen. Furthermore, the large size of the HSC clone (sum of cells in spleen and bone) could lead to a less efficient retention in the spleen. Besides, assuming a longer production time or delayed production of B-cells at the single cell level, the migration of HSC could always start at a specific time after start of differentiation (when HSC do already produce B cells), or the migration to, and homing in bone could take a timespan similar to the initiation of B-cell production.

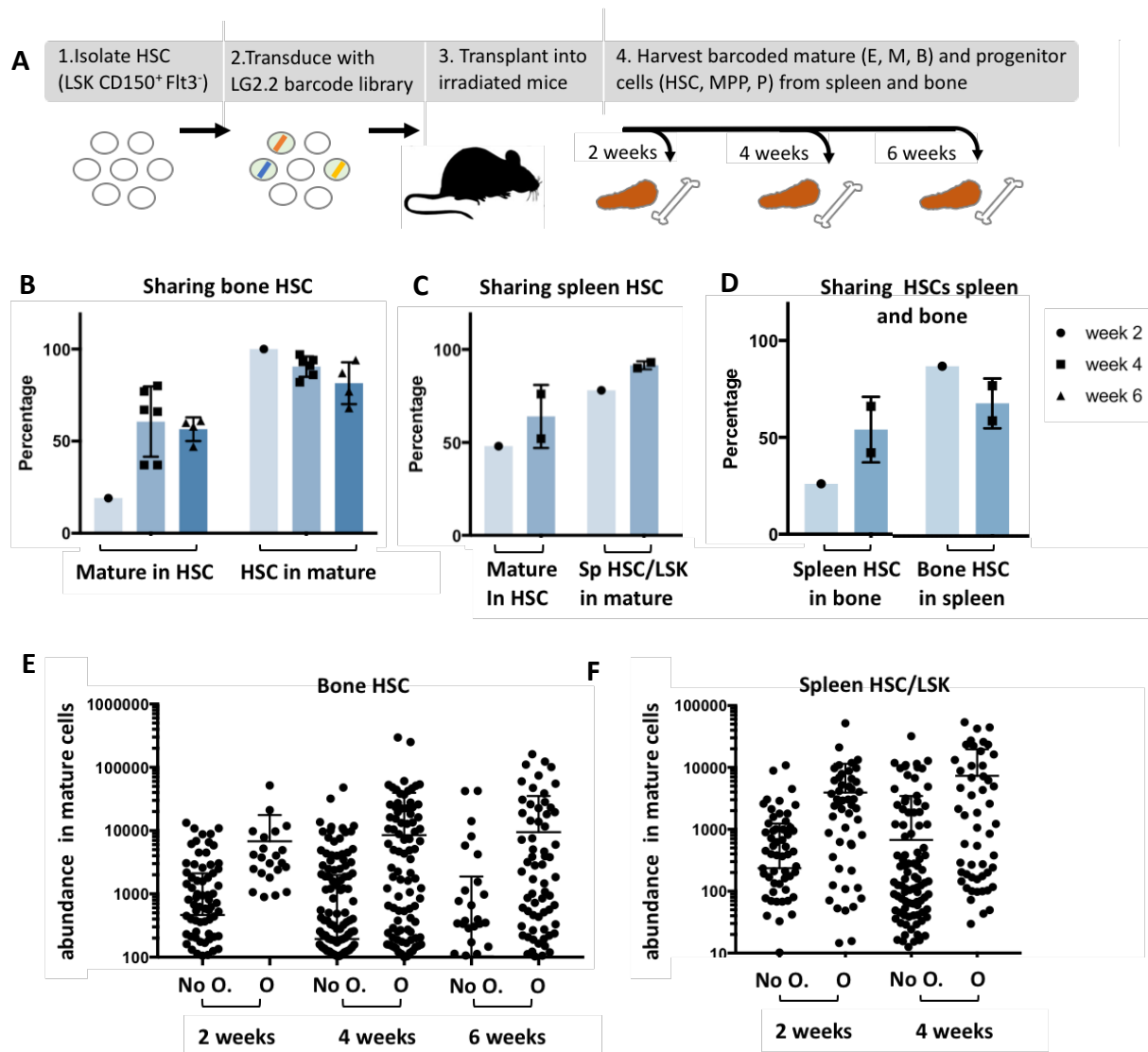


Figure 7: HSC self-renewal vs differentiation, and localization early after transplantation. (A-F) HSC were sorted from Rosa26CreER^{T2}; mTdTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and

transplanted into sublethal-irradiated mice. The barcode identity in the E, M, and B lineage was assessed at week 2, week 4, and week 6 in spleen and compared to the barcode identity in HSC in bone and spleen (A) Scheme of experimental set-up. (B-D) Percentage of barcodes shared between indicated cell subsets at 2, 4, and 6 weeks after transplantation. (E-F) summed abundance in the three cell types for barcodes found back (symbol O) or not (symbol No O.) in HSC of bone (E) and spleen (F). Shown are values from individual animals for (B-D) and all animals (E-F) (n=1 week 2, n=6 (or 10) week 4, n=4 week 6, collected over 5 different experiments). For plots mean and S.D. between mice (B-D) or barcodes (E-F) are depicted.

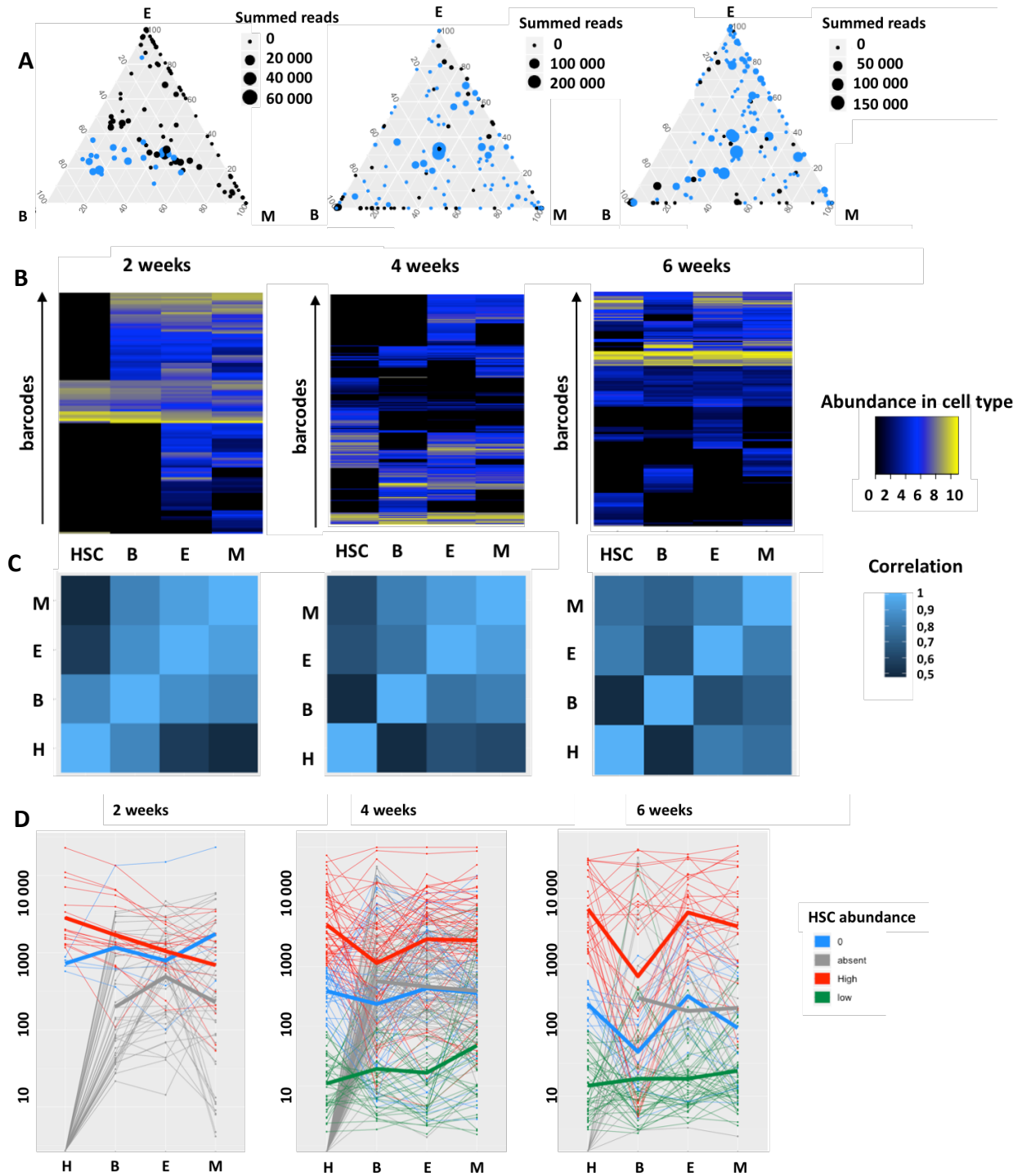


Figure 8: HSC self-renewal vs differentiation in the different lineages early after transplantation. (A-D) HSC were sorted from Rosa26CreER^{T2}; mTdTtomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. The barcode identity in the E, M, and B lineage was assessed at week 2, week 4, and week 6 in spleen and compared to the barcode identity in HSC in bone (A) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output at week 2, 4, and 6 after transplantation for barcodes found back in bone HSC (blue) or not (black). (B) Heatmaps showing the output of individual barcodes (rows) in different samples as indicated. Data is log transformed and clustered by complete linkage using Euclidean distance. No output is represented in black. (C) Pearson correlation between the indicated cell types at the indicated timepoints (H=HSC). (D) Abundance in indicated lineages for all barcodes classified and colored according to their abundance in bone HSC (High >1000> Middle>100 >low, 0=absent) (H=HSC). Mean of each category is represented in bold. Shown throughout are values from individual animals (n=1 week 2, n=6 (or 10) week 4, n=4 week 6, collected over 5 different experiments).

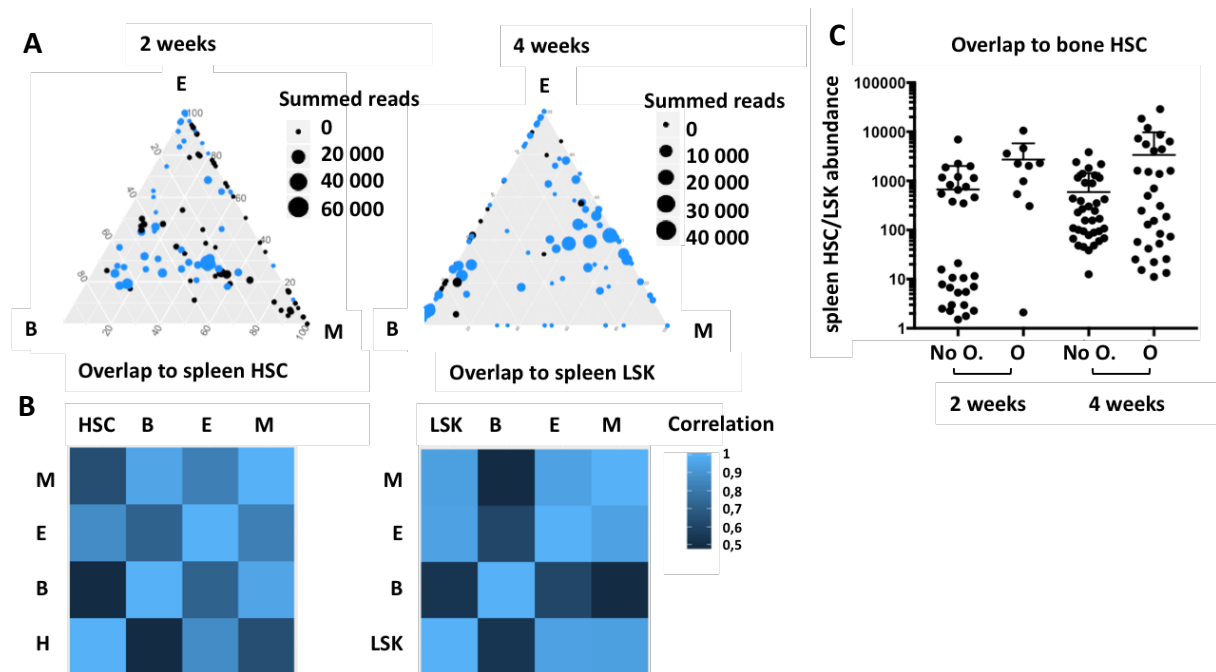


Figure 9: HSC self-renewal vs differentiation, and localization early after transplantation. (A-C) HSC were sorted from Rosa26CreER^{T2}; mTdTtomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. The barcode identity in the E, M, and B lineage was assessed at week 2 and 4 in spleen and compared to the barcode identity in HSC in bone and spleen (A) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output at week 2 and 4 after transplantation for barcodes found back in spleen HSC or LSK (blue) or not (black). (B) Pearson correlation between the indicated cell types at the indicated timepoints (H=HSC). (C) Abundance in the spleen HSC or LSK for barcodes found back (symbol O) or not (symbol No O.) in HSC of bone. Shown are values from individual animals for all animals (n=1 week 2, n=2 week 4, collected over 2 different experiments). For plots mean and S.D. between barcodes are depicted.

The relation of HSC, MPP, and other progenitors during differentiation

To further study the possibility of high amplification steps as origin for the differences of correlations between HSC and different lineages, we went on to analyze the overlap of barcodes in HSC with two progenitor categories: a MPP subset (LSK Flt3⁺ CD150⁻) (“MPP4”) and other multipotent progenitors (Lin⁻ c-kit⁺ Sca-1⁻) (Figure 10 A-E and Figure S1 and S9). In general, we observed a very high overlap (>40% at any timepoint) between presence in HSC, MPP, and P in bone (Figure 10 A-C and Figure S9). Few barcodes were found only in HSC, MPP, or progenitors, and in all other progenitor combinations at week 4 and 6 (Figure 10 A-C). At week 2, very few barcodes were present only in HSC, few only in MPP, and a larger fraction was present only in progenitors. Besides, no barcodes were shared only by HSC and MPP, or HSC and progenitors (Figure 10 A-C). Interestingly, the overlap to spleen HSC was different between these subsets (Figure 10 B). Barcodes present only in progenitors, were for example not found back among spleen HSC. This could be in line with clones differentiating without self-renewal. The abundance of this category among progenitors and mature cells was however very low (Figure 10 B-C). It is possible that these clones were also present among spleen progenitors which we did not assess. Also at week 4 and 6, high abundance in HSC, MPP, and P (>1% of subset) was mainly restricted to the barcodes present in all three analyzed progenitor categories, although some HSC+P barcodes, HSC+MPP, and MPP+P barcodes were abundant too (Figure 10 B). In general, there was also a good correlation between abundance in HSC, MPP, and P (Figure 10 B). This would speak for a rather uniform amplification of HSC clones on the MPP, and P level.

Indeed, the analysis of the output patterns of clones with an especially high or low amplification at the MPP, or P level, or present only in specific progenitor combinations, did not allow for other strong conclusions (Figure 10 D-E). Among clones present in all progenitor subsets (HSC, MPP, and P), clones with a balanced abundance in HSC, MPP and P were in general more abundant in mature cells than other clones but the relative abundance in the different mature cell lineage was not clearly or consistently changed. A low P level relative to HSC was correlated with a more even B to M ratio. Likewise, a high MPP to P level was correlated with a less “ME-biased” output. This was also true for barcodes present only in HSC and MPP. Thereby, a higher amplification at especially the progenitor stage could lead to a “ME-biased” output of HSC clones. Strong conclusions on this point can however not be drawn from the data.

The relation of clonal stability and HSC self-renewal

Finally, we assessed how the detection of clones over time (blood at week 2 and 4) relates to their detection in the bone HSC subset at week 6. Barcodes present at week 6 in HSC but not in mature cells did mostly not correspond to barcodes detected at previous timepoints (Figure S10). Only for one mouse two barcodes detected only at week 4 could be found back in HSC at week 6. This could be in line with

a real extinction of HSC clones over time. The chance to find back a mature cell clone among HSC at week 6, was not significantly related to the detection at previous timepoints (Figure S10).

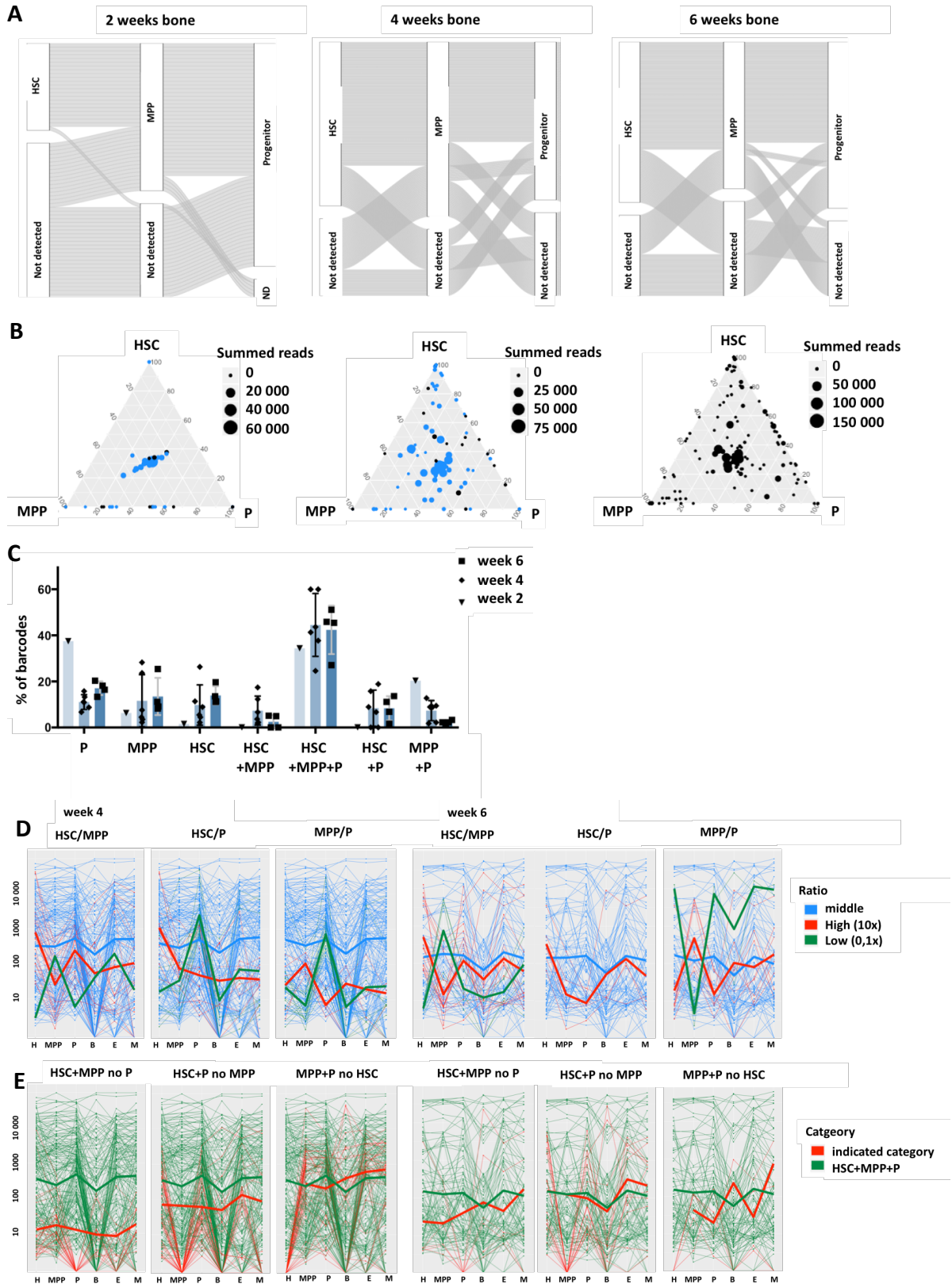


Figure 10: HSC self-renewal vs presence in MPP, progenitors, and mature cells early after transplantation. (A-E) HSC were sorted from Rosa26CreER^{T2}; mTdtomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. The barcode identity in the E, M, and B lineage was assessed at week 2, week 4, and week 6 in spleen and compared to the barcode identity in HSC in bone. (A) Sankey diagrams showing detection of barcodes in HSC, MPP, and progenitors at 2,4, and 6 weeks for all mice combined (ND=not detected). (B) Triangle plots showing the relative abundance of barcodes (dots) in the HSC, MPP, and progenitor (P) lineage with respect to the summed output at week 2, 4 and 6 after transplantation. For week 2 and 4 barcodes found back in spleen HSC or LSK are colored in blue. (C) Bar-graph showing variability between mice in detection for data in (A). Mean and S.D. between mice are depicted. (D) Abundance in indicated lineages for barcodes present in HSC, MPP, and P, classified and colored according to the indicated ratio. Mean of each category is represented in bold. (E) representation as in (D) for barcodes present in HSC, MPP, and P, or the indicated subsets. Shown throughout are values from individual animals (n=1 week 2, n=6 (or 10) week 4, n=4 week 6, collected over 5 different experiments).

3.3 Discussion

In this chapter we studied the differentiation of HSC into the erythroid, myeloid and B-cell lineage at the single cell level over the first six weeks after bulk transplantation. To do so, we applied a new lentiviral cellular barcoding library, and established the detection of cellular barcodes from RNA of erythroid cells in blood. We could observe that early high erythroid cell production after transplantation was maintained by distinct short-lived HSC clones. In contrast, high myeloid cell production after transplantation was maintained by long-lived balanced multi-outcome HSCs. A high overlap of barcodes in HSC and other mature cells implied a close link of self-renewal and differentiation early after transplantation-e.g. few HSC only self-renewing. In contrast some transplanted clones seemed indeed to be devoid of self-renewal activity and to be lost from the HSC pool over time. The higher number of HSC in the spleen early after transplantation and their overlap with HSC in bone implied that early differentiation occurs in the spleen, and that HSC move from spleen to bone marrow over time.

Both clonal succession (Wu et al., 2014) (Koelle et al., 2017), as well as clonal stability have been suggested to occur after bulk HSC transplantation (E. Verovskaya et al., 2013) (Brewer et al., 2016b) (Sun et al., 2014) (Sanggu Kim et al., 2014). Here, we analyzed cellular barcodes in mature cells over time as well as in hematopoietic progenitor populations at different timepoints to assess these phenomena in the first six weeks after transplantation. In line with previous studies, we could observe that the bulk production kinetics of different lineages differed, notably that the erythroid and myeloid lineage were produced earlier than B-cells after transplantation. At the single cell level, we could observe that both clonal succession and clonal stability were underlying this mature cell production waves, implying the presence of short and long-lived HSCs. Interestingly, the importance of the two phenomena for different lineages differed. Notably, in contrast to the myeloid and B-cell lineage, early high erythroid cell production was only maintained by distinct (non-stable) clones. High myeloid cell production after transplantation was more stable and maintained by long-lived multi-outcome HSCs.

Thereby erythroid-producing short-lived HSCs which we detected as erythroid-restricted and multi-lineage long-lived HSC dominated the early post-transplantation hematopoietic reconstitution.

Underlying the most abundant clones, we could detect all possible uni- and multi-lineage outcome HSCs. Also, among multi-lineage outcome HSCs, we observed a broad spectrum of different lineage biases. Lineage restrictions were frequently changing over the different analysis timepoints which can reflect different single cell production kinetics of multi-lineage outcome HSCs (Yamamoto et al., 2013a) (Carrelha et al., 2018). We can however not exclude that this is due to sampling in blood (week 2 and 4) and differences in migration between blood and spleen (week 4 and 6). Clearly, B-restricted HSC were not detected at the earliest timepoint in spleen, implying for the least a delayed differentiation of this subset. Besides, we observed few occurrences of long-lived uni-lineage outcome HSCs and uniformly strongly-biased HSCs. Our findings are in line with previous single-cell studies describing E-, EM-, and EMB- restricted HSC in blood (Yamamoto et al., 2013a) (Carrelha et al., 2018) and cellular barcoding studies describing M-, B-, and MB- restricted HSC in blood, spleen or bone marrow (despite the additional analysis of the erythroid lineage) (Lu et al., 2011) (Lu et al., 2019) (Brewer et al., 2016b) (Wu et al., 2014) (E. Verovskaya et al., 2013) (Evgenia Verovskaya, Broekhuis, et al., 2014) (Koelle et al., 2017). Foremost they underline the importance to consider sampling, kinetics, and localization in the lineage-outcome characterization.

Previously, clonal succession and stability on the mature cell level have indirectly been linked to presence, stability, or extinction of clones among HSCs. The outcome of secondary transplantations of different restricted HSC subsets has been the major evidence for this relation (Yamamoto et al., 2013a). We observed here a high overlap of barcodes between HSC and mature cells in line with such relation early after transplantation. This implies that few HSC extensively self-renew without differentiation and that the loss of a clone from mature cells is related to the extinction of this clone at the HSC level. This was also supported by the fact that the few HSC barcodes not found back among mature cells did not correspond to clones detected at previous timepoints. We did observe clones only detected in mature cells which implies differentiation without self-renewal. These represented however only few cells in each lineage. At later timepoints after transplantation the sharing of HSC barcodes with mature cells has repeatedly been described to be low, although not all analyses have been detailed (Evgenia Verovskaya, van Os, et al., 2014) (E. Verovskaya et al., 2013) (Lu et al., 2019) (Sun et al., 2014). Clearly, Aiuti et al. and Biasco et al. reported a low sharing of CD34⁺ cells and mature cells several months after bone marrow transplantation in human (Aiuti et al., 2013) (Biasco et al., 2016). Also in our data, the sharing of barcodes between HSC and mature cells decreased slowly over time (from 100% at week 2 to an average of 91% and 82% at week 4 and 6 respectively) (see Chapter III for 4 months). This could imply that self-renewal without differentiation is a very slow process, present but occurring under the detection limit early after transplantation.

We could observe evidence for a first engraftment of HSC in the spleen and a later switch of the major hematopoietic activity to the bone marrow. Indeed the engraftment of HSC in the spleen after transplantation has been described before (Connie J Eaves, 2015) (Cao et al., 2004) (Malide, Métais, & Dunbar, 2012), and is known to be faster than in the bone marrow (Ahn et al., 2017; S Kim, Lin, Brown, Hosaka, & Scott, 2017; Le et al., 2017; Lo Celso et al., 2009; Malide et al., 2012; L. Wang et al., 2013). It is interesting to consider how this first HSC engraftment could impact the single HSC differentiation kinetics observed. It has previously been described that the rate of maturation of erythroid precursors is around 10 time higher in spleen as compared to bone marrow, due to an increased cycling of bone marrow vs splenic erythroid progenitor cells (Barminko, Bernitz, & Ghaffari, 2013). Thereby the splenic localization might be crucial for the fast and high production of erythroid cells by HSCs we observed.

The initial phase of anemia is one of the most critical phases for the success of bone marrow transplantation in the clinics (Tomblyn et al., 2009) (Radia & Pamphilon, 2011). Improved transplantation protocols allowing a faster erythroid recovery could greatly increase survival rates and shorten hospitalization of transplant patients. We observed here that early high erythroid production was maintained by distinct HSCs. The enrichment of this HSC subsets in the transplant could be one step towards a shorter anemic phase after transplantation. However, it would have to be monitored carefully how an increase in short-lived HSC clones in the transplant would influence long-term outcomes. Also the influence of splenic localization on differentiation kinetics could be a point of attack to improve transplantation protocols. It could be thought to direct specific HSC to the spleen to fasten their differentiation. Also the expansion of HSC niches in the spleen or ectopic sites could be envisaged (Inra et al., 2015) (Short, Lim, Tan, & O'Neill, 2019). As tested with endothelial cells, the co-infusion of splenic cells during transplantation might be a further tool to test. Traditionally the importance of the spleen for hematopoiesis has mainly been considered in murine models. Different first clinical trials show however that also during clinical bone marrow transplantation, spleen function can be related to treatment outcome (Kroger et al., 2009) (Short et al., 2019).

We established here the detection of cellular barcodes from RNA of erythroid cells in blood and applied it to study the kinetics of single cell HSC differentiation early after transplantation. Longer kinetic studies will allow to analyze long-term single HSC contribution the erythroid lineage. The detection of barcodes from RNA, paves the way for new applications of cellular barcoding in the future, especially its combination with scRNAseq profiling to link transcriptome and lineage tracing information (see also Chapter IV). CRISPR Cas9 based lineage tracing systems have already allowed such linkage in zebrafish (Spanjaard et al., 2018), (Raj et al., 2018), (Alemany, Florescu, Baron, Peterson-Maduro, & Van Oudenaarden, 2018). The detection of cellular barcodes from RNA could allow to match lineage tracing information to the emerging scRNAseq profiles of HSCs and other hematopoietic progenitors.

2.4 Materials and methods

Mice

Male C57BL/6J CD45.1⁺ (in house breeding or Charles River) and C57BL/6J CD45.2⁺ (Charles River) donor and recipient mice were maintained at the animal facility of Institute Curie. Heterozygous *Rosa26CreER^{T2};mT/mG* mice were generated by crossing of *Rosa26mTmG* (<https://www.jax.org/strain/007576>) and *ROSA26CreERT2-TG* (<https://www.jax.org/strain/008463>) mice. Experiments were performed between 7-13 weeks of age. All procedures were approved by the responsible national ethics committee.

Lentivirus production

A new barcode library, named LG2.2, was generated. Oligo DNA stretches of 180 bp including a 20 bp “N”-stretch were ordered. DsDNA was generated by 10 PCR rounds and cloned into the XhoI-EcoRI site of the lentiviral pRRL-CMV-GFP plasmid (original description Dull et al., 1998). Subsequently ElectroMaxStbl4 cells were transformed, and approximately 16 000 colonies picked for amplification by Maxiprep. A colony PCR showed approximately 15% of barcode negative colonies which led to an estimated final diversity of 13 000 barcodes for the new LG2.2 library. Lentivirus was produced by transfection of HEK293T cells in DMEM-Glutamax supplemented with 10% FCS (Gibco), 1% MEM NEAA, and 1% sodium pyruvate. Polyethylenimine-mediated transfection together with psPAX2 and pVSVG. Supernatant was 0,45 um filtered, concentrated by 1h30 ultracentrifugation 31 000g and frozen at -80°C.

HSC isolation

Donor mice were sacrificed by cervical dislocation, femur, tibia and iliac bone isolated, and kept at 4°C in RPMI-1640-Glutamax (Gibco) supplemented with 10% FCS (Gibco) and 1% P/S (Gibco) (10%RPMI). Bone marrow was flushed using a 21G needle. Cells were washed with 10%RPMI by centrifugation for 6 minutes and 300g at 4°C and stained with α -c-kit magnetic beads (Miltenyi) at a dilution of 1:6 in 150 μ l 10%RPMI on ice for 20 minutes. After 5 minutes of incubation α -c-kit antibody was added for competitive staining. After staining, cells were washed as above and c-kit⁺ cells enriched by MACS (one LS column for three mice). An insulin syringe was used to generate a single cell suspension. Cells were maintained in 10%RPMI on ice at 4°C overnight. The next day, cells were washed as above and c-kit, Flt3, CD150 and Sca-1 stained in 200 μ l 10%RPMI for 35 minutes at 4°C. Cells were washed and filtered into 5 ml polystyrene round bottom tubes with cell strainer cap (Falcon) and HSC (LSK flt3⁻ CD150⁺) (Figure S1) sorted into FCS-precoated Eppendorf tubes.

HSC barcoding and transplantation

Sorted HSC were washed with HBSS (Gibco) by centrifugation for 6 minutes and 300 g at 22°C and resuspended in StemSpanMedium SFEM (STEMCELL Technologies) supplemented with 50 ng/ml mSCF (STEMCELL Technologies) and 1% P/S (Gibco) (SS⁺) at a maximal concentration of 100 000 cells/ 100 µl in a 96-well round bottom plate. 1,3 µl (tested to give a 10% transduction efficiency) of lentivirus from the lentiviral barcode library were added and the cells centrifuged for 1,5 hours at 300g and 4°C with slow deceleration and acceleration. This was followed by a 4,5 hours incubation at 37°C. Cells were washed as before in 10% RPMI and resuspended in 200 µl SS⁺ cells and incubated for 16 hours at 37°C. After the incubation, the cells were washed as before once in 10%RPMI and once in HBSS, and finally resuspended in HBSS for transplantation. Recipient mice were sub-lethally irradiated at 6Gy. 2500 cells (of which approximately 250 barcoded cells) were injected in the tail vein of each mouse with an insulin syringe.

Cell progeny isolation from blood, spleen, and bone

At the indicated timepoints after transplantation (2 weeks, 4 weeks, or 6 weeks), blood was taken, and/or spleen, and/or bones were isolated from recipient mice. For the analysis of barcodes in HSC and other progenitor populations a c-kit-enrichment was performed on bones as during initial HSC isolation (see above). Blood was taken retro-orbital, immediately mixed 1:5 with citrate concentrated solution (Sigma) at RT and diluted in 10% RPMI. After cervical dislocation, splenic cells were isolated by meshing of the spleens through 100 µm cell strainers in 10%RPMI. From here on blood and spleen cells were processed similarly. Cells were washed by centrifugation for 6 minutes at 300 g and 4°C and stained in 150 µl 10%RPMI supplemented with 50 µg/ml DNase I (Invitrogen) with α -Ter119-biotinylated antibody (Table 1) for 20 minutes on ice. After 5 minutes, α -Ter119 PEcy7 antibody was added for competitive staining. After staining, 50 µl biotin-magnetic beads (Miltenyi) (final dilution 1:6) and 100 µl 10%RPMI were added and cells incubated for 20 min on ice. Cells were washed as above and Ter119⁺ and Ter119⁻ cells separated by MACS (one LS column per mouse). An insulin syringe was used to generate a single cell suspension. Cells were counted using a Neubauer chamber and kept on ice at 4°C overnight. The next day, cells were washed as above and stained. Ter119⁺ cells were stained for TER119 and CD44. Ter119⁻ cells were stained for CD45.1 CD11b, CD11c, CD19, and Ter119 in 100 µl 10%RPMI for 25 minutes at 4°C (Table 1). Cells were washed, resuspended in 10%RPMI. Viable donor (CD45.1⁺ or Tom⁺) barcoded (GFP⁺) B-cells (B; Ter119⁻ CD19⁺), myeloid cell (M; Ter119⁻CD19⁻ CD11c⁻ CD11b⁺), and erythroid cell (E; Ter119⁺ CD44⁺) lineage were sorted into FCS-precoated Eppendorf tubes (Figure S1). Propidium iodine (Sigma) was added at a concentration of 1:5000 directly before sorting. The volume before and after sorting was carefully determined for total

cell output calculations (the total cellular output was considered to be equal to the number of cells sorted multiplied by the total volume before sorting, divided by the volume actually sorted). For the analysis of progenitors from spleen, a c-kit-enrichment was performed on spleen cells as on bones (see above). The c-kit⁺ fraction was further processed for HSC and progenitor isolation (MPP; LSK Flt3⁺ CD150⁻, multipotent progenitors P; Lin⁻ c-kit⁺ Sca-1⁻) (Figure S1). On the c-kit⁻ fraction, Ter119-enrichment was performed as described before.

Table 1: Fluorescently labelled antibodies used for FACS

Antibody target	Clone	Conjugate	Manufacturer
CD45.1	A20	PE	BD
Ter119	TER119	PECy7	BD
CD11c	N418	APC	eBio
CD19	1D3	APCCy7	BD
CD11b	M1/70	PerCPCy5.5	eBio
CD117 (c-kit)	2B8	APC	Biogened
CD135 (flt3)	A2F10	PE	eBio
CD135 (flt3)	A2F10	PECy5	Life technologies
Sca1	D7	Pacific Blue	Biologend
CD150	TC15-12F12.2	PECy7	Biologend
Ter119	TER119	biotin	BD
CD44	IM7	PE	BD

Cell progeny lysis, barcode amplification and sequencing

After sorting, cells were concentrated by centrifugation as above and supernatant removed. For barcode amplification from DNA, cells were transferred into the wells of a 96-well PCR plate and lysed in 40 µl Viagen/Euromedex Direct PCR (cell) lysis buffer supplemented with 0,5 mg/ml proteinase K solution RNA grade (Invitrogen) in a thermic cycler: (55°C for 120 min, 85°C for 30 min, 95°C for 5 min, indefinite at 4°C). After lysis, samples were frozen at -20°C till further handling. For barcode amplification from RNA, cells were resuspended in 20 ul of Superscript III RT buffer (Invitrogen) and transferred into the wells of a 96-well PCR plate. Lysis buffer, and reagents for RT by SuperScript III RT (Invitrogen) were added and lysis+RT performed using the primer top-LIB (5'TGCTGCCGTCAACTAGAACA-3') (1h at 50°C and 20 min at 70°C). After lysis (+RT), barcodes were amplified by a newly established three step nested PCR. Barcode sequences were first amplified using the primers top-LIB (5'TGCTGCCGTCAACTAGAACA-3') and bot-LIB (5'GATCTCGAATCAGGCGCTTA-3'). In a second round PCR, the Illumina sequencing primer sequences were added as well as a unique 4bp plate index. Primers used were as follows: forward (as 5'ACACTCTTCCCTACACGACGCTCTTCCGATCTNNNNCTAGAACA CT CGAGATCAG) and reverse (5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGATC

TCGAATCAGGCGCTTA). In a third round PCR, the P5 and P7 flow cell attachment sequences as well as one of 96 sample indices of 7bp were added. Primers were as follows: P5 (5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT) and P7 (5'CAAGCAGAAGACGGCATACGAGANNNNNNGTGACTGGAGTTCAGACGTGCTCTTCCGATC) (PCR1 and 2 30 cycles : hot start 5 min 95°C, 15 s at 95°C ; 30 s at 57.2°C; 30 s at 72°C, 5 min 72°C. for PCR3 15 cycles). Both index sequences (sample and plate) were designed based on (Faircloth & Glenn, 2012) such that sequences differed by at least 2 bases, homopolymers or more than 2 bp, hairpins and complementary regions with the rest of the primer sequence were absent (sequences available on request). To ease sequencing at least 4 different plate indices were used for each sequencing run. Primers were ordered desalted, as high-performance liquid chromatography (HPLC) purified. During lysis and each PCR, a mock control was added. The DNA amplification by the three PCRs was monitored by the run on a large 2% Agarose gel. Samples were pooled in order to guarantee a sequencing depth of 50 reads/cell. Five µl of the products of PCR3 for each sample and replicate were pooled and sequenced on a HiSeq system (Illumina) (SR-65bp) at the sequencing facility of Institute Curie (10% of Phix Illumina phage genome library were added to generate a more diverse set of clusters). Before sequencing, DNA sequences of adequate length (324 bp) were purified using the Agencourt AMPure XP system, analyzed on a Bioanalyzer, and diluted to a concentration of 5 nM.

Barcode sequence analysis

Sequencing results were analyzed using R-3.4.0, Excel, and GraphPad Prism. Firstly, reads were compared to a newly established barcode reference list (Figure S11) and filtered for a 100% match in the input index- and common- sequences using XCALIBR (<https://github.com/NKI-GCF/xcalibr>). Next, the mean read count per sample and plate index combination was analyzed. All samples displaying a mean read count above 5000 were allowed for further analysis. The reads belonging to a same sample were summed (same sample index, and plate indices of a same plate). Because read counts were variable, reads were normalized to 10^5 per sample. The Pearson correlation in read numbers for all barcodes between the two replicates for every sample was calculated. Samples with a correlation above 90% were considered for further analysis. Next, barcodes with reads in only one of the two replicates were discarded. For further analysis, the mean of the technical replicates was used. The sharing of barcodes between all mice transplanted from a same transduction batch over sequencing runs was determined. A read threshold was implemented to equalize the barcode sharing between mice transplanted from a same transduction batch within each sequencing run. When indicated Student's T test was used to test statistical significance. When indicated, heatmaps were generated using R on log10 transformed data using complete linkage and Euclidean distance (samples without reads were attributed the lowest

occurring values before log10 transformation -> color coding black). When indicated, barcodes were categorized in progenitor classes either defining output in a lineage as presence of reads or >10% of a barcode's reads. When indicated, data was arcsine transformed for representation.

Fluorescence activated cell sorting

FACS was performed at the flow cytometry facility of Institute Curie on a FACS ARIA. FACSDiva™ software (BD) was used for measurements. Data analysis was performed using FlowJo v.10 (TreeStar). Cells were sorted using a 70 µm nozzle at precision 0/16/0 and high efficiency.

2.5 Supplementary information

2.5.1 Gating strategies

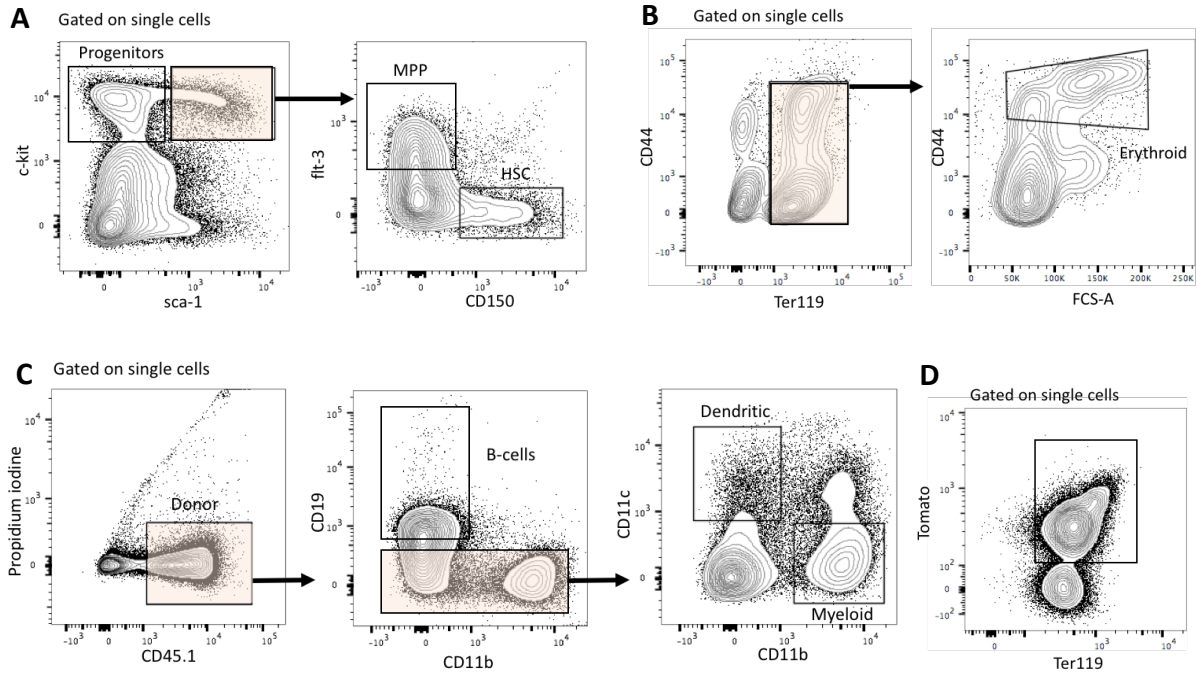


Figure S1: Gating strategies for hematopoietic progenitor and mature cell subsets for barcode analysis. (A-D) (A) HSC were gated as viable single c-kit⁺ Sca-1⁺Flt3⁻ CD150⁺ cells, MPP as c-kit⁺ Sca-1⁺ Flt3⁺ CD150⁻, and progenitors as c-kit⁺ Sca-1⁻ subset of c-kit-enriched (Lin⁻) bone marrow cells. (B) Erythroid cells for barcode analysis were gated as Ter119⁺CD44⁺ and FSC high cells on Ter⁺ enriched cells from bone marrow, spleen, or blood. (C) Gating strategy for B-cells (CD19⁺ CD11b⁻), dendritic cells (CD19⁻ CD11b⁻CD11c⁺), and myeloid cells (CD119⁻CD11c⁻CD11b⁺) on Ter119⁻, live single, donor cells (Tom⁺ or, as depicted here CD45.1⁺) on Ter119-depleted cells from bone marrow, spleen, or blood. (D) The Tomato expression of Ter-enriched cells in blood of unperturbed Rosa26CreER^{T2}; mTdT/mGFP mice.

2.5.2 The variability between mice

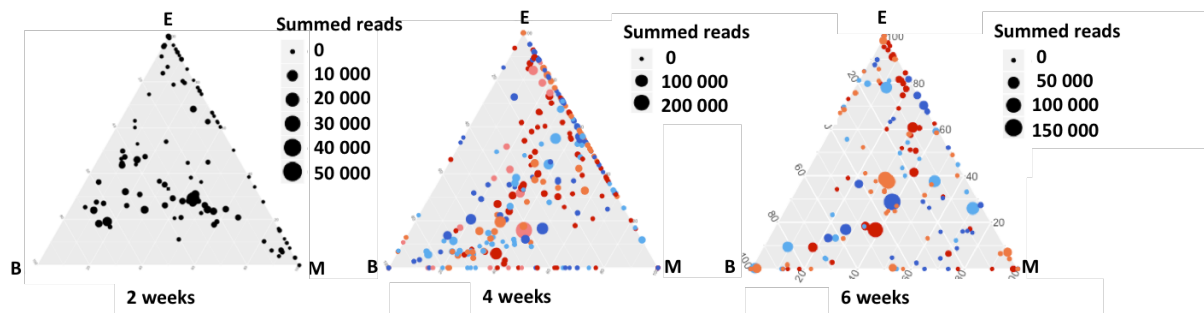


Figure S2: The variability between mice regarding barcode results. HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen of individual mice sacrificed at week 2, 4, or 6 post-transplantation. (A-B) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output for the different experimental groups as indicated. Different colors are used for individual mice in each group.

2.5.3 The read distribution in different uni- and multi-lineage outcome categories

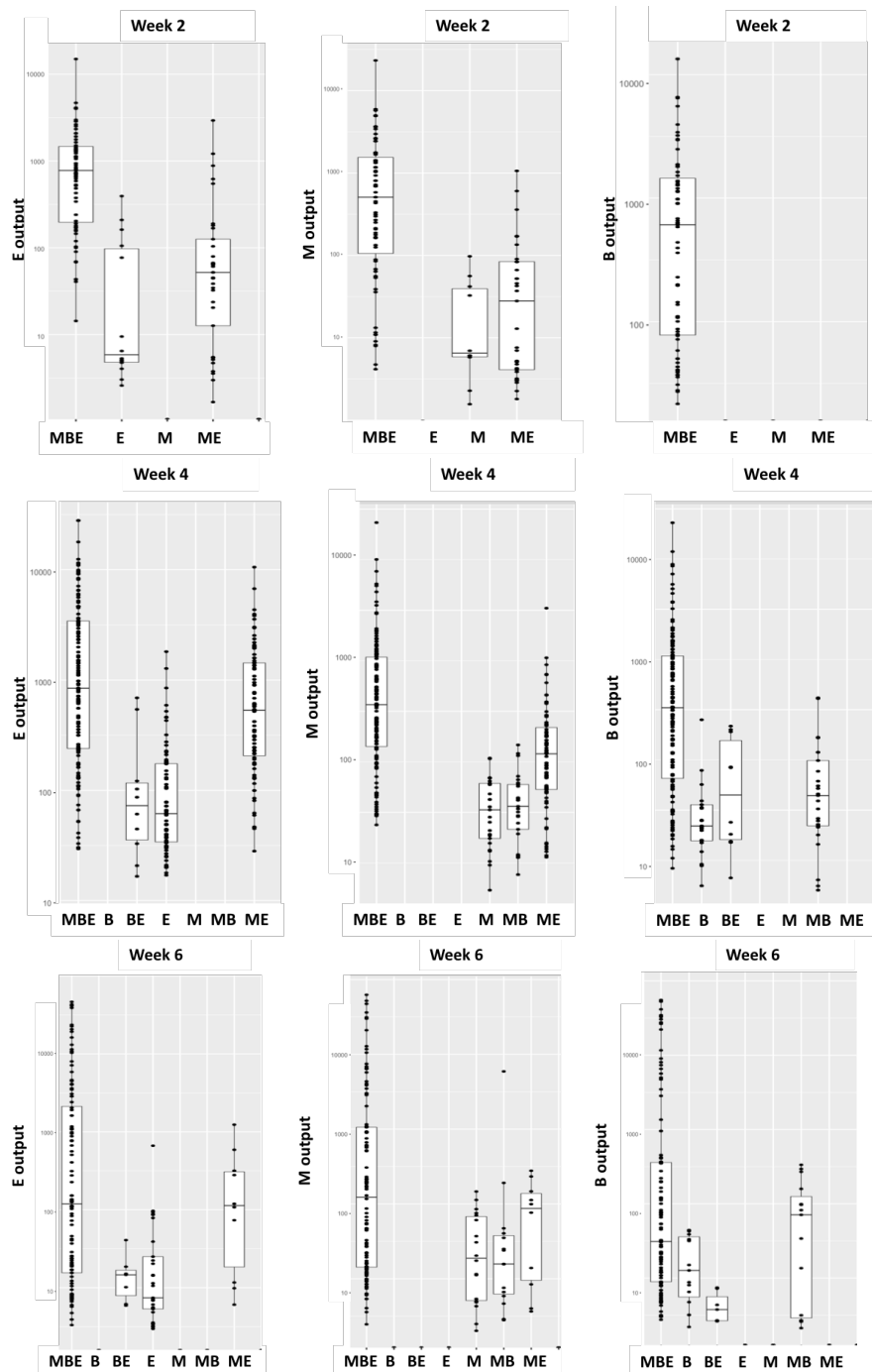


Figure S3: The read distribution in different lineages over lineage outcome categories. HSC were sorted from Rosa26CreER^{T2}; mTdTTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen or blood of individual mice sacrificed at week 2, 4, or 6 post-transplantation. (A-B) Box plots showing the relative abundance of barcodes (dots) of different uni- and multi-lineage outcome categories in the E, M, and B lineage as indicated.

2.5.4 Comparison of blood and spleen barcoding profiles

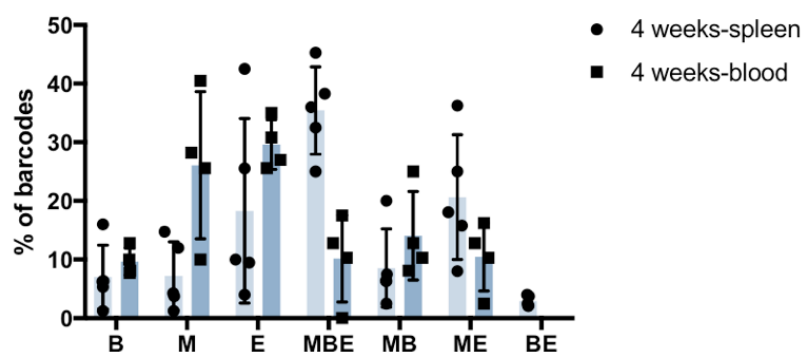


Figure S4: Comparison of blood and spleen barcoding profiles. HSC were sorted from Rosa26CreER^{T2}; mTdTTomato/mGFP mice or CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes 4 weeks post-transplantation in the E, M, and B lineage from blood samples compared to barcode profile in spleen (of different cohorts). Percentage of barcodes found back in the indicated lineage combinations at week 4 after transplantation in spleen or blood. Shown are values from individual animals (n=4 for blood and n=5 for spleen collected over 2 different experiments). For all bar graphs mean and S.D. between mice are depicted.

2.5.5 The influence of sampling on barcoding profiles

We tested two different subsampling approaches. In a first step we assumed the full blood would correspond to the full spleen in terms of barcode composition. As we sorted all B- and myeloid cells from spleen, we took the sampling of blood from total blood as the subsampling factor for these lineages (around 10%). For the erythroid lineage we used data from Boyer et al to estimate a 0,25% sampling as compared to spleen (Boyer et al., 2019). The resulting uni- and multi-lineage outcome HSC distribution did not correspond to the distribution in blood (Figure S5 A and Figure S4). Rather a uniform subsampling across the three lineages, which we set off for in a second step, resulted in a uni- and multi-lineage outcome HSC distribution similar to the distribution in blood (Figure S5 B). Such uniform subsampling does enhance lineage biases, meaning strong lineage biases will be detected as lineage restrictions.

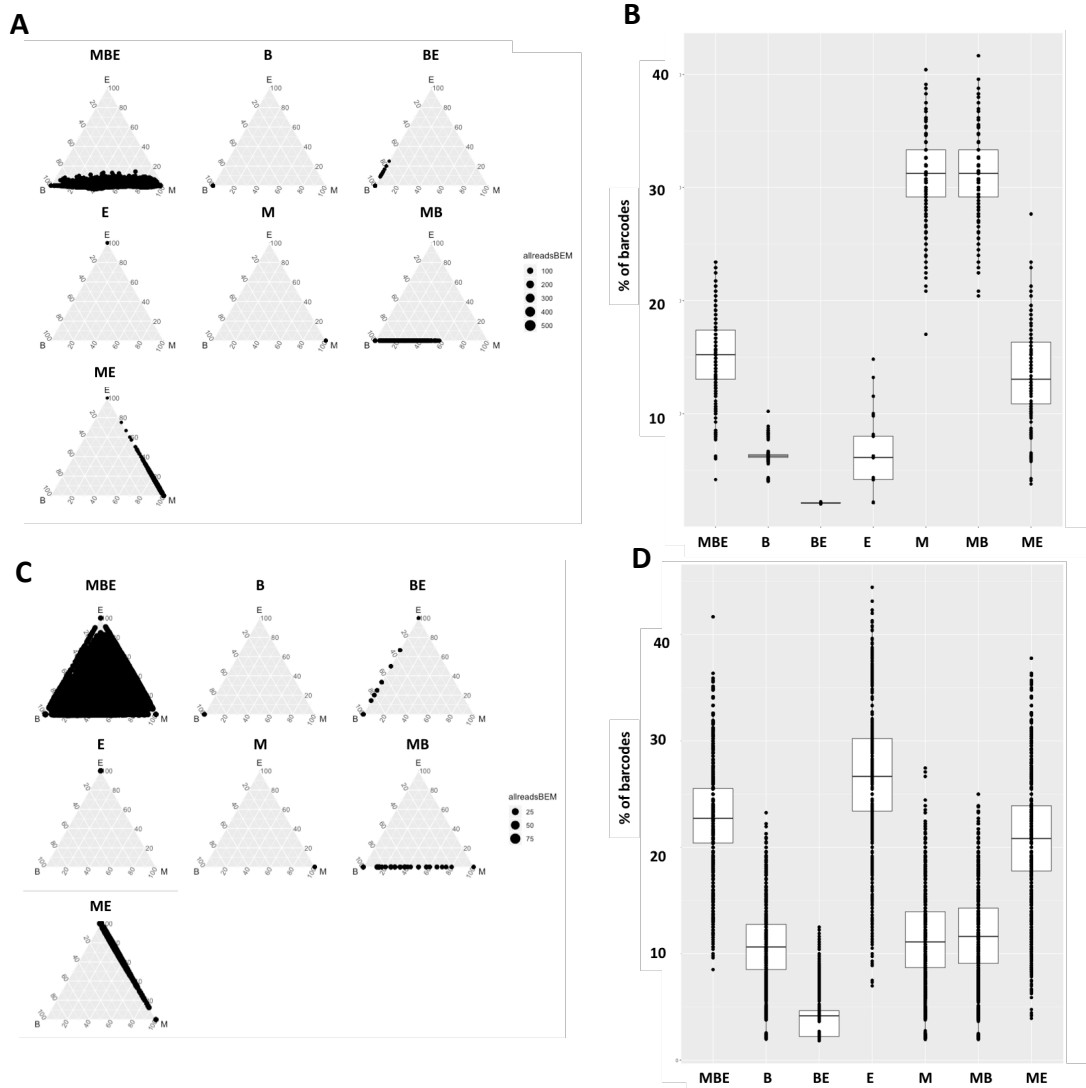


Figure S5: Comparison of blood and spleen barcoding profiles. (A-D) HSC were sorted from Rosa26CreER^{T2}; mTdtomato/mGFP mice or CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes 4 weeks post-transplantation in the E, M, and B lineage from spleen. (A) Barcode data was subsampled 10% in the M and B lineage, and 0,25% in the E lineage (1000 repeats). For barcodes of each original uni- and multi-lineage outcome category the distribution of the resulting barcode profiles is depicted in a triangle plot showing the relative abundance of barcodes in the E, M, and B lineage with respect to the summed output in the three lineages. (B) Percentage of barcodes found back in the indicated lineage combinations after subsampling as in (A). (C-D) Representations as in (A-B) for barcode data subsampled 10% in all three lineages (10 000 repeats). Analysis was repeated for 5 mice, one representative mouse is shown. For all bar graphs mean and S.D. between repeats are depicted.

2.5.6 Changes in lineage restriction of individual HSC over time

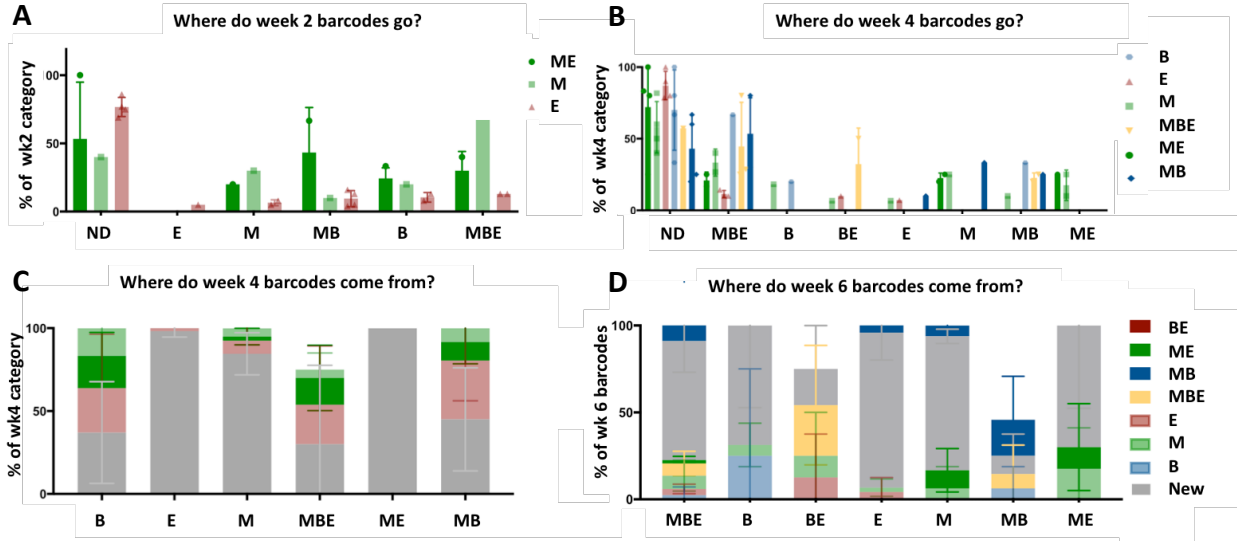


Figure S6: Changes in lineage restriction of individual HSC over time. (A-D) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen 6 in the same individual mice. (A-D) Bar-graph showing the fate (A, B) and origin (C, D) of barcodes detected with the indicated lineage restriction at the indicated timepoint. Fate and origin are defined based on the detection and lineage restriction at the respectively previous (origin) and next (fate) analysis timepoint. Shown are values from the same animals throughout (n=4 from one experiment). For all bar graphs mean and S.D. between mice are depicted.

2.5.7 The overlap of bone HSC with mature hematopoietic cells

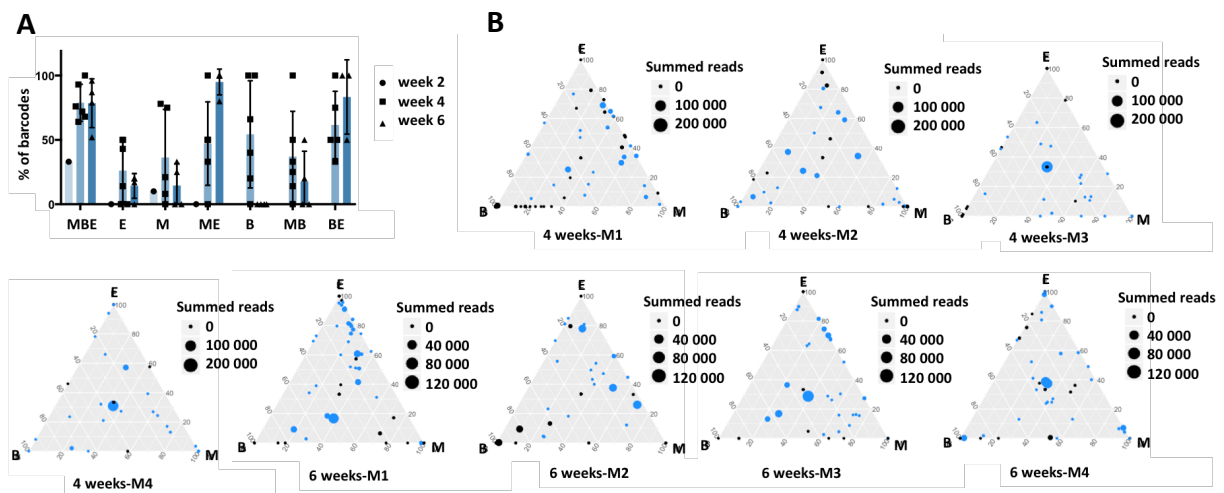


Figure S7: The percentage of different lineage outcome categories found back in bone HSC. (A-B) HSC were sorted from Rosa26CreERT²; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen, and HSC in bone at week 2, 4, or 6 post-transplantation. (A) Percentage of barcodes found back in bone HSC for the indicated

lineage combinations. Mean and S.D. between mice is depicted. Shown throughout are values from individual animals (n=1 week 2, n=6 (or 10) week 4, n=4 week 6, collected over 5 different experiments). (B) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output at week 4, and 6 after transplantation for barcodes found back in bone HSC (blue) or not (black) for single mice.

2.5.8 The overlap of spleen HSC with mature cells-variability between mice

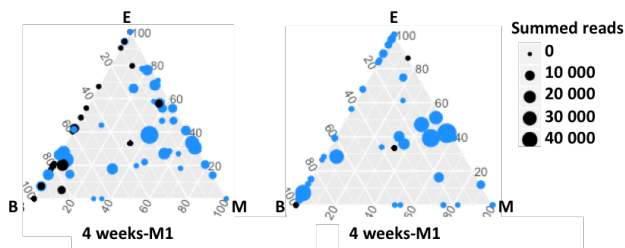


Figure S8: The variability between mice in overlap of spleen HSC with mature hematopoietic cells. (A-B) HSC were sorted from Rosa26CreER^{T2}; mTdTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage and LSK in spleen, 4 weeks post-transplantation. Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output at week 4, and 6 after transplantation for barcodes found back in spleen LSK (blue) or not (black) for single mice.

2.5.9 The variability between mice in hematopoietic progenitor subset abundance

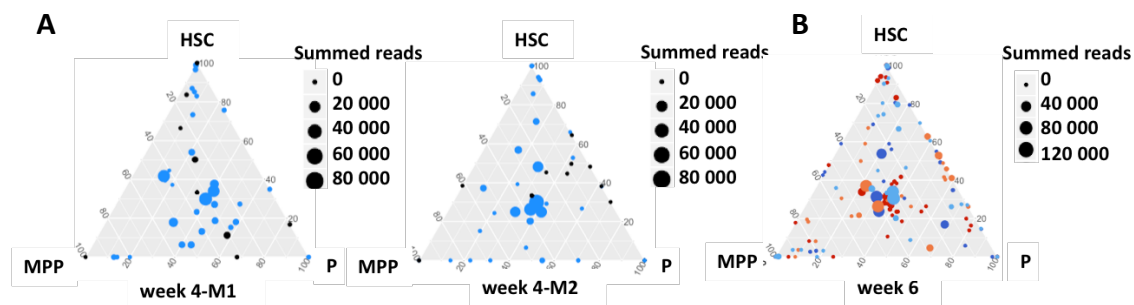


Figure S9: The variability between mice in hematopoietic progenitor subset abundance. (A-B) HSC were sorted from Rosa26CreER^{T2}; mTdTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the HSC, MPP, and P lineage in bone and LSK in spleen, 4 or 6 weeks post-transplantation. (A) Triangle plots showing the relative abundance of barcodes (dots) in the HSC, MPP, and P cells with respect to the summed output at week 4 for barcodes found back in spleen LSK (blue) or not (black) for single mice. (B) Triangle plots showing the relative abundance of barcodes (dots) in the HSC, MPP, and P cells with respect to the summed output at week 4 colored by individual mice.

2.5.10 The influence of previous detection on detection of barcodes in HSC

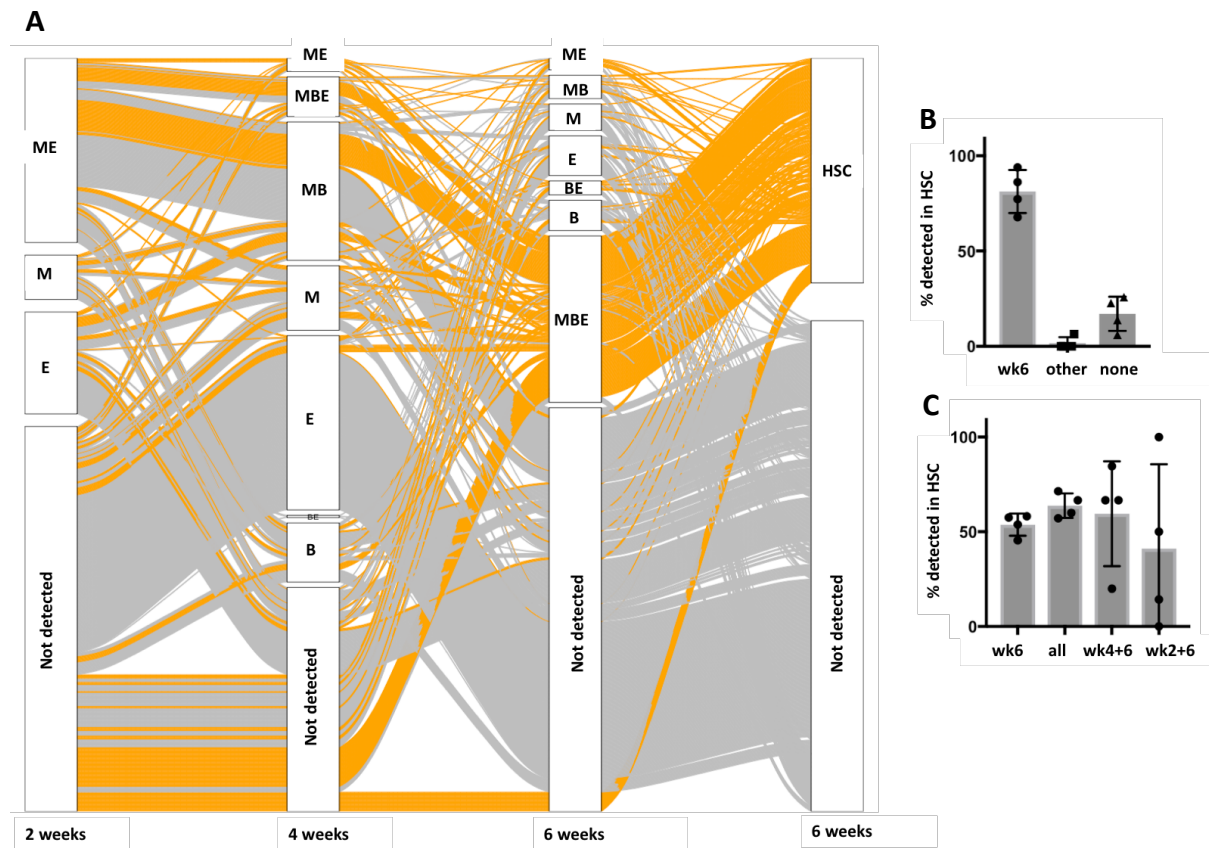


Figure S10: Influence of previous detection of barcodes on detection in HSC at week 6. (A-C) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen at week 6, as well as HSC in bones at week 6, in the same individual mice. (A) Sankey diagram showing detection of barcodes in different lineages over time for all mice combined. Orange color is used for barcodes detected in HSC at week 6. (B) Percentage of barcodes detected in bone HSC at week 6, which were found at week 6, another timepoint, or no timepoint in mature hematopoietic cells (E, M, B) in spleen. (C) Percentage of barcodes detected in mature hematopoietic cells (E, M, B) at week 6 only, or as indicated which was found back among HSC in bone. Mean and S.D. between mice (n=4) is depicted.

2.5.11 Barcode reference list

Creation of a barcode reference list as in (Gerlach et al., 2013). Briefly, the entire library previously cloned from 16 000 barcodes of unknown sequence, underwent both PCR and sequencing in duplicate. This gave unique sequences, the majority of which were presumably a result of PCR and sequencing errors. The read numbers were normalized between replicates and sequences present in both replicates were sorted by frequency. The cumulative read number was plotted as a function of the

number of sequences observed and barcode sequences that made up less than 92,5% of the cumulative reads were discarded (Figure S11 A). barcodes that were more than 1,5-fold more prevalent in one of replicates were discarded (Figure S11 B). Finally, the most abundant barcodes were discarded (Figure S11 C).

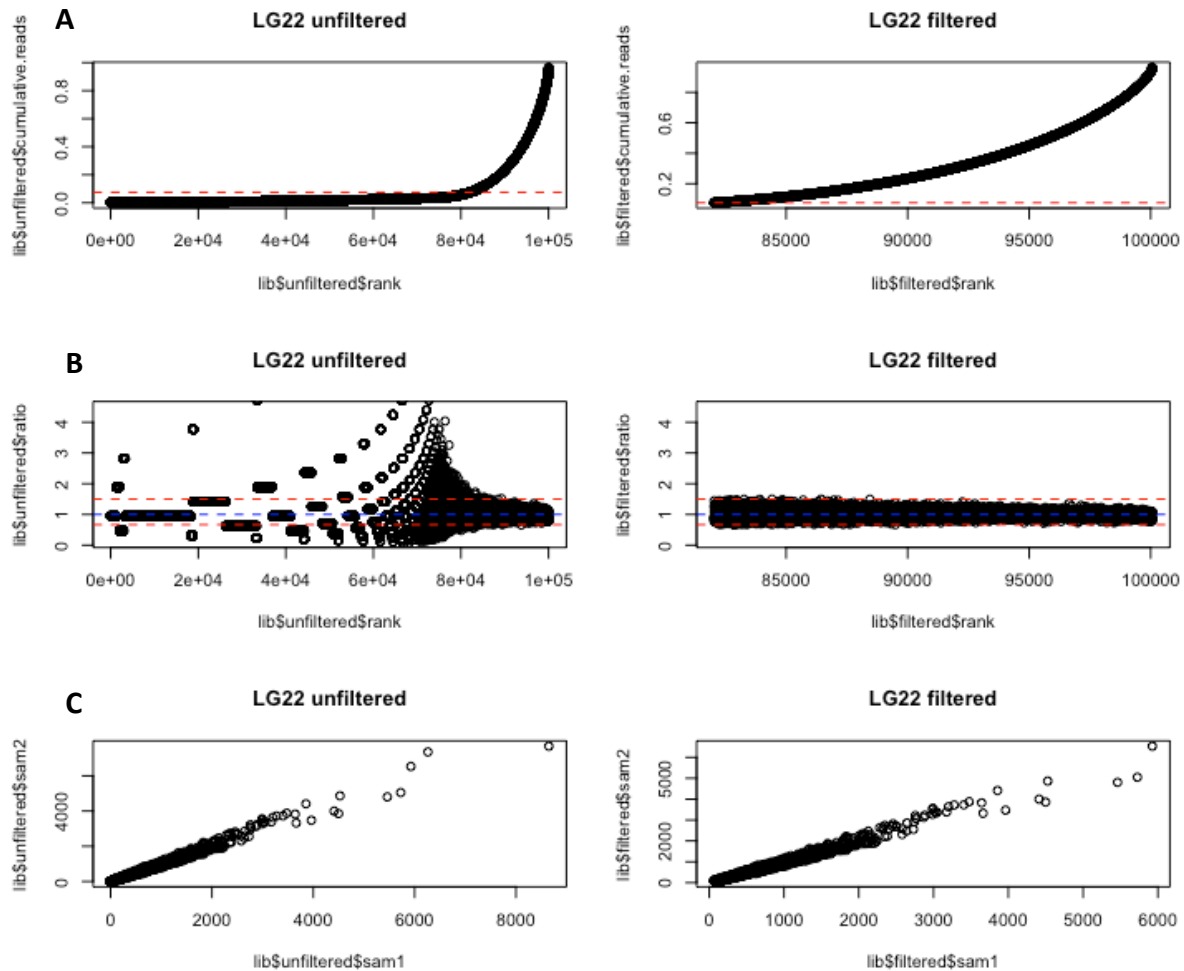


Figure S11: Filtering for the creation of the LG2.2 reference library. (A-C) (A) cumulative distribution of all barcodes. The red line represents a filter, below which all barcodes are removed from the reference library. (B) ratio of replicates with the blue dotted line representing perfect agreement between replicates and the dotted red lines representing thresholds for the ratio. Barcodes that exceed this threshold are removed. (C) a plot further showing the agreement between replicates before and after removal of the most abundant barcodes (Creation of reference library by J. Cosgrove).

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Chapter 3: Erythropoietin affects hematopoietic stem cell clonality after transplantation

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3.0 Abstract

The cytokine EPO is a potent inducer of erythrocyte development and one of the most prescribed biopharmaceuticals worldwide. EPO action has been attributed to committed erythroid progenitors which highly express the EPO receptor, but an effect on HSC has also been suggested. EPO-induced changes in HSC clonality could potentially lead to adverse long-term treatment effects. Here we studied the influence of *in vitro* EPO exposure on the lineage commitment of hundreds of single HSC after bone marrow transplantation using a lentiviral cellular barcoding system for lineage tracing. Over the first six weeks after transplantation the clonal output of control and EPO-exposed HSC was assessed in erythroid cells, macrophages, neutrophils, eosinophils, monocytes, dendritic cells (DC), megakaryocyte progenitors (MkP) and B-cells. In both conditions, reconstitution kinetics of the different hematopoietic lineages after transplantation were characterized by co-occurrence of clonal stability and clonal succession, and distinctive lineage production kinetics and restrictions at the single cell level. However, after transplantation of EPO-exposed HSC long-lived unbiased multi-outcome HSCs lost preponderance with respect to cellular output. Rather, changing clones of two types of highly biased HSCs, myeloid-erythroid (ME)-biased and myeloid B-cell (MB)-biased HSC, produced now the majority (>60%) of erythroid, myeloid and B-cells. This effect was transient but stable over different EPO concentrations and after *in vivo* EPO treatment during transplantation. At the progenitor level, a higher number of HSC was detected and self-renewing without differentiation early after transplantation of EPO-exposed HSCs, suggesting that the high output ME-biased and MB-biased HSC could occur through functional compensation mechanisms. We hope the detailed description of the effect of EPO exposure on single HSC will improve and further the clinical use of EPO and be an important step towards the elucidation of the general mechanisms by which cytokines influence HSC behavior in steady state and different pathologies.

3.1 Introduction

Erythrocytes are the most numerous hematopoietic cells in our body and are constantly renewed (Sender et al., 2016). The major regulator of this process is the cytokine EPO which is a potent inducer of erythroid cell development in steady state and during anemic conditions when its levels increase (Richmond et al., 2005). Recombinant EPO is widely used in the clinics to counteract anemia in many different settings, and one of the most sold biopharmaceuticals in the world (Walsh, 2014). Recently, adverse effects of the clinical use of EPO have been reported as hypertension, thromboembolism, stroke, leukemic disease, and death in cancer patients (Rainville, Jachimowicz, & Wojchowski, 2016) (Ma et al., 2010) (Halawi, Cappellini, & Taher, 2017).

The action of cytokines to enhance production of specific hematopoietic cell subsets in times of need is classically considered to be restricted to mature hematopoietic cells or committed hematopoietic progenitors. Accordingly, EPO's main action has been considered to be an increase in viability of the erythroid committed progenitors CFU-E and ProEB. Over the last years however some evidence accumulated that less committed hematopoietic progenitors, and even HSC could be affected by cytokines too, and this has also been suggested for EPO (Rieger et al., 2009) (De Bruin et al., 2012) (Grover et al., 2014).

For mature hematopoietic cells and committed hematopoietic progenitors, cytokines are considered to act through general induction of proliferation to increase cell numbers of a specific lineage. For HSC, two other mechanisms of cytokine action have been suggested: the selective induction of proliferation in a pre-existing lineage-biased HSC subset, and a de novo lineage instruction. Indeed for both principles first evidence exists (Matatall et al., 2014) (Challen et al., 2010) (Mossadegh-Keller et al., 2013) (Etzrodt et al., 2019) (Grover et al., 2014). Two studies, strengthened the importance of the first working mechanisms by suggesting a selective proliferation of myeloid-biased HSC after IFN γ and TGF- β exposure respectively (Matatall et al., 2014) (Challen et al., 2010). Two other studies highlighted the second principle of cytokine action on HSC. Firstly, the lineage biasing of HSC by M-CSF has been suggested and debated (Mossadegh-Keller et al., 2013) (Etzrodt et al., 2019). Secondly, high systemic levels of EPO have been implicated in the biasing of HSC fate (Grover et al., 2014).

High systemic levels of EPO (short or chronic) and in vitro culture with EPO have repeatedly been shown to change the transcriptome of HSC. In line with older studies (Cheshier et al., 2007) (Erslev, 1971) (Dubart et al., 1994), several recent scRNAseq studies reported an increase of HSC proliferation through high systemic EPO levels (Giladi et al., 2018) (Tusi et al., 2018), sometimes in conjunction with an increased expression of immune response genes unrelated to lineage differentiation (J. Yang et al., 2017) and/or genes linked to erythroid development (Singh et al., 2018) (Grover et al., 2014). Also the induction of genes related to bone homeostasis by EPO in HSC has been reported

(Shiozawa et al., 2010). In some studies EPO increased the expression of lineage related genes rather in different MPP subsets, than in HSCs. The induction of genes linked to erythroid/ megakaryocytic development (Tusi et al., 2018), to myeloid, megakaryocytic and erythroid development (J. Yang et al., 2017), as well as an induction of innate immune response and myeloid genes by EPO in MPP (mix, “MPP1”, or “MPP3” and “MPP4” respectively) has been reported (Singh et al., 2018). How these transcriptional changes induced in HSC by EPO relate to actual lineage differentiation, has been studied in Grover et al. Here, high systemic EPO levels induced expression of erythroid genes and decreased myeloid gene expression in HSC, but this was also linked to a changed differentiation. In vivo EPO-exposed HSC produced upon transplantation higher erythroid and less myeloid cells, which suggested a biasing of HSC towards the erythroid lineage by EPO similar to the effect of M-CSF on HSC reported. The creation of an “erythropoietic superhighway” was suggested with a biasing towards the erythroid lineage at every bifurcation of the classical hematopoietic tree (Grover et al., 2014). As EPO was administered systemically, it remains however unclear if the effects of EPO on HSC were really direct, or maybe via effects on bone marrow or through feedback mechanisms. Both have been described previously (C. Li et al., 2015) (Singbrant et al., 2011) (American Society of Hematology et al., 2017) (Zhang et al., 2014) (Ito et al., 2017).

In this report we have assessed the direct effects of EPO on the differentiation of single HSC. The lineage commitment of hundreds of single HSC in up to eight hematopoietic cell subsets form five lineages after EPO-exposure and transplantation was assessed using a new cellular barcoding library.

3.2 Results

EPO influence on HSC engraftment and bulk differentiation

Cellular barcoding was used to study the effect of *in vitro* EPO priming on HSC differentiation after transplantation. HSC (LSK Flt3⁻ CD150⁺) (Figure S1) were isolated from tibia, ilium, and femur of Rosa26CreER^{T2}; mTdTomato/mGFP or CD45.1 donor mice and labelled with unique genetic barcodes by lentiviral transduction. A new lentiviral barcode library (LG2.2) of over 10 000 barcodes was applied, which's barcodes consist of random stretches of 20 nucleotides which are integrated in transduced cell in conjunction with GFP, both expressed through the CMV promoter. The transduction protocol, and controls for single integration of barcodes were applied as previously described, which included a low transduction efficiency of 10%, and a low number of transduced cells relative to the capacity of the barcode library (Naik et al., 2013). Transduced HSC were cultured with or without EPO at 1000 ng/ml for 16 hours in vitro (Figure S2) and transplanted at 2500 cells/mouse (of which around 250 barcoded HSC) without helper cells in sub-lethally irradiated mice. At day 30 after transplantation, barcoded (GFP⁺) erythroid (E; Ter119⁺ CD44⁺), myeloid (M; Donor Ter119⁻ CD19⁻ CD11c⁻ CD11b⁺),

and B-cells (B; Donor Ter119⁻ CD19⁺) (Figure S1) were sorted from the spleen and their barcode identity was analyzed through nested PCR and NGS detection. At the same timepoints the E, M, and B lineage chimerism was assessed in blood samples (Figure 1 A).

As a first indication towards a changed behavior of in vitro EPO exposed and Ctrl HSC, we analyzed the survival of transplanted mice (Table S1). Throughout all cohorts a similar number of mice died before the analysis endpoint in the Ctrl and EPO group (9,7% and 12% of mice respectively). However, we could see a slight increase in the number of mice with a very low engraftment (spleen Ter119⁻ chimerism of under 10%) in the EPO group which were excluded from further analysis (1,8% vs 8,5% of mice had a low engraftment in Ctrl and EPO group respectively) (Table S1). When analyzing chimerism in the different lineages, we did not detect significant differences between EPO and Ctrl group (Figure 1 B-D). Especially in the erythroid lineage we could not observe a difference in chimerism between Ctrl and EPO group in spleen and blood. We did however detect a decreased B-cell chimerism in mice transplanted with EPO-exposed HSC, although not significant (Figure 1 B).

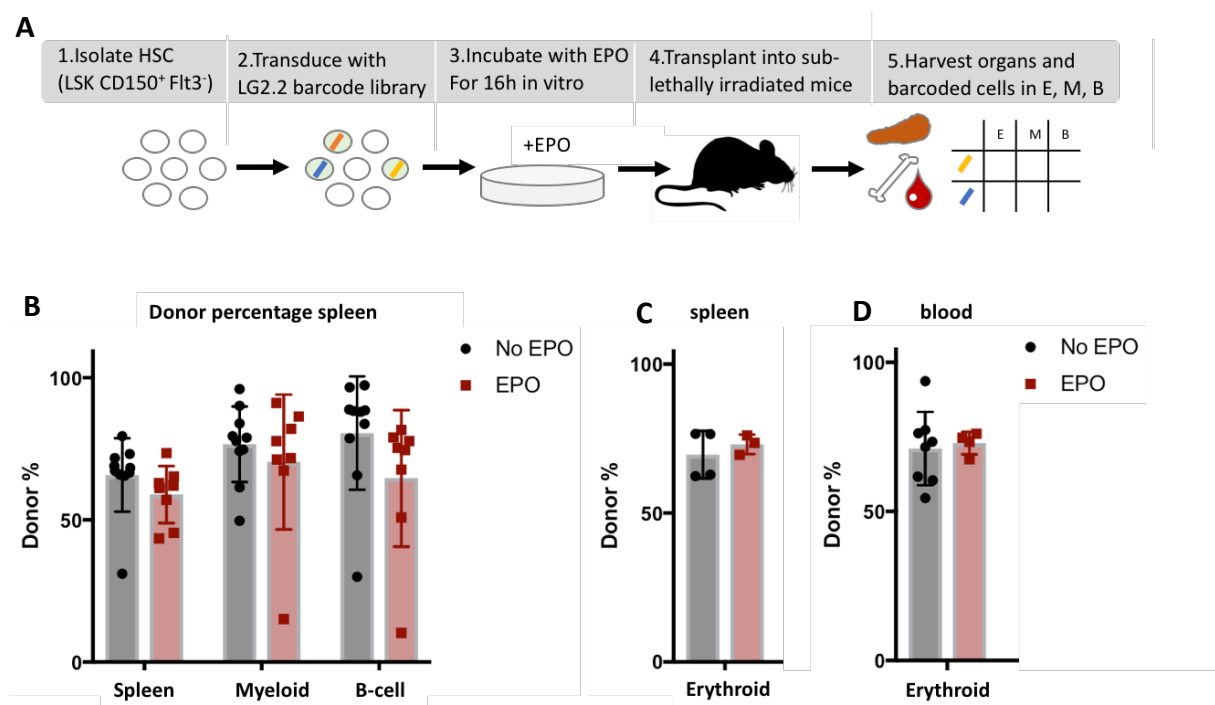


Figure 1: Chimerism in the myeloid, B-cell, Erythroid subsets after EPO exposure and transplantation. (A-D) HSC were sorted from Rosa26CreER^{T2}; mTdT/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without EPO for 16h, and transplanted into sublethal-irradiated mice. After 4 weeks the chimerism (based on Tom or CD45.1 expression) was assessed in the spleen or blood of sacrificed individual mice. (A) Scheme of experimental set-up. (B) The fraction of CD45.1⁺ or Tom⁺ cells among the indicated cell types in spleen of control and EPO group. (C-D) The fraction of Tom⁺ cells among erythroid cells in spleen (C) and blood in control and EPO group (D). (Shown throughout are values from individual animals (n= 8 EPO, n= 10 Ctrl in (B), n= 3 EPO, n=4 Ctrl in (C), n= 4 EPO, n=8 Ctrl in (D), collected over 5 different experiments). For all plots mean and S.D. between mice are depicted.

EPO changes clonality of single HSC after transplantation

To analyze if in vitro EPO exposure changed the differentiation of individual HSCs, we went on to analyze the presence and overlap of cellular barcodes in the E, M, and B lineages in spleen at week 4 after transplantation of control and EPO-exposed HSC (Figure 2 A-G and Figure S4). We observed no significant change in the number or relative percentage of barcodes in the analyzed lineages, meaning the same number of HSC had differentiated after transplantation, and an equal number of HSC had generated B, E, and M output. (Figure 2 A). Also, the percentage of different uni- and multi-lineage detections of barcodes, corresponding to the contribution of uni- and multi-lineage outcome HSC to the different lineages, did not significantly change in the EPO group (Figure 2 C). Notably, the percentage of barcodes detected only in the erythroid lineage was not increased in the EPO group, meaning the number of transplanted HSC which had produced only erythroid cells was not different from the control group. When considering the output generated by the HSC classified according to their lineage restrictions, we could see that, as in the control group, the majority of cells in all lineages was contributed by barcodes present in all three cell subsets analyzed (multi-outcome HSC) (Figure 2 D). The triangle plots of the barcode data in both groups, revealed however a subtle switch in the exact localization of barcodes present in all three cell subsets in the EPO group, implying a changed lineage bias of multi-lineage outcome HSC after EPO-exposure, which we explored next.

To analyze the lineage biases of HSC, we went on to introduce a new classifier. The uni- and multi-lineage outcome categories were now re-applied using a 10% threshold of relative output for defining output/presence in a lineage. This meant for example that the ME category now consisted of HSC having above 10% of their output in the M, and E lineage and no, or under 10% of their output in the B lineage (“ME-biased” HSC) (Figure 2 E) (Figure S3 for other thresholds). Also using this threshold, the percentage of the different output categories were not significantly different. However, the contribution of the different uni- and multi-lineage outcome categories to the overall lineages was changed (Figure 2 F-G and Figure S4). In the control group, barcodes classified as “unbiased” and present in all analyzed lineages produced the majority of each lineage. After transplantation of EPO-exposed HSC, ME- and MB- or B-biased HSC were the categories contributing the most cells to each analyzed lineage (Figure 2 G): ME-biased HSC contributed the majority of erythroid cells, MB- or B-biased HSC contributed the majority of B-cells, and ME-, and MB-biased HSC contributed the majority of myeloid cells. To test the significance of this effect, we made use of a permutation test, which compares the effect size between control and EPO-group to the one of all random groupings of mice (Tak, 2019).

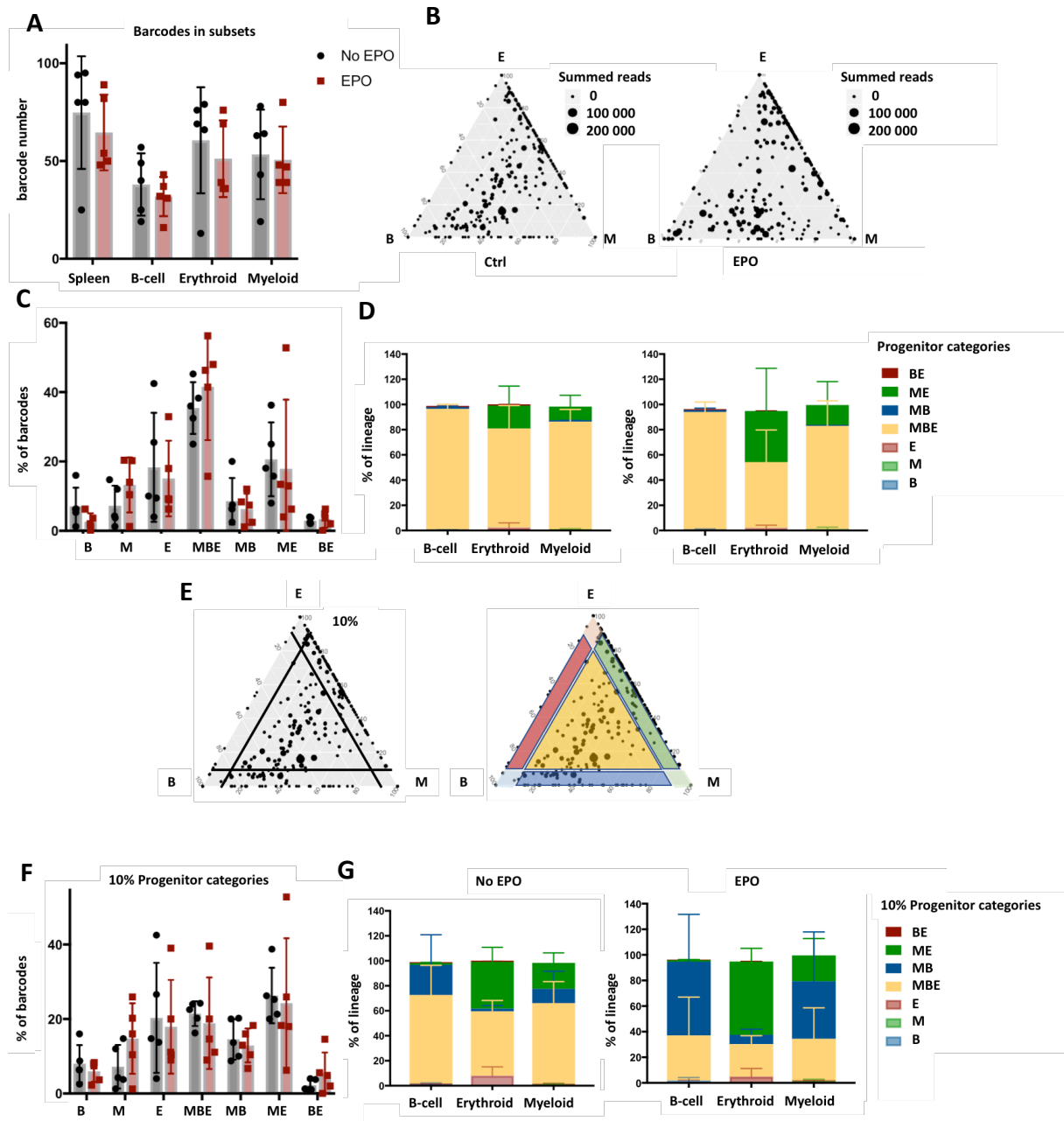


Figure 2: Clonality in the E, M, and B lineage of EPO-exposed HSC after transplantation. (A-G) HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/or without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen of individual mice sacrificed at week 4 post-transplantation. (A, C) Percentage of barcodes found back in the indicated lineage (A) and lineage combinations (C) at week 4 after transplantation in Ctrl and EPO group. (B) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output over the 3 lineages for Ctrl and EPO group. (D) The percentage of each lineage produced by the indicated uni-, or multi-lineage outcome HSC. (E) Explanatory plot for a categorization of HSCs based on bias using a 10% threshold (10% progenitor categories). (F-G) Representations as in (C-D) using a 10% threshold for categorization as explained in (E). Shown are values from several animals (n=5 for both groups, see additional mice in Figure S4 (collected over 2 different experiments)). For all bar graphs mean and S.D. between mice are depicted.

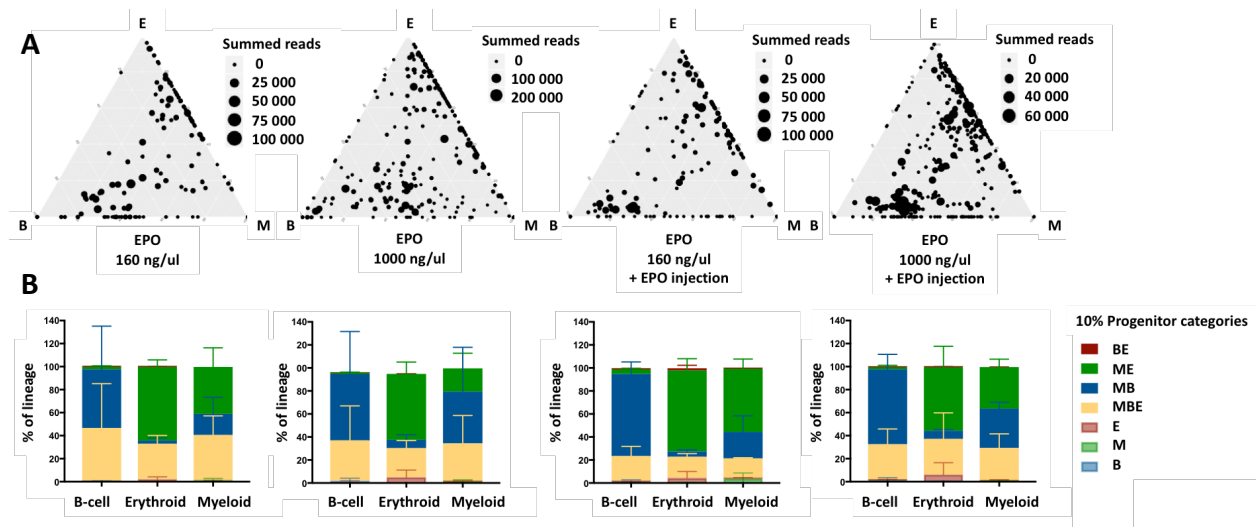


Figure 3: Concentration dependency of changes in the clonality of EPO-exposed HSC after transplantation. (A-G) HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without different concentrations of EPO (160 ng/ml or 1000 ng/ml) for 16h, and transplanted into sublethal-irradiated mice with or without a single additional injection of EPO at the moment of transplantation. Analysis of barcodes in the E, M, and B lineage in spleen of individual mice sacrificed at week 4 post-transplantation. (A) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output over the 3 lineages for the different experimental groups as indicated. (B) The percentage of each lineage produced by the HSC categorized by bias using a 10% threshold. Shown are values from several animals (n=2 for 160 ng/ml, n=5 for 1000 ng/ml see also Figure S4, n=2 for 160 ng/ml +injection, n=4 for 1000 ng/ml +injection for both groups (collected over 4 different experiments). For all bar graphs mean and S.D. between mice are depicted.

Applying this test, both the changed contribution of the ME- and of the MB- or B-biased categories was highly significant (p between 0,007 and 0,01) (Figure S5). We repeated the experiment with different EPO concentrations. Both, a lower concentration during the in vitro EPO-exposure, as well as an additional single injection of EPO during transplantation of in vitro EPO-exposed HSC, resulted in a similarly changed HSC clonality after 4 weeks (Figure 3 A-B and Figure S4). Also at these concentrations a permutation test confirmed a significantly increased contribution of the ME-biased HSC category to the myeloid and erythroid lineage, and of the MB-or B-biased HSC category to the myeloid and B-lineage, as compared to the control group (Figure S5). To test the duration of the effect, we repeated the experiment and analyzed barcodes in the E, M, and B lineage at 4 months after transplantation of control or EPO-exposed HSC (two different EPO concentrations) (Figure 4 and Figure S6). We detected no significant changes at this timepoint, implying that the effect of direct EPO-exposure of HSC is transient.

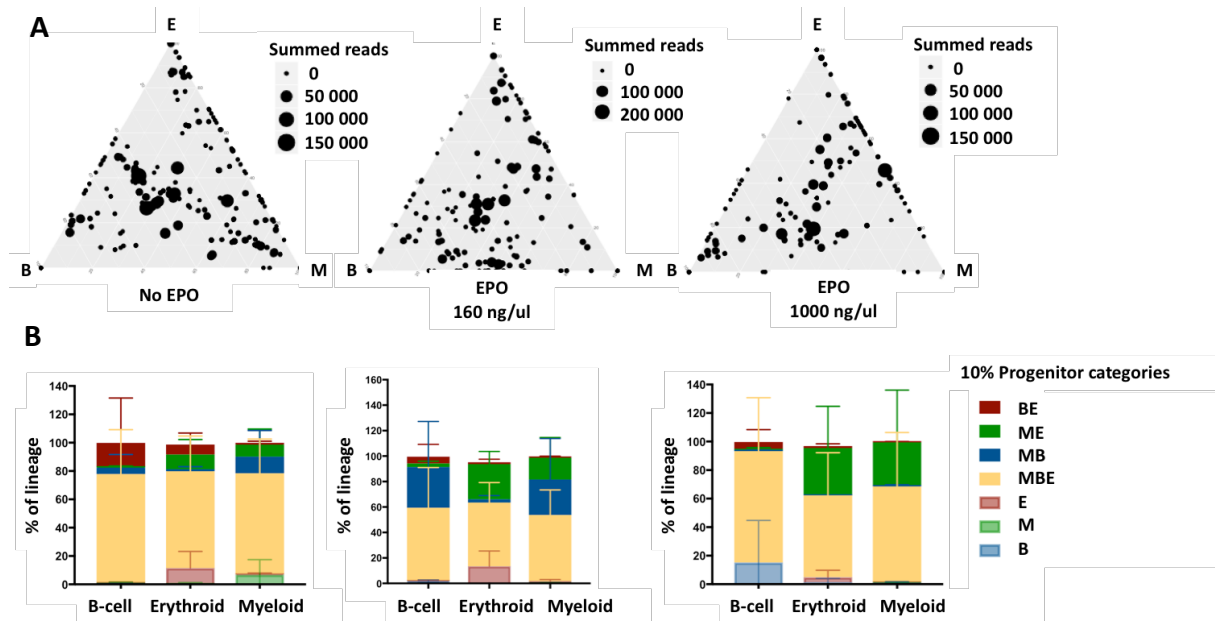


Figure 4: Clonality in the E, M, and B lineage of EPO-exposed HSC 4 months after transplantation. (A-B) HSC were sorted from Rosa26CreER^{T2}; mTdT/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 160 ng/ml or 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen and HSC in bone of individual mice sacrificed at month 4 post-transplantation. (A) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output over the 3 lineages for Ctrl and EPO group. (B) The percentage of each lineage produced by the indicated uni-, or multi-outcome barcodes. Shown are values from several animals (n=4/3 for control and EPO-group respectively (collected over two experiments)). For all bar graphs mean and S.D. are depicted.

EPO-exposure induced bias of HSC in additional lineages

After having established the changed contribution of ME- and MB- or B- biased HSC to the E, M, and B lineages 4 weeks after transplantation of EPO-exposed HSC, we wanted to analyze these ME- and MB- biased HSC in more detail. More specifically we went on to analyze in more detail the contribution of these cell subsets to other lineages, namely DC, and MkP cells, and specific myeloid cell subsets, as Macrophages, Monocytes, Neutrophils, and Eosinophils.

Firstly, we analyzed the contribution of ME- and MB- biased HSC, and “unbiased” MBE HSC to the DC lineage (Figure 5 A-B). To do so barcoded (GFP⁺) DC (DC; Donor Ter119⁻ CD19⁻ CD11c⁺ CD11b⁻) were sorted together with M, E, and B cells, 4 weeks after transplantation of control or EPO-exposed HSC (Figure S1). Both in the control and EPO group a high percentage of HSC classified as MBE-, ME-, or MB-biased was found back in DC (Figure 5 A), meaning most HSC do produce DC 4 weeks after transplantation of both control and EPO-exposed HSC. To analyze possible biases to the DC lineage, of the different subsets we compared the abundance of barcodes in DC and E, M, and B lineages for the different biased HSC categories (Figure 5 B). For all categories, the abundance of

barcodes in DC correlated highly with presence in M and B lineage, meaning cells with a high contribution to M, and B lineage had also produced many DC. The correlation of abundance of barcodes in DC and erythroid lineage was less pronounced, and especially low in the EPO group, where ME-biased HSC had the highest erythroid output (Figure 5 B). This meant not all high erythroid producing ME-biased HSC did (highly) produce DC, or in other words, the high output ME-biased HSC present 4 weeks after transplantation of EPO-exposed HSC were also ME-biased with respect to DC production.

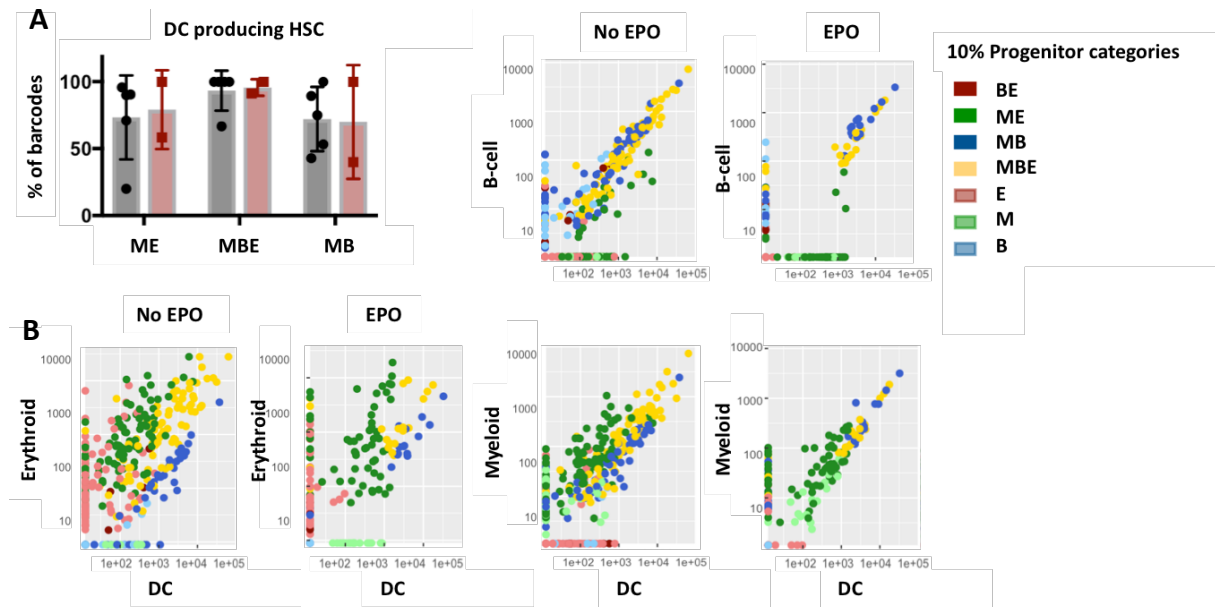


Figure 5: Production of DC by HSC after EPO-exposure and transplantation. (A-B) HSC were sorted from Rosa26Cre^{ERT2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, B, and DC lineage in spleen of individual mice sacrificed at week 4 post-transplantation. (A) Percentage of barcodes classified based on their presence in M, E, and B lineage using a 10% threshold which is also detected in DC. (B) Abundance of barcodes in E, M, B, and DC lineage as indicated, colored based on presence in E, M, and B lineage using a 10% threshold. Shown are values from several animals (n=5 for ctrl and n=2 for EPO group (collected over one experiment)). For all bar graphs mean and S.D. are depicted.

Next, we went on to study the production of MkP, and specific myeloid cell subsets by ME- and MB- biased HSC, and “unbiased” MBE HSC (Figure 6, 7, and 8). MkP were chosen as proxy for the production of platelets not suitable for barcode analysis. Four weeks after the transplantation of control and EPO-exposed HSC, MkP (MkP; c-kit⁺ Sca-1⁻ CD150⁺ CD41⁺) and barcoded myeloid subsets (GFP⁺ Donor Ter119⁻ CD19⁻ CD11c⁻ CD11b⁺), namely macrophages (Ma; CD115⁻ SiglecF⁻ Ly6G⁻), eosinophils (Eo; CD115⁻ SiglecF⁺ Ly6G⁺), monocytes (Mo; CD115⁺ cells), and neutrophils (Neu; CD115⁻ SiglecF⁻ Ly6G⁺), were sorted together with B, and E cells as before for further analysis (Figure S1). To be able

to classify HSC into the ME- and MB-biased, and “unbiased” MBE-category despite the subsampling of the myeloid cell subset, we chose to use the B/E output ratio of these categories as a new classifier (Figure 6 A-B).

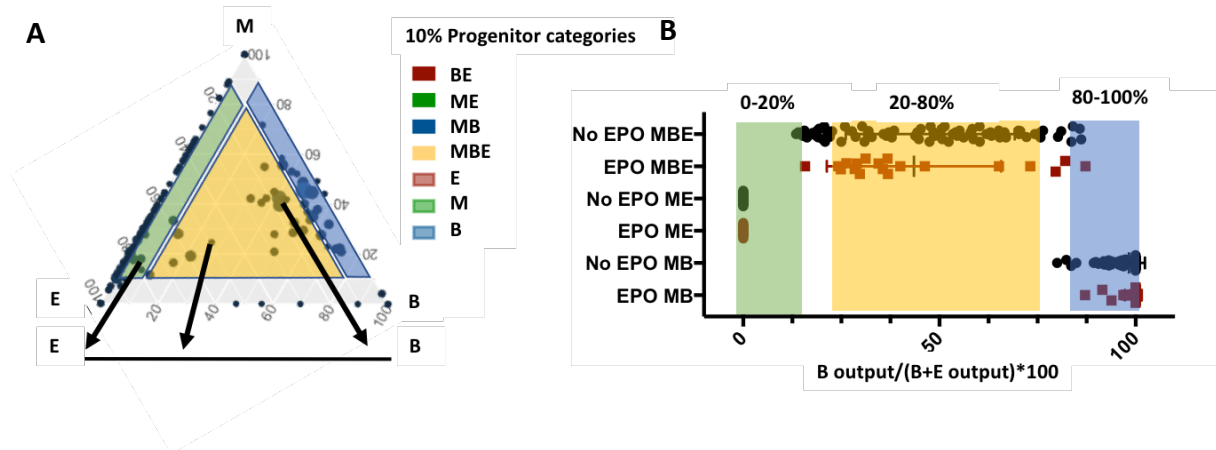


Figure 6: Production of DC by HSC after EPO-exposure and transplantation. (A-B) HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen of individual mice sacrificed at week 4 post-transplantation. (A-B) Barcodes classified based on their presence in M, E, and B lineage using a 10% threshold where analyzed for their relative contribution to the B and E lineage (A) Explanatory scheme for the analysis of B/E contribution of classified barcodes. (B) Based on the B/E contribution of MBE-, and MB-, and ME-biased HSC in ctrl and EPO group a 20% threshold in the B/E contribution was chosen to distinguish these three subsets without the requirement for barcode analysis of the myeloid subset.

In a first instance, we analyzed the MkP production of the ME-, MB- and MBE-biased categories defined in this way (Figure 7 A-B). Compared to DC, an overall lower overall percentage of barcodes was found in MkP, and this was not uniform between the different categories. While MBE-, and ME-biased HSC mostly did produce MkP, few barcodes of the MB-biased HSC category were also found in MkP (Figure 7 A). The abundance of a barcode in the MkP subset was in general much less correlated to the abundance in other subsets, than for DC (Figure 7 B). In line with the low percentage of MB-biased HSC producing MkP, this was especially prominent for the B-cell lineage; the abundance of a barcode in the B-cell lineage was uncorrelated to its abundance in MkP. Throughout all these findings we did not detect changes between control and EPO-group. Both in the control and EPO-group, MB-biased HSC did not produce (high) numbers of MkP, while ME-biased HSC did. Thereby, while high output ME-biased HSC present 4 weeks after transplantation of EPO-exposed HSC were also ME biased with respect to DC production, our data did not allow to conclude so for MkP; or in other words, MkP are part of the bias of the ME-biased HSC (are “MkPME-biased”).

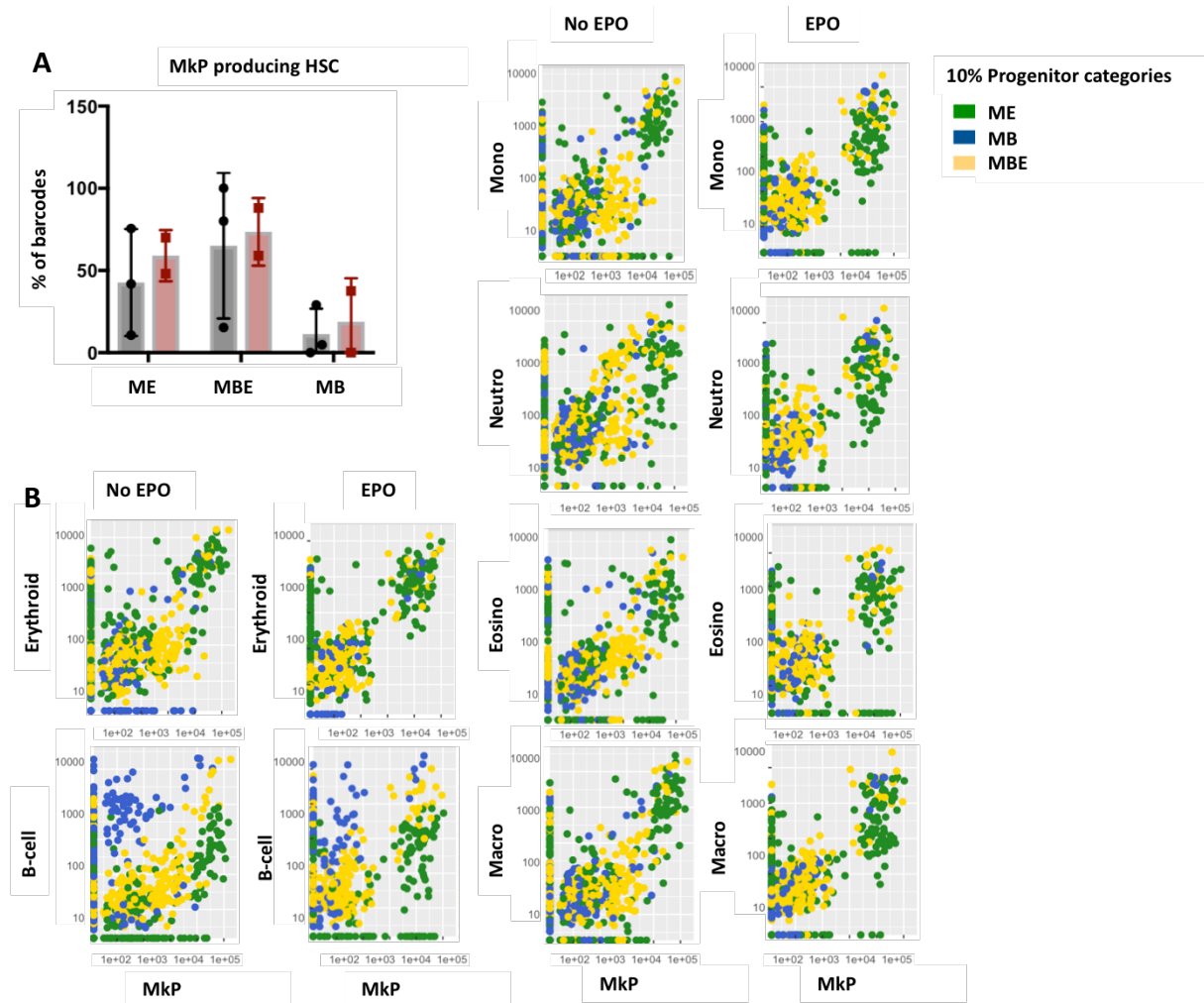


Figure 7: Production of MkP by HSC after EPO-exposure and transplantation. (A-B) HSC were sorted from Rosa26CreERT²; mTdTtomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, B, MkP, and different myeloid subsets in spleen of individual mice sacrificed at week 4 post-transplantation. (A) Percentage of barcodes classified based on their presence in E, and B lineage using a 20% threshold (see Figure X) which was detected in MkP. (B) Abundance of barcodes in the indicated lineages, colored based on classification as in (A). Shown are values from several animals (n=5 for ctrl and n=2 for EPO group (collected over one experiment). For all bar graphs mean and S.D. are depicted.

Finally, we analyzed the production of specific myeloid cell subsets by ME-, and MB- biased HSC, and “unbiased” MBE HSC (Figure 8 A-D and Figure S7). The general donor contribution in the myeloid eosinophil, neutrophil, macrophage, and monocyte subsets did not change between control and EPO group. Most donor myeloid cells recovered 4 weeks after transplantation of control and EPO-exposed HSCs were neutrophils (Figure 8 A). On the single-cell level, most HSC, and especially HSC with a high myeloid contribution (> 1% of myeloid output), produced all of the different myeloid subsets analyzed (lineage restriction analyzed) (Figure S7). Among the HSC producing all myeloid subsets different biases were present both in control and EPO group (Figure S7). To analyze these biases further,

we applied, as before for the M, B, E, output (Figure 2 E), a 10% threshold to define output/presence in the neutrophil, macrophage, monocyte, eosinophil subsets. Using this classifier again the majority of HSC was found in the “unbiased” category with >10% output in each myeloid lineage, both in control and EPO-group (Figure 8 B). This category also contributed most cells in each myeloid lineage in both conditions (Figure 8 D and Figure S7). Given, the changed contribution of ME-, MB- or B-, and MBE-biased HSC to the myeloid lineage 4 weeks after transplantation of EPO-exposed HSC described before (Figure 2), this finding already implied few changes in the detailed myeloid output of the ME-, MB- and MBE-biased HSC categories. Indeed, when analyzing the contribution of these three subsets to the different myeloid subsets, we could not detect significant changes in number or output of myeloid restricted or biased HSC between groups or condition (Figure S7). Notably, the “unbiased” MBE-category also had an especially “unbiased” myeloid output (Figure S7).

EPO effect and kinetics-week 2, 4 and 6

After having established a higher contribution of ME- and MB-biased HSC to the E, M, and B lineages after transplantation of EPO-exposed HSC and their contribution to DC and MkP, we wanted to analyze in more detail the kinetics of the EPO effect. More in detail we wanted to establish the state of the high output ME- and MB-biased HSC clones present 4 weeks after transplantation of EPO-exposed HSC at a close earlier (2 weeks), and later timepoints (6 weeks). In a first step we sorted barcoded M, E, and B cells from spleen as before, at 2, 4, and 6 weeks after transplantation of control or EPO-exposed barcoded HSC for further analysis.

At the same timepoints the E, M, and B lineage chimerism was assessed in spleen and blood samples (Figure 9 A). As determined before for the 4-week timepoint, we could not observe differences in chimerism between Ctrl and EPO group in spleen and blood at the other analysis timepoints (Figure 9 B-C). The chimerism increased in the B and E lineage from week 2 to 4 in both the control and EPO group, while the myeloid chimerism was high from the first analysis timepoint on. The analysis of the clonality of single HSC at the week 2 and 6 timepoints in the EPO-group, revealed again as for week 4 no change to the control group when considering lineage restrictions only (Figure 9 D, data not shown and Chapter 2 Figure 2). As for the control group, barcodes at 2 weeks were not B- or MB-restricted, the percentage of ME-restricted HSC was reduced at week 6, and the MBE category contributed the majority of cells in all lineages at all timepoints in the EPO-group.

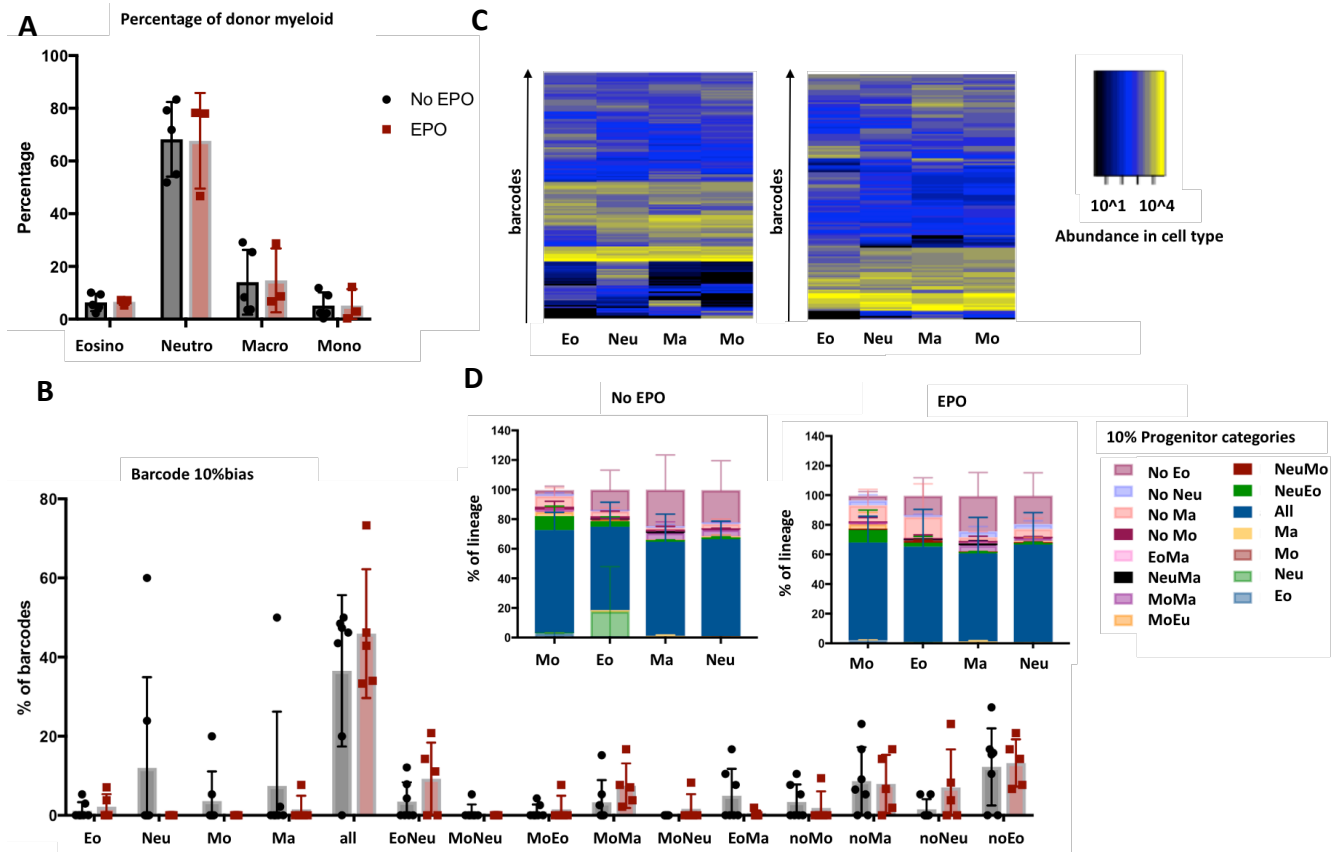


Figure 8: Production of macrophages, monocytes, neutrophils and eosinophils by HSC after EPO-exposure and transplantation. (A-D) HSC were sorted from Rosa26CreER^{T2}; mTdTTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, B, MKP, and different myeloid subsets in spleen of individual mice sacrificed at week 4 post-transplantation. (A) The contribution of different cell types to the overall donor myeloid subset in control and EPO group. (B). Percentage of barcodes classified based on their presence in myeloid lineages using a 10% threshold for clones with a contribution of 1% to the overall myeloid output (red=EPO group, black= control). (C) Heatmaps showing the output of individual barcodes (rows) in different samples as indicated. Data is normalized by cell subset, log transformed and clustered by complete linkage using Euclidean distance. No output is represented in black. (D) The percentage of each lineage produced by the indicated uni-, or multi-outcome barcode categories. Shown are values from several animals (n=5 for both groups (collected over two experiments)). For all bar graphs mean and S.D. between mice are depicted.

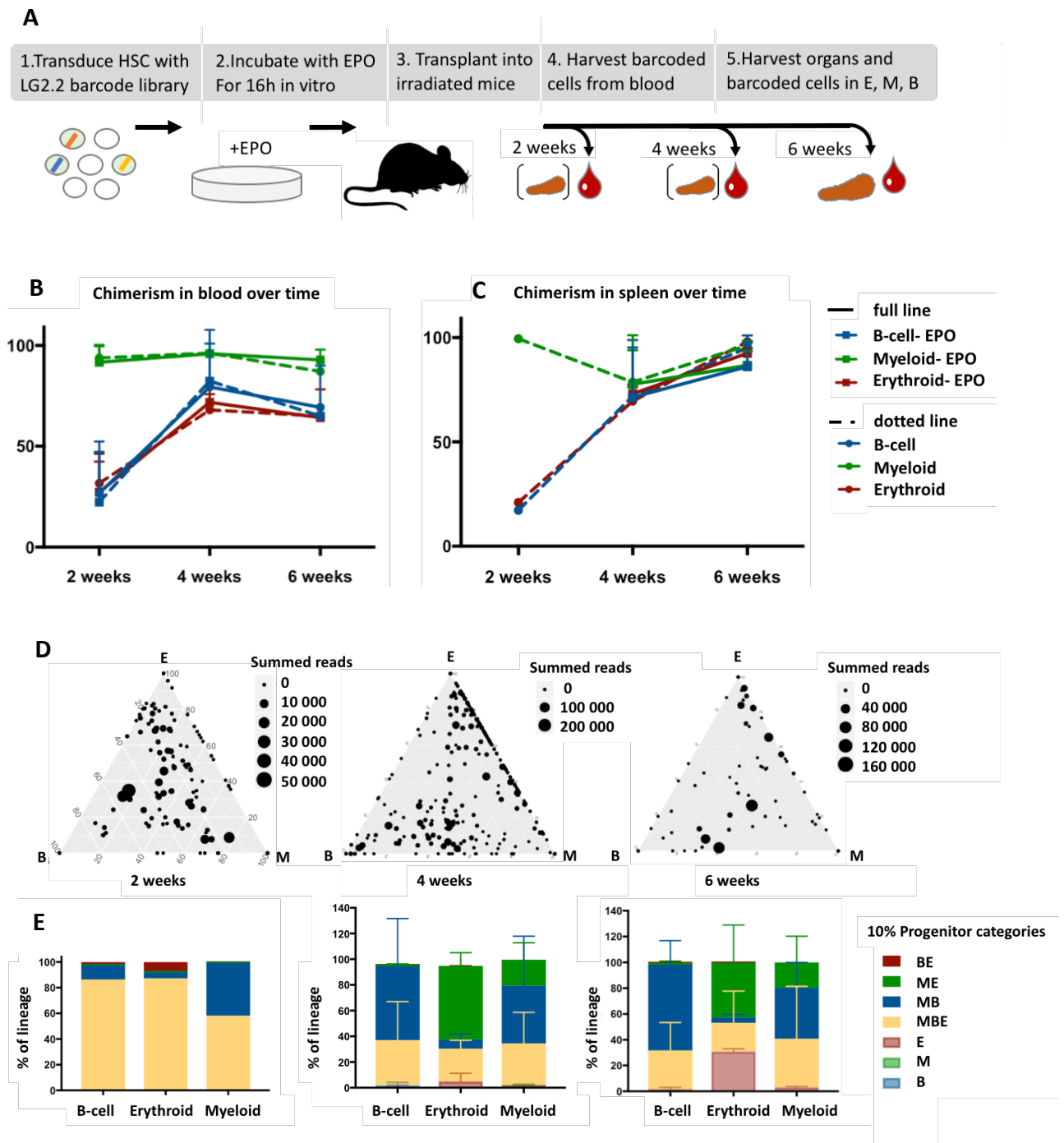


Figure 9: Chimerism and clonality of HSC at week 2 and 6 after EPO-exposure and transplantation. (A-E) HSC were sorted from Rosa26CreER^{T2}; mTdTtomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Chimerism in the E, M, and B lineage (based on Tom expression) was assessed at week 2, week 4, and week 6 in PB and spleen of sacrificed individual mice. Barcodes were analyzed in the E, M, and B lineage in spleen. (A) Scheme of experimental set-up. (B-C) The fraction of Tom⁺ cells among the indicated cell types in PB (B) and spleen (C). (D) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output over the 3 lineages at week 2, 4, and 6 after transplantation for the EPO-group. (E) The percentage of each lineage produced by barcodes classified according to the indicated uni-, or multi-outcome using a 10% threshold. Shown are values from several animals (n=1 week 2, n=5 week 4, n=4 week 6 (collected over 4 different experiments)). For all bar graphs mean and S.D. between mice are depicted.

When re-applying the 10% threshold to classify HSC according to their lineage bias, we could however determine also changes at week 2 and 6 in the EPO-group as compared to control. At week 2 MB-biased HSC contributed 50% of the myeloid lineage. At week 6, as previously established for week 4, ME- and MB-biased HSC contributed the majority of cells in the E and M, or, M and B lineages respectively. We previously established that both clonal stability and clonal succession do occur in the first weeks after transplantation, and that strong lineage biases correlate with clonal extinction in this phase (Chapter 2). This queries how the high output MB- and ME-biased HSC present at week 2, 4, and 6 are related. We set of to assess this next.

EPO effect and kinetics-clonal stability and succession

We started a cohort for which blood samples were taken at week 2 and 4 and, the spleen analyzed at week 6 after transplantation of control and EPO-exposed HSC, to assess changes of individual barcodes in the E, M, and B lineage, in both groups over time. Specifically, we wanted to determine the clonal stability of high output MB- and ME-biased HSCs present after transplantation of EPO-exposed HSC. We could determine that as in the control-group, both clonal succession and clonal stability do occur after transplantation of EPO-exposed HSC. Indeed, both the number and percentage of barcodes detected at one, two, or all three timepoints in any lineage was unchanged in both conditions (Figure S8 and Figure 10 A). Also, when assessing each lineage independently, the clonal kinetics in lineage detection and switches over the first 6 weeks were similar between control and EPO-group (Figure S9 and Figure S10).

As for each single timepoint analyzed, changes between control and EPO-group became however apparent when considering the clones contributing highly to the different lineages over time (Figure 10 A-C). When considering the summed output in E, M, and B lineage, it became apparent that among the clones present at all three analysis timepoints, few were or stayed highly contributing (Figure 10 A). Also, when considering each lineage independently this effect was apparent (Figure 10 C). In the EPO-group the percentage of high output clones among the clones present at all three analysis timepoints was significantly lower than in the control group (Figure 10 B). A higher percentage of high output clones was also apparent among clones present at more than one timepoint, although this was not significant. When analyzing high output MB- and ME- biased HSC in particular, this effect remained. Some highly MB-contributing clones remained from week 2 to 4. At week 6 distinct clones contributed highly to the M and B lineage. The highly ME-contributing clones were seldomly (highly) detected at other timepoints (Figure 10 C).

The influence of EPO on HSC self-renewal and differentiation

After having established that the high contributing ME- and MB-biased HSC are changing over the first six weeks after transplantation of EPO-exposed HSC, we wanted to analyze how this “EPO effect” relates to HSC self-renewal and differentiation. We analyzed barcode profiles in HSC besides the E, M, and B lineage at week 2, 4, and 6 after transplantation of control and EPO-exposed HSC (Figure 11 A and Figure S12). After the transplantation of control HSC, we detected similar amounts of barcodes in HSC at all timepoints. In contrast we recovered a very high number of barcodes at week 2 ($n=1$) after the transplantation of EPO-exposed HSC, after which barcode numbers decreased to be equal to control at week 4 and significantly lower than control at week 6 (Figure 11 B). The percentage of HSC shared with mature cells, was lower than control at week 2 and 4 (Figure 11 C). This implied, that a larger number of HSC had self-renewed without differentiating at week 2 and 4 after transplantation of EPO-exposed HSC. At week 2, some of the barcodes detected in HSC but not mature cells were abundant ($>1\%$ of HSC reads), suggesting that the corresponding HSC had undergone a high number of self-renewal divisions without differentiation (Figure 11 D). At week 4, barcodes recovered only in HSC were not among the abundant barcodes anymore. Nevertheless, both at week 2 and week 4, the correlation of HSC, and the mature cell lineages in the EPO-group was lower than for control (Figure 11 E and Chapter II). Only at week 6, the correlation between HSC, E, M, and B lineage was similar to control again, being higher for the E and M lineage than for the B lineage (Figure 11 E and Chapter II).

As the number of barcodes detected in HSC after transplantation of EPO-exposed HSC was lower than control at week 6, we wanted to assess how the numbers developed at a later timepoint. We repeated the experiment and analyzed barcodes in the HSC, E, M, and B lineage at 4 months after transplantation of control or HSC EPO-exposed at two different concentrations. Both in control and EPO-groups the number of barcodes detected in HSC was lower than at the early timepoints after transplantation. However, we did not detect differences in the HSC number between the groups (Figure 11 B). Also, the percentage of HSC shared with mature cells was as high ($>90\%$) in the EPO-groups as at all timepoints (week 2, 4, 6 and month 4) in control (Figure 11 C). This reflected in the correlation between HSC, E, M, and B lineage to be, as for control (week 4-month 4) high between HSC, E and M lineage and lower for the B lineage (Figure S12 and Chapter II). Altogether, the analysis of barcodes in HSC besides the E, M, and B lineage at 4 months after transplantation of EPO-exposed HSC again suggested that the direct effect of EPO-exposure on HSC is transient.

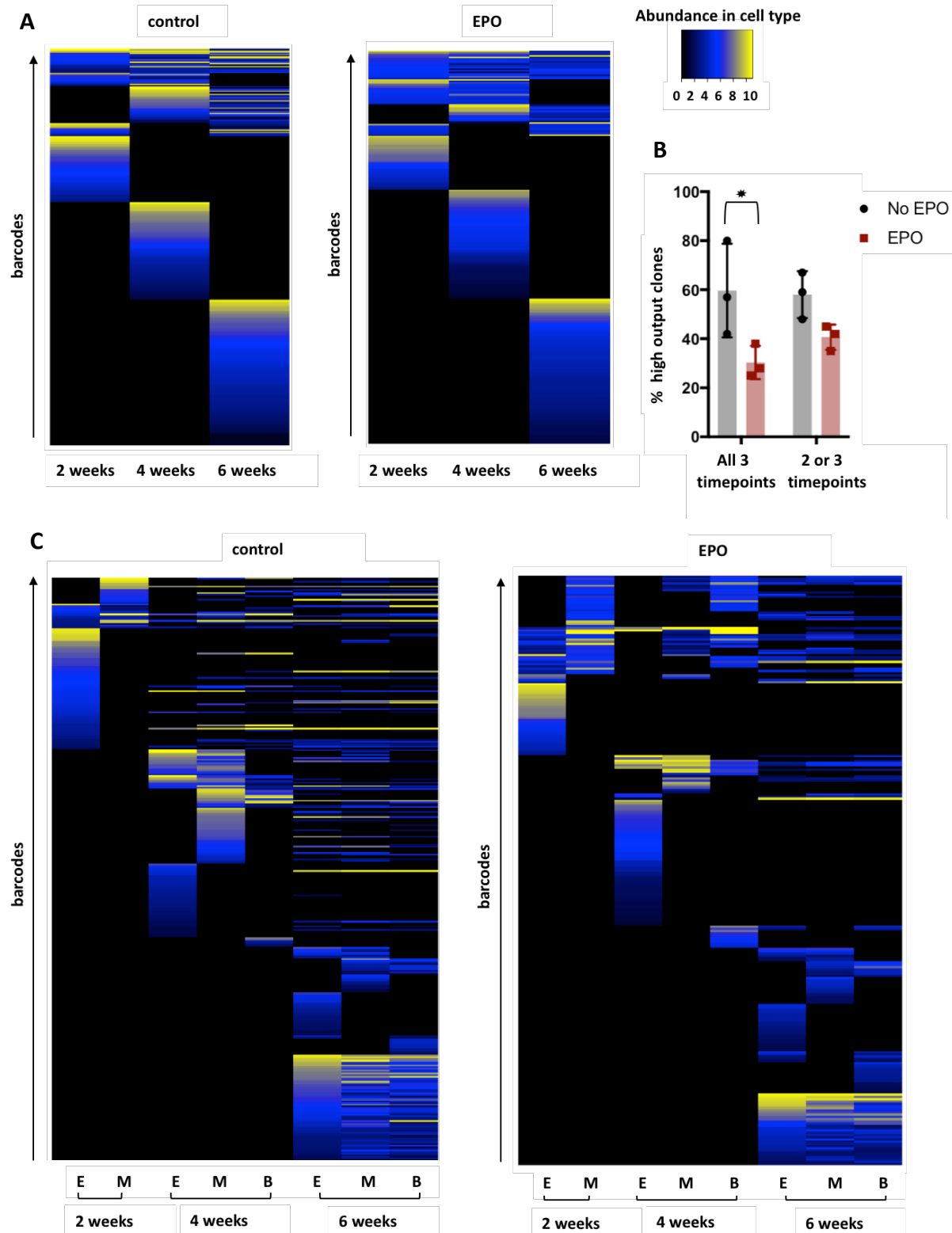


Figure 10: Stability of high abundance clones after transplantation of EPO-exposed HSC. (A-C) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen 6 in the same individual mice. (A) Heatmaps showing the output of

individual barcodes (rows) over time for all mice combined, summing all lineages. Barcodes are clustered according to their abundance at the week 2 timepoint. Data is log transformed. No output is represented in black. (B,) Bar graph showing variability between mice in percentage of high output barcodes (> 1% of a lineage at a timepoint) among barcodes detected at 3 or more than one timepoint in control and EPO-group. Mean and S.D. between mice are depicted. (C) Heatmaps showing the output of individual barcodes (rows) over time for all mice of control or EPO-group as indicated. Barcodes are clustered according to their abundance at the week 2 timepoint Data is log transformed. No output is represented in black. Shown are values from the same animals throughout (n=3 from one experiment for both groups).

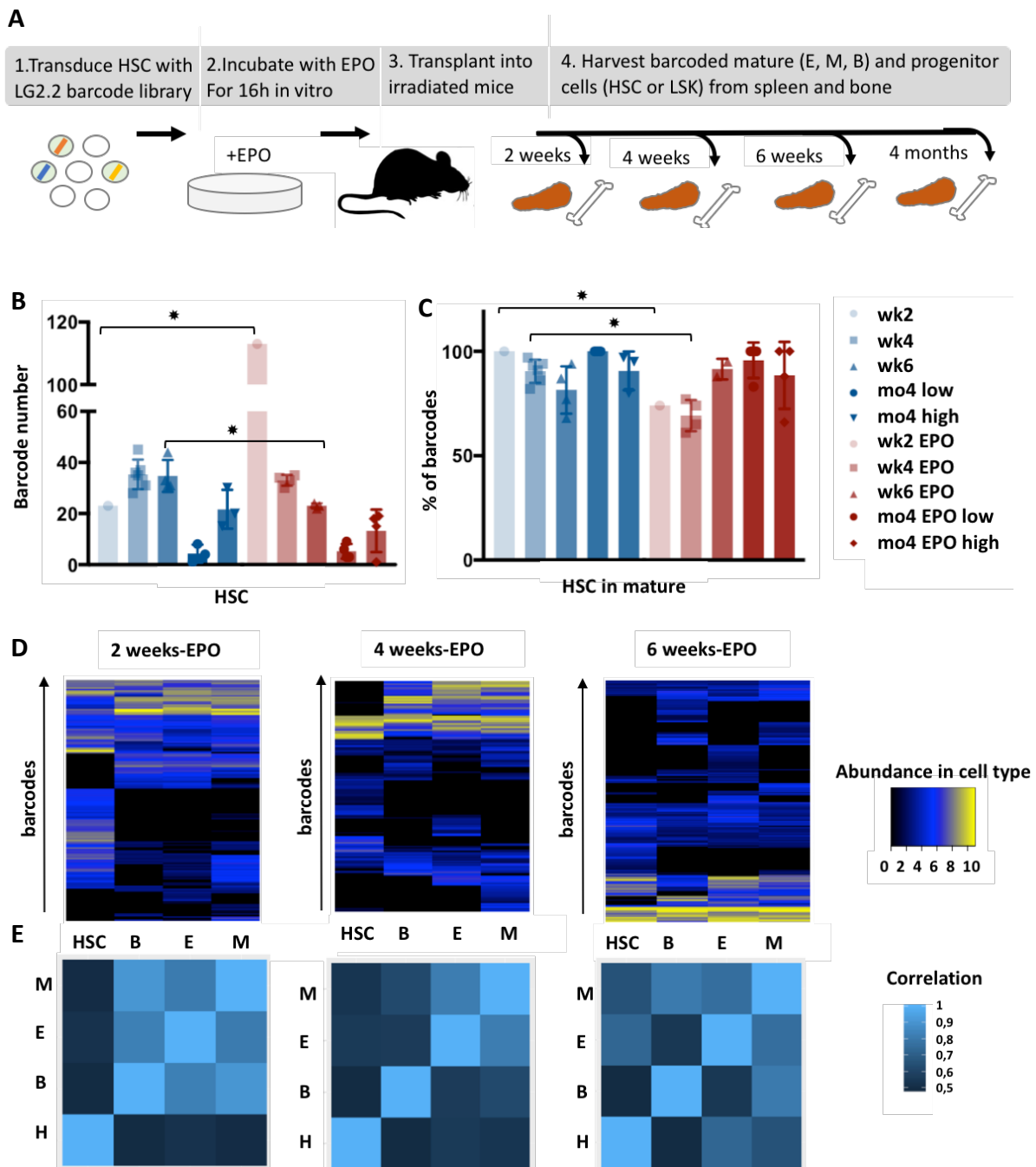


Figure 11: HSC self-renewal vs differentiation after transplantation of EPO-exposed HSCs. (A-E) HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured *in vitro* with/without 1000 ng/ml (no indication and EPO high) or 160 ng/ml EPO (EPO low) for 16h, and transplanted into sublethal-irradiated mice. The barcode identity in the E, M, and B lineage was assessed at week 2, week 4, week 6, or month 4 in spleen and compared to the barcode identity in HSC (or LSK month 4 EPO high) in bone. (A) Scheme of experimental set-up. (B) The number of barcodes found back in HSC (or LSK month 4 EPO high) at the indicated timepoint for control and EPO-group. (C) The percentage of barcodes in HSC (or LSK month 4 EPO high) detected in the mature cell subsets (E, M, and B) at the indicated timepoint for control and EPO-group. (D) Heatmaps showing the output of individual barcodes (rows) in different samples as indicated. Data is log transformed and clustered by complete linkage using Euclidean distance. No output is represented in black. (E) Pearson correlation between the indicated cell types at the indicated timepoints (H=HSC). Shown are values from several animals (n=1 week 2, n=5 week 4, n=3 week 6, month 4 n=4 EPO and n=3 control (collected over 6 different experiments)). For all bar graphs mean and S.D. between mice are depicted.

3.3 Discussion

In this chapter we studied the direct effect of erythropoietin on HSC differentiation after transplantation at the single cell level using a new lentiviral cellular barcoding library. Over the first six weeks after transplantation the clonal output of control and EPO-exposed HSC was assessed in erythroid cells, macrophages, neutrophils, eosinophils, monocytes, dendritic cells (DC), megakaryocyte progenitors (MkP) and B-cells. The reconstitution kinetics of the different hematopoietic lineages after transplantation of EPO-exposed HSC remained dominated by co-occurrence of clonal stability and clonal succession, and distinctive lineage production kinetics and restrictions at the single cell level. However, HSC clonality was changed in that ME-biased and MB-biased HSC, produced the majority (>60%) of erythroid, myeloid and B-cells. Both biased subsets produced similar levels of macrophages, neutrophils, eosinophils, monocytes. The ME-biased HSC subset produced however high MkP and low DC numbers, while the MB-biased HSC subset produced few MkP, but many DC. The changed clonality after transplantation of EPO-exposed HSC was transient but stable over different EPO concentrations and after *in vivo* EPO treatment during transplantation. At the progenitor level, a higher number of HSC was detected, and self-renewing without differentiation early after transplantation of EPO-exposed HSCs, suggesting that the high output ME-biased and MB-biased HSC could occur through functional compensation mechanisms.

Previously, reduced EPOR signaling has been described to lead to enhanced CD34⁺ HSPC engraftment (Aljitawi et al., 2016). In line with this report, we detected here a slightly reduced hematopoietic reconstitution after EPO exposure, in that a higher percentage of mice transplanted with EPO-exposed HSCs (1,8% vs 8,5% of mice in Ctrl vs EPO group) had a very low chimerism (spleen Ter119⁻ chimerism of under 10%) which did not allow for further barcode analysis. Nevertheless, the vast majority of mice transplanted with EPO-exposed HSC had a normal hematopoietic reconstitution derived from a similar number of HSC which had differentiated (a similar number of barcodes in mature cells). Furthermore, on the HSC level, we detected higher number of barcodes at early timepoints after

transplantation of EPO-exposed HSC. Notably these additional barcodes were not found back in mature cells at the same analysis timepoints. This implied EPO-exposed HSC had engrafted better, but a higher number of HSC had self-renewed without differentiation leading to a similar hematopoietic reconstitution in mature hematopoietic lineages.

High systemic levels of EPO (short or chronic) and in vitro culture with EPO have repeatedly been linked to an increased HSC proliferation (Cheshier et al., 2007) (Erslev, 1971) (Dubart et al., 1994) (Giladi et al., 2018) (Tusi et al., 2018) (J. Yang et al., 2017) (Singh et al., 2018). Besides, several studies described that high systemic levels of EPO and in vitro culture with EPO lead to an increased expression of erythroid genes (Singh et al., 2018) (Grover et al., 2014), and decreased expression of myeloid genes in HSC which was linked to an increased erythroid and myeloid cell production four weeks after transplantation (Grover et al., 2014). Here, after direct EPO-exposure in vitro, transplanted HSC did not produce changed amounts of erythroid, myeloid, or B-cells. Also the number of HSC producing each, and only each of these lineages (lineage restricted HSC) did not change. However, we did detect a changed HSC clonality. Besides, the higher amount of HSC not differentiating, we could detect the co-occurrence of changing high output ME- and MB-biased HSCs. Different scenarios could link these observations to the transcriptional changes observed previously. Functional compensation mechanisms as previously suggested by Nguyen et al. after transplantation of lymphoid-deficient HSC might be important (L. Nguyen et al., 2018). The higher number of HSC self-renewing without differentiation, could be in line with and EPO-induced proliferation (and specifically self-renewal proliferation) of specific HSCs. As a possible compensation for the lack of differentiation of these HSC, ME- and MB-biased HSCs could increase their output. Alternatively, also the appearance of high-output ME-biased HSC could be directly linked to EPO-exposure. The increased expression of erythroid genes in HSC after EPO exposure has been suggested to bias differentiation (Grover et al., 2014). This bias might be broader, encompass erythroid, megakaryocyte, and myeloid differentiation, and result in the occurrence of high output ME-biased HSCs. Indeed, although Grover et al. previously suggested a reduced expression of myeloid genes in HSC other studies reported an increased expression of erythroid/megakaryocytic and myeloid genes in MPP1 falling within the HSC gate applied by Grover et al. and ourselves (J. Yang et al., 2017) (Grover et al., 2014). High output MB-biased HSC might appear to compensate for directly EPO-induced high-output ME-biased HSC. Co-transplantation of barcoded EPO-exposed and control HSC in a same mouse could help to distinguish between these mechanisms. Different EPO receptors might mediate different effects.

We could establish here, that EPO does have a direct effect on HSCs. In vivo, this effect will not stand alone. It will be interesting to study how it integrates with the effect of EPO on other cell subsets, including other hematopoietic progenitors (most prominently the erythroid committed progenitors CFU-E and ProEB) and bone marrow components. Many different bone marrow components have been

described to react to EPO exposure. EPO has for example repeatedly been involved in bone homeostasis and MSC differentiation (C. Li et al., 2015) (Singbrant et al., 2011) (Shiozawa et al., 2010) . But also adipocytes and endothelial cells have been described as EPO-targets (American Society of Hematology et al., 2017) (Zhang et al., 2014) (Ito et al., 2017). Especially interesting is a report by Singbrant et al. which links the effects of EPO on the bone marrow to the erythroid response (Singbrant et al., 2011). In this study, blocking of EPO-induced osteoclast activity reduced the EPO-induced erythroid response. The dependence of HSC on their bone marrow microenvironment would make it interesting to study such interactions for the effect of EPO on HSC.

Recombinant EPO is widely used in the clinics to counteract anemia in many different settings, and one of the most sold biopharmaceuticals in the world (Walsh, 2014). Recently, adverse effects of the clinical use of EPO have been reported as hypertension, thromboembolism, stroke, leukemic disease, and death in cancer patients (Rainville et al., 2016) (Ma et al., 2010) (Halawi et al., 2017). Besides, higher leukemia prevalence has been described in thalassemia patients (Halawi et al., 2017) and erythropoietin gene polymorphisms have been linked to the occurrence of myelodysplastic syndromes (Ma et al., 2010). The direct effect of EPO on HSC we describe here could be one of the underlying factors for the development of these adverse side effects and co-morbidities. Although the effect we describe here is transient, chronic exposure to EPO could result in severe alterations at the HSC level. Besides, EPO has recently been described to change the epigenome of erythroid cells (Perreault, Benton, Koury, Brandt, & Venters, 2017). This could also be the case for HSC and other hematopoietic progenitors, leading to effects beyond the time of EPO exposure. Over the last years the number of indications for the clinical use of EPO has been increasing. New EPO formulations have been developed. Some of these have been shown to have less effects on bone marrow cells for example (Dewamitta, Russell, Nandurkar, & Walkley, 2013). It will be interesting to study if other EPO formulations also affect HSC to the same extent, or could be a way to avoid unwanted alterations at the stem cell level when applying EPO in the clinics.

The effect of cytokines on HSC has previously been suggested to consist in selective induction of proliferation in a pre-existing lineage-biased HSC subset, and a de novo lineage instruction (Matatall et al., 2014) (Challen et al., 2010) (Mossadegh-Keller et al., 2013) (Etzrodt et al., 2019) (Grover et al., 2014). Two studies, strengthened the importance of the first working mechanisms by suggesting a selective proliferation of myeloid-biased HSC after IFN γ and TGF- β exposure respectively (Matatall et al., 2014) (Challen et al., 2010). Two other studies highlighted the second principle of cytokine action on HSC. Firstly, the lineage biasing of HSC by M-CSF has been suggested and debated (Mossadegh-Keller et al., 2013) (Etzrodt et al., 2019). Secondly, high systemic levels of EPO have been implicated in the biasing of HSC fate and the induction of an “erythropoietic superhighway” (Grover et al., 2014). Here, by studying the differentiation of EPO-exposed HSC after transplantation at the single cell level

we can confirm a direct effect of EPO on HSC differentiation. We cannot confirm however, a direct biasing of HSC to the erythroid lineage. First and foremost, our results highlight the underestimated complexity of single cell behavior after transplantation- and the necessity to consider this complexity when describing cytokine-induced HSC biases.

3.4 Materials and methods

Mice

Male C57BL/6J CD45.1⁺ (in house breeding or Charles River) and C57BL/6J CD45.2⁺ (Charles River) donor and recipient mice were maintained at the animal facility of Institute Curie. Heterozygous *Rosa26CreER^{T2};mT/mG* mice were generated by crossing of *Rosa26mTmG* (<https://www.jax.org/strain/007576>) and *ROSA26CreERT2-TG* (<https://www.jax.org/strain/008463>) mice. Experiments were performed between 7-13 weeks of age. All procedures were approved by the responsible national ethics committee.

Lentivirus production

A new barcode library, named LG2.2, was generated. Oligo DNA stretches of 180 bp including a 20 bp “N”-stretch were ordered. DsDNA was generated by 10 PCR rounds and cloned into the XhoI-EcoRI site of the lentiviral pRRL-CMV-GFP plasmid (original description Dull et al., 1998). Subsequently ElectroMaxStbl4 cells were transformed, and approximately 16 000 colonies picked for amplification by Maxiprep. A colony PCR showed approximately 15% of barcode negative colonies which led to an estimated final diversity of 13 000 barcodes for the new LG2.2 library. Lentivirus was produced by transfection of HEK293T cells in DMEM-Glutamax supplemented with 10% FCS (Gibco), 1% MEM NEAA, and 1% sodium pyruvate. Polyethylenimine-mediated transfection together with psPAX2 and pVSVG. Supernatant was 0,45 um filtered, concentrated by 1h30 ultracentrifugation 31 000g and frozen at -80°C.

HSC isolation

Donor mice were sacrificed by cervical dislocation, femur, tibia and iliac bone isolated, and kept at 4°C in RPMI-1640-Glutamax (Gibco) supplemented with 10% FCS (Gibco) and 1% P/S (Gibco) (10%RPMI). Bone marrow was flushed using a 21G needle. Cells were washed with 10%RPMI by centrifugation for 6 minutes and 300g at 4°C and stained with α -c-kit magnetic beads (Miltenyi) at a dilution of 1:6 in 150 μ l 10%RPMI on ice for 20 minutes. After 5 minutes of incubation α -c-kit antibody was added for competitive staining. After staining, cells were washed as above and c-kit⁺ cells enriched by MACS (one LS column for three mice). An insulin syringe was used to generate a single cell suspension. Cells were maintained in 10%RPMI on ice at 4°C overnight. The next day, cells were washed as above and c-kit, Flt3, CD150 and Sca-1 stained in 200 μ l 10%RPMI for 35 minutes at 4°C. Cells were washed and filtered into 5 ml polystyrene round bottom tubes with cell strainer cap (Falcon) and HSC (LSK flt3⁻ CD150⁺) (Figure S1) sorted into FCS-precoated Eppendorf tubes.

HSC barcoding, EPO treatment, and transplantation

Sorted HSC were washed with HBSS (Gibco) by centrifugation for 6 minutes and 300 g at 22°C and resuspended in StemSpanMedium SFEM (STEMCELL Technologies) supplemented with 50 ng/ml mSCF (STEMCELL Technologies) and 1% P/S (Gibco) (SS⁺) at a maximal concentration of 100 000 cells/ 100 µl in a 96-well round bottom plate. 1,3 µl (tested to give a 10% transduction efficiency) of lentivirus from the lentiviral barcode library were added and the cells centrifuged for 1,5 hours at 300g and 4°C with slow deceleration and acceleration. This was followed by a 4,5 hours incubation at 37°C. Cells were washed as before in 10% RPMI and resuspended in 400 µl SS⁺. At this point the sample was split into 2x200 µl for control- and EPO-condition. For EPO-priming human recombinant EPO (Eprex, erythropoietin alpha, Janssen) at a final concentration of 1000 or 160 ng/ml was added and cells incubated for 16 hours at 37°C. After the incubation, the cells were washed as before once in 10%RPMI and once in HBSS, and finally resuspended in HBSS for transplantation. Recipient mice were sub-lethally irradiated at 6Gy. 2500 cells (of which approximately 250 barcoded cells) were injected in the tail vein of each mouse with an insulin syringe. If indicated cells were injected together with additional EPO (16 000 IU/kg) at the moment of transplantation.

Blood analysis and cell progeny isolation

At the indicated timepoints after transplantation (2 weeks, 4 weeks, 6 weeks, or 4 months), blood was taken, and/or spleen, and/or bones were isolated from recipient mice. For the analysis of barcodes in HSC a c-kit-enrichment was performed on bones as during initial HSC isolation (see above). And additional staining for CD41 allowed the isolation of MkP (MkP; c-kit⁺ Sca-1⁻ CD150⁺ CD41⁺). Blood was taken retro-orbital, immediately mixed 1:5 with citrate concentrated solution (Sigma) at RT and diluted in 10% RPMI. After cervical dislocation, splenic cells were isolated by meshing of the spleens through 100 µm cell strainers in 10%RPMI. From here on blood and spleen cells were processed similarly. Cells were washed by centrifugation for 6 minutes at 300 g and 4°C and stained in 150 µl 10%RPMI supplemented with 50 µg/ml DNase I (Invitrogen) with α-Ter119-biotinylated antibody (Table 1) for 20 minutes on ice. After 5 minutes, α-Ter119 PECy7 antibody was added for competitive staining. After staining, 50 µl biotin-magnetic beads (Miltenyi) (final dilution 1:6) and 100 µl 10%RPMI were added and cells incubated for 20 min on ice. Cells were washed as above and Ter119⁺ and Ter119⁻ cells separated by MACS (one LS column per mouse). An insulin syringe was used to generate a single cell suspension. Cells were counted using a Neubauer chamber and kept on ice at 4°C overnight. The next day, cells were washed as above and stained. Ter119⁺ cells were stained for TER119 and CD44.

Table 1: Fluorescently labelled antibodies used for FACS

Antibody target	Clone	Conjugate	Manufacturer
CD45.1	A20	PE	BD
CD45.1	A20	Pacific Blue	Biolegend
CD45.2	104	Pacific Blue	Biolegend
Ter119	TER119	PECy7	BD
CD11c	N418	APC	eBio
CD19	1D3	APCCy7	BD
CD11b	M1/70	PerCPCy5.5	eBio
CD117 (c-kit)	2B8	APC	Biogened
CD135 (flt3)	A2F10	PE	eBio
CD135 (flt3)	A2F10	PECy5	Life technologies
Scal	D7	Pacific Blue	Biolegend
CD150	TC15-12F12.2	PECy7	Biolegend
Ter119	TER119	biotin	BD
CD44	IM7	PE	BD
CD41	MVVREG30	BV510	BD
SiglecF	E50-2440	PE-CF594	BD
Ly6G	1A8	BV510	Biolegend
CD115	AFS98	PE	Biolegend

Ter119⁻ cells were stained for CD45.1 CD11b, CD11c, CD19, and Ter119 (and if adequate CD115, SiglecF, Ly6G) in 100 μ l 10%RPMI for 25 minutes at 4°C (Table 1). Cells were washed, resuspended in 10%RPMI. Viable donor (CD45.1⁺ or Tom⁺) barcoded (GFP⁺) B-cells (B; Ter119⁻ CD19⁺), myeloid cell (M; Ter119⁻ CD19⁻ CD11c⁻ CD11b⁺), and erythroid cell (E; Ter119⁺ CD44⁺) lineage were sorted into FCS-precoated Eppendorf tubes (Figure S1). For some cohorts additional subsets sorted were dendritic cells (DC; Ter119⁻ CD19⁻ CD11c⁺ CD11b⁻) and myeloid subsets (Ter119⁻ CD19⁻ CD11c⁻ CD11b⁺) macrophages (Ma; CD115⁻ SiglecF⁺ Ly6G⁻), eosinophils (Eo; CD115⁻ SiglecF⁺ Ly6G⁺), monocytes (Mo; CD115⁺ cells), and neutrophils (Neu; CD115⁻ SiglecF⁺ Ly6G⁺). Propidium iodine (Sigma) was added at a concentration of 1:5000 directly before sorting. The volume before and after sorting was carefully determined for total cell output calculations (the total cellular output was considered to be equal to the number of cells sorted multiplied by the total volume before sorting, divided by the volume actually sorted).

Cell progeny lysis, barcode amplification and sequencing

After sorting, cells were concentrated by centrifugation as above and supernatant removed. For barcode amplification from DNA, cells were transferred into the wells of a 96-well PCR plate and lysed in 40 μ l Viagen/Euromedex Direct PCR (cell) lysis buffer supplemented with 0,5 mg/ml proteinase K solution RNA grade (Invitrogen) in a thermic cycler: (55°C for 120 min, 85°C for 30 min, 95°C for 5 min, indefinite at 4°C). After lysis, samples were frozen at -20°C till further handling. For barcode

amplification from RNA, cells were resuspended in 20 µl of Superscript III RT buffer (Invitrogen) and transferred into the wells of a 96-well PCR plate. Lysis buffer, and reagents for RT by SuperScript III RT (Invitrogen) were added and lysis+RT performed using the primer top-LIB (5'TGCTGCCGTCAACTAGAACAA-3') (1h at 50°C and 20 min at 70°C). After lysis (+RT), barcodes were amplified by a newly established three step nested PCR. Barcode sequences were first amplified using the primers top-LIB (5'TGCTGCCGTCAACTAGAACAA-3') and bot-LIB (5'GATCTCGAATCAGGCGCTTA-3'). In a second round PCR, the Illumina sequencing primer sequences were added as well as a unique 4bp plate index. Primers used were as follows: forward (as 5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNCTAGAACACT CGAGATCAG) and reverse (5'GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGATC TCGAATCAGGCGCTTA). In a third round PCR, the P5 and P7 flow cell attachment sequences as well as one of 96 sample indices of 7bp were added. Primers were as follows: P5 (5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG ATCT) and P7 (5'CAAGCAGAAGACGGCATACGAGANNNNNNGTGACTGGAGTTC AGACGTGCTCTTCCGATC) (PCR1 and 2 30 cycles : hot start 5 min 95°C, 15 s at 95°C ; 30 s at 57.2°C; 30 s at 72°C, 5 min 72°C. for PCR3 15 cycles). Both index sequences (sample and plate) were designed based on (Faircloth & Glenn, 2012) such that sequences differed by at least 2 bases, homopolymers or more than 2 bp, hairpins and complementary regions with the rest of the primer sequence were absent (sequences available on request). To ease sequencing at least 4 different plate indices were used for each sequencing run. Primers were ordered desalted, as high-performance liquid chromatography (HPLC) purified. During lysis and each PCR, a mock control was added. The DNA amplification by the three PCRs was monitored by the run on a large 2% Agarose gel. Samples were pooled in order to guarantee a sequencing depth of 50 reads/cell. Five µl of the products of PCR3 for each sample and replicate were pooled and sequenced on a HiSeq system (Illumina) (SR-65bp) at the sequencing facility of Institute Curie (10% of Phix Illumina phage genome library were added to generate a more diverse set of clusters). Before sequencing, DNA sequences of adequate length (324 bp) were purified using the Agencourt AMPure XP system, analyzed on a Bioanalyzer, and diluted to a concentration of 5 nM.

Barcode sequence analysis

Sequencing results were analyzed using R-3.4.0, Excel, and GraphPad Prism. Firstly, reads were compared to a newly established barcode reference list (Chapter II Figure S11) and filtered for a 100% match in the input index- and common- sequences using XCALIBR (<https://github.com/NKI-GCF/xcalibr>). Next, the mean read count per sample and plate index combination was analyzed. All samples displaying a mean read count above 5000 were allowed for further analysis. The reads

belonging to a same sample were summed (same sample index, and plate indices of a same plate). Because read counts were variable, reads were normalized to 10^5 per sample. The Pearson correlation in read numbers for all barcodes between the two replicates for every sample was calculated. Samples with a correlation above 90% were considered for further analysis. Next, barcodes with reads in only one of the two replicates were discarded. For further analysis, the mean of the technical replicates was used. The sharing of barcodes between all mice transplanted from a same transduction batch over sequencing runs was determined. A read threshold was implemented to equalize the barcode sharing between mice transplanted from a same transduction batch within each sequencing run. When indicated Student's T test was used to test statistical significance. When indicated, heatmaps were generated using R on log10 transformed data using complete linkage and Euclidean distance (samples without reads were attributed the lowest occurring values before log10 transformation -> color coding black). When indicated, barcodes were categorized in progenitor classes either defining output in a lineage as presence of reads or >10% of a barcode's reads. When indicated, data was arcsine transformed for representation.

Fluorescence activated cell sorting

FACS was performed at the flow cytometry facility of Institute Curie on a FACS ARIA. FACSDiva™ software (BD) was used for measurements. Data analysis was performed using FlowJo v.10 (TreeStar). Cells were sorted using a 70 µm nozzle at precision 0/16/0 and high efficiency.

3.5 Supplementary information

3.5.1 Gating strategies for different hematopoietic cell subsets

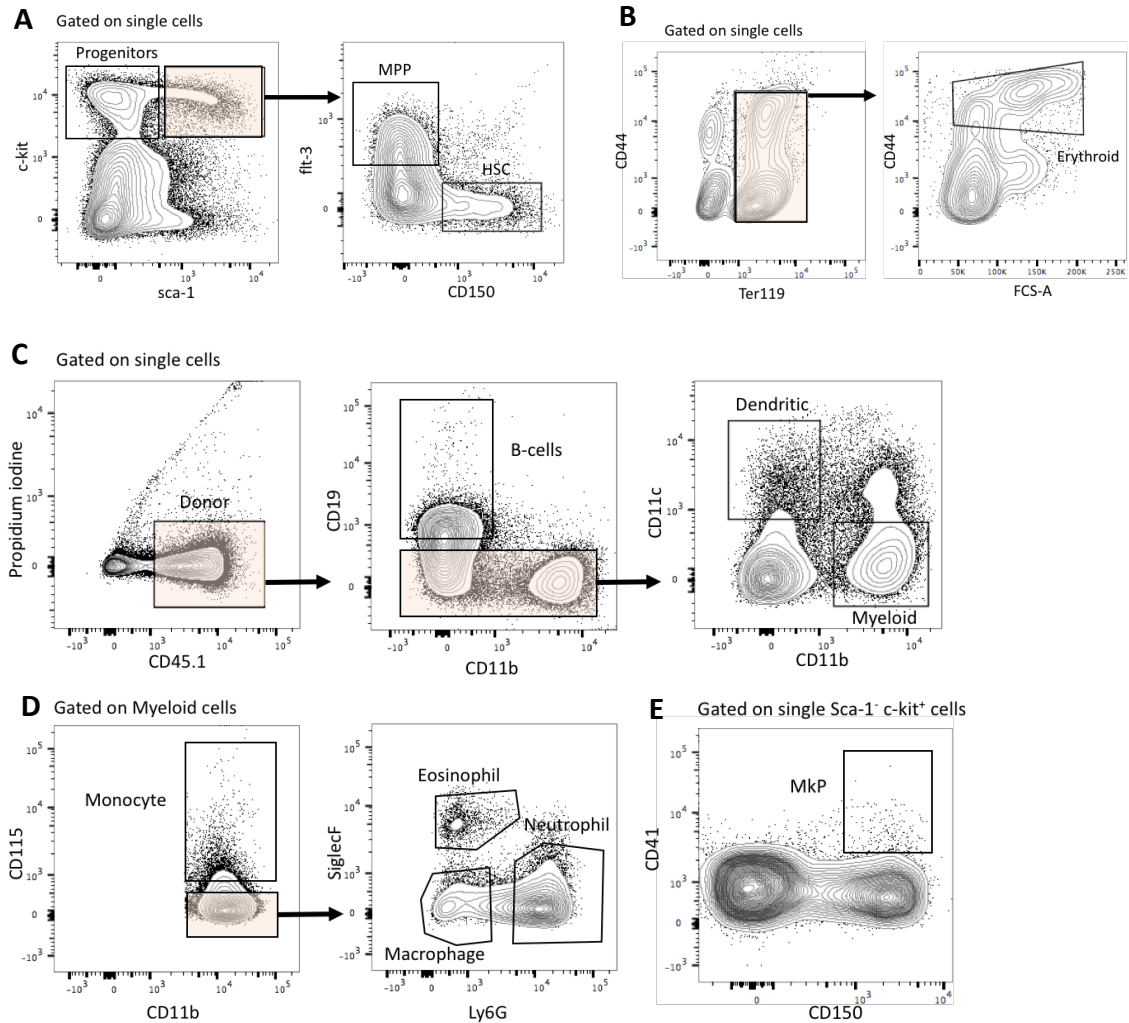


Figure S1: Gating strategies for hematopoietic progenitor and mature cell subsets for barcode analysis. (A-E) (A) HSC were gated as viable single c-kit⁺ Sca-1⁺ Flt3⁻ CD150⁺ cells, MPP as c-kit⁺ Sca-1⁺ Flt3⁺ CD150⁻, and progenitors as c-kit⁺ Sca-1⁻ subset of c-kit-enriched (Lin⁻) bone marrow cells. (B) Erythroid cells for barcode analysis were gated as Ter119⁺ CD44⁺ and FSC high cells on Ter⁺ enriched cells from bone marrow, spleen, or blood. (C) Gating strategy for B-cells (CD19⁺ CD11b⁻), dendritic cells (CD11c⁺ CD11b⁻), and myeloid cells (CD11c⁻ CD11b⁺) on Ter119⁻, live single, donor cells (Tom⁺ or, as depicted here CD45.1⁺) on Ter119 depleted cells from bone marrow, spleen, or blood. (D) further gating for detailed myeloid subsets on myeloid cells as in (C). Monocytes were sorted as CD115⁺ cells, Eosinophils as CD115⁺ SiglecF⁺ Ly6G⁺, Macrophages as CD115⁺ SiglecF⁻ Ly6G⁻, and Neutrophils as CD115⁺ SiglecF⁻ Ly6G⁺ cells. (E) Gating for MkP from ckit enriched (Lin⁻) bone marrow cells.

3.5.2 Changes in HSC marker expression after in vitro culture

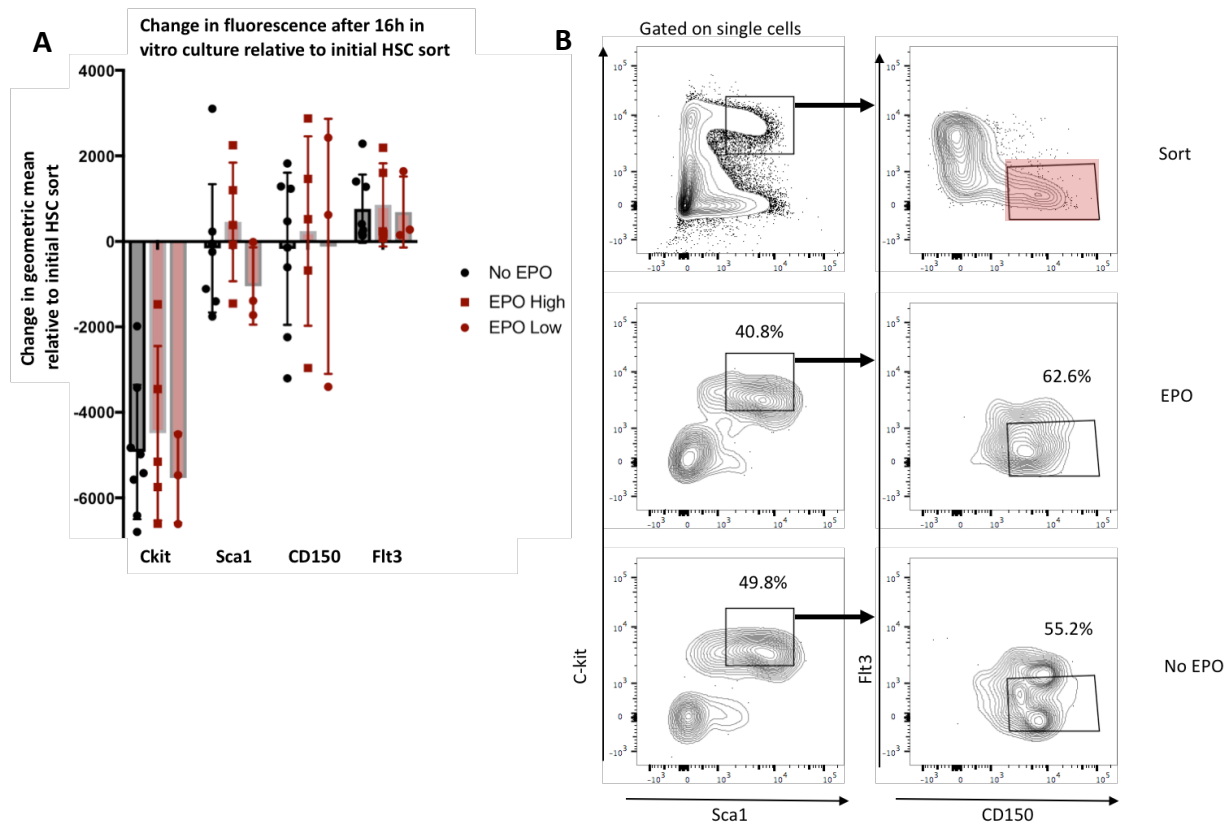


Figure S2: Changes in HSC marker expression after lentiviral transduction and in vitro culture with or without EPO. (A-B) HSC were sorted from Rosa26CreER^{T2}; mTdT/mGFP mice, barcoded by a 6h lentiviral transduction using the LG2.2 cellular barcoding library, and cultured in vitro with/without 160 (low) or 1000 ng/ul (high) EPO for 16h. (A) Changes in geometric mean relative to initial HSC sort for the markers c-kit, Sca-1, CD150, and Flt3. Shown are values from several experiments (transduction batches) (n=8 for control, n=5 for EPO high, and n=3 for EPO low group). (B) Representative flow cytometry plots of HSC sort (HSC are sorted as final red gate), and cells after 6h lentiviral transduction and 16h in vitro incubation with or without EPO.

3.5.3 Overview of mice survival and engraftment after transplantation

Table S1: Overview of mice survival and engraftment after transplantation in the different experiments. No engraftment was defined as a chimerism of under 10% in spleen Ter119 depleted cells.

Experiment	Mice in EPO and ctrl group	Deaths in EPO group	Deaths in ctrl group	No engr. in EPO group	No engr. in ctrl group
1	3/3	0	0	0	0
2	4/4	0	0	1	0
3	5/5	0	0	1	0
4	4/4	0	1	0	0
5	4/4	0	1	0	0
6	4/4	0	0	1	0
7	4/4	0	0	0	0
8	4/4	1	2	0	0
9	4/4	0	0	0	0
10	4/4	2	0	0	1
11	4/4	1	0	2	0
12	4/4	0	0	0	0
13	5/5/5	0	1	0	0
14	5/5	2	1	0	0
15	5/4	2	0	0	0
Sum		8	6	5	1

3.5.4 The influence of the threshold defining bias

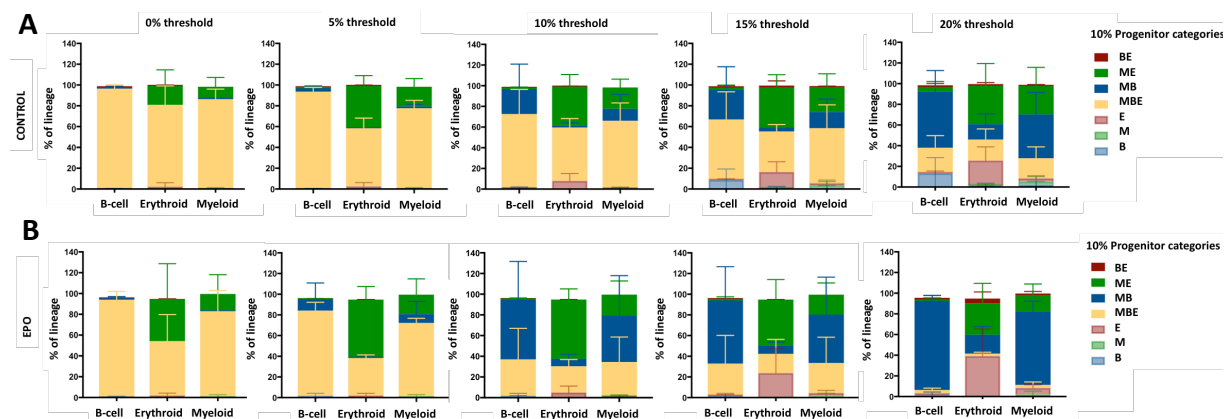


Figure S3: The influence of the threshold defining bias on the output of progenitor categories in different lineages (A-B) HSC were sorted from Rosa26CreERT²; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ul EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen of individual mice sacrificed at week 4 post-transplantation. (A-B) Categorization of barcodes based on bias using a threshold of 0%, 5%, 10%, 15% and 20% as indicated in different uni-, or multi-outcome progenitor categories, and representation of the percentage of each lineage produced by barcodes in these categories for the control (A) and EPO (B) group. Shown are values

from several animals (n=5 for control group and n=2 for EPO group (collected over 1 experiment). For all bar graphs mean and S.D. are depicted.

3.5.5 The variability between mice in ctrl and EPO groups

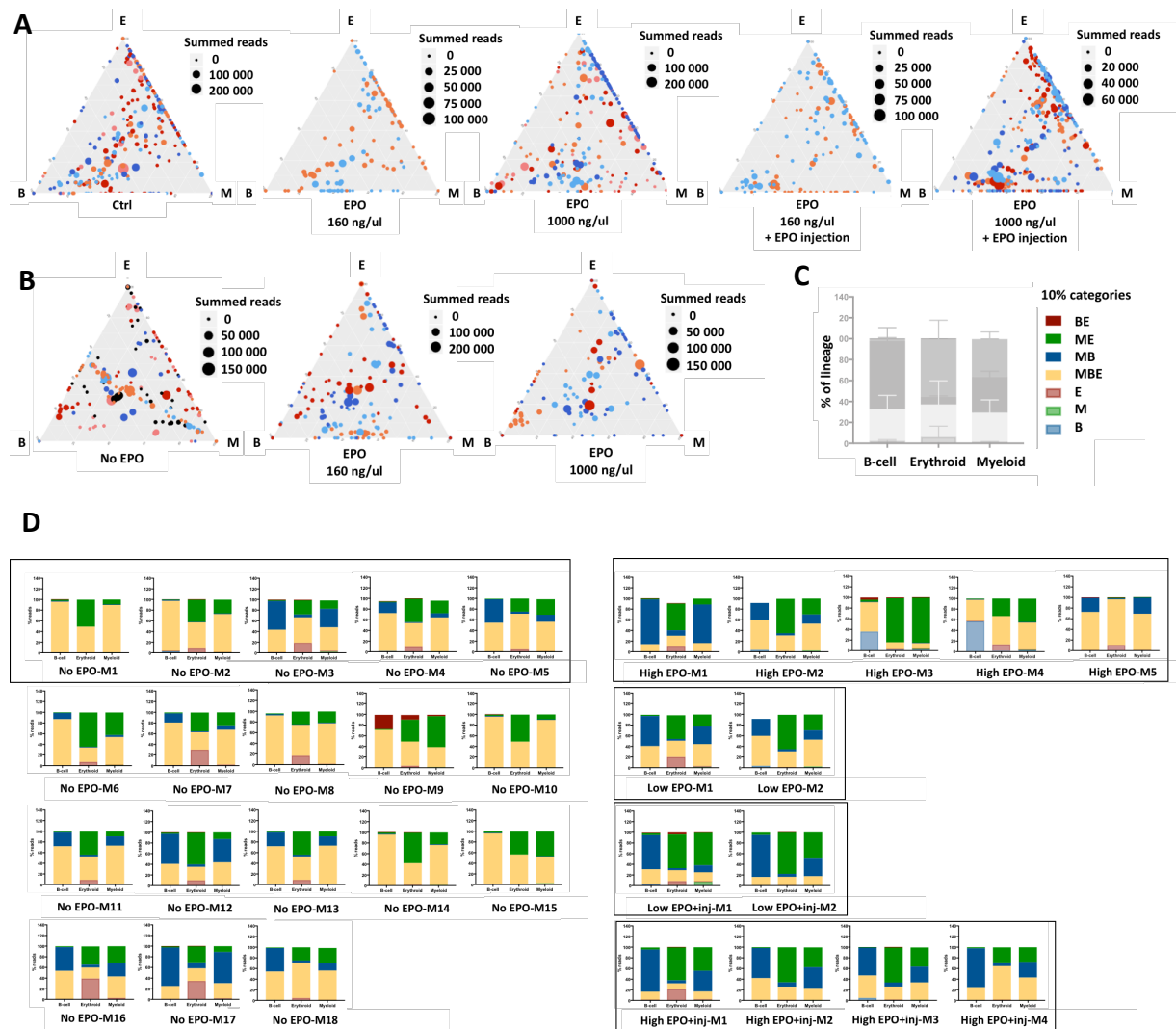


Figure S4: The variability between mice in ctrl and EPO groups regarding barcode results (A-C). HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/or without 160 ng/ul or 1000 ng/ul EPO for 16h, and transplanted with or without a single additional injection of EPO into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen of individual mice sacrificed at week 4 or month 4 post-transplantation. (A-B) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output for the different experimental groups as indicated. (B) 4-month data. Different colors are used for individual mice in each group (n=5 for control, n=2 for 160 ng/ul, n=5 for 1000 ng/ul, n=2 for 160 ng/ul +injection, n=4 for 1000 ng/ul +injection, n=3 160 and 1000 ng/ul 4 months, n=6 for control 4 months (collected over 6 different experiments). (C) exemplary plot to show lineage order and category legend for all plots in (D) The percentage of each lineage

produced by the HSC categorized by bias using a 10% threshold for individual mice. Encircled are plots of mice depicted in (A).

3.5.6 Permutation tests for 10% ME and MB progenitor output

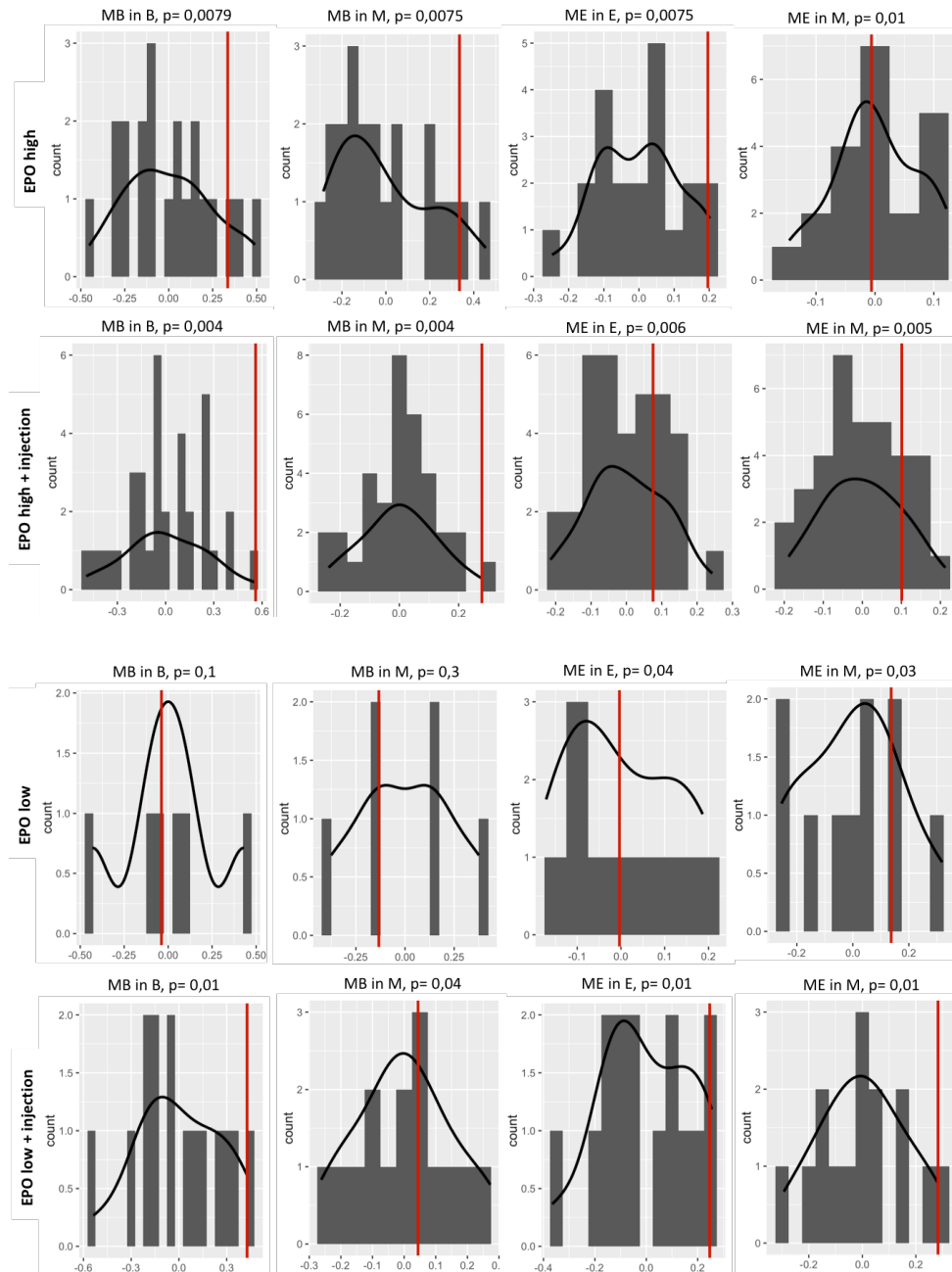


Figure S5: The variability between mice in ctrl and EPO groups regarding barcode results. HSC were sorted from Rosa26CreERT²; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 160 ng/ul or 1000 ng/ul EPO for 16h, and transplanted with or without a single additional injection of EPO into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen of individual mice sacrificed at week 4 post-transplantation. Barcodes were classified into progenitor categories

using a 10% threshold. The output of MB and ME progenitors to the B and M, and E and M lineages respectively was analyzed. By permutating the mice of ctrl and EPO group, the random distribution of this output was generated (histogram and density in black and gray) and compared to the real output difference between ctrl and EPO group (red line). A p-value was generated as in (Tak, 2019).

3.5.7 The HSC clonality 4 months after transplantation of EPO-exposed HSC

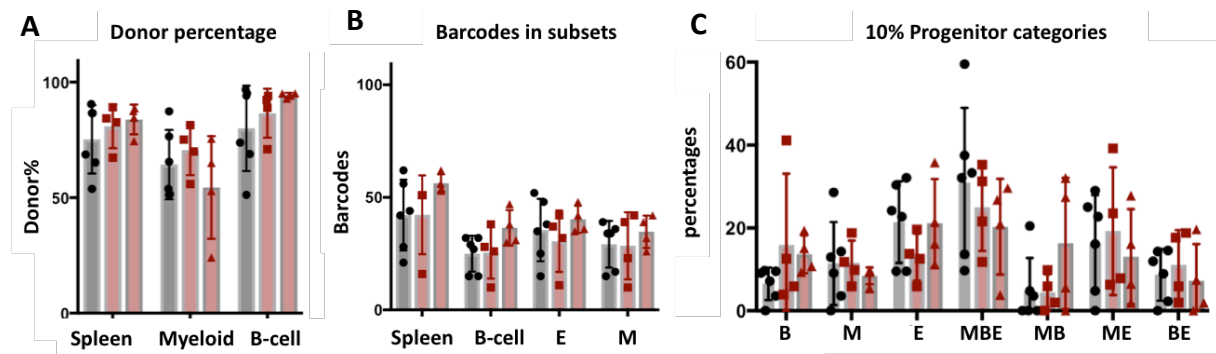


Figure S6: Clonality in the E, M, and B lineage of EPO-exposed HSC 4 months after transplantation. (A-C) HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/or without 160 ng/ml or 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, M, and B lineage in spleen and HSC in bone of individual mice sacrificed at month 4 post-transplantation. (A) The fraction of donor cells among the indicated cell types in spleen. (B, C) Percentage of barcodes found back in the indicated lineage (B) and lineage combinations (C) at month 4 after transplantation in Ctrl and EPO group. Shown are values from several animals (n=4/3 for control and EPO-group respectively (collected over two experiments)). For all bar graphs mean and S.D. between mice are depicted.

3.5.8 Detailed myeloid output of transplanted HSC

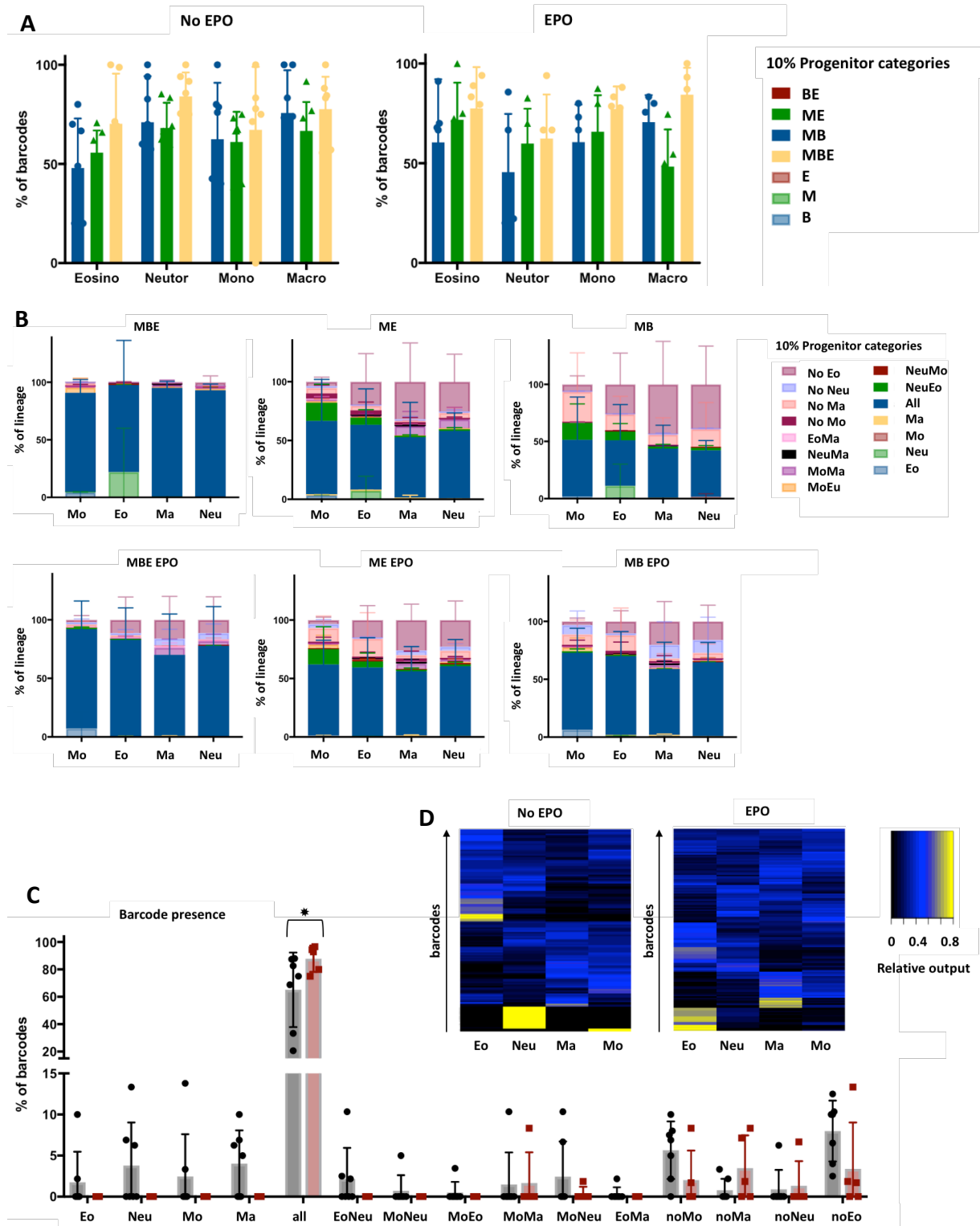


Figure S7: Production of macrophages, monocytes, neutrophils and eosinophils by HSC after EPO-exposure and transplantation. (A-D) HSC were sorted from Rosa26CreER^{T2}; mTdtTomato/mGFP mice, barcoded using the

LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ul EPO for 16h, and transplanted into sublethal-irradiated mice. Analysis of barcodes in the E, B, MkP, and different myeloid subsets in spleen of individual mice sacrificed at week 4 post-transplantation. (A) A 20% threshold in the E/B contribution was chosen to distinguish the ME-, MB-, and MBE-biased HSCs. The contribution of barcodes in these three categories to the indicated myeloid subsets is represented for control and EPO-group. (B) The percentage of each lineage produced by the indicated uni-, or multi-outcome barcode categories. (C) Percentage of barcodes classified based on their presence in myeloid lineages using a 0% threshold for clones with a contribution of 1% to the overall myeloid output (red=EPO-group, black=control). (D) Heatmaps showing the output of individual barcodes (rows) in different samples as indicated. Data is normalized by barcode, log transformed and clustered by complete linkage using Euclidean distance. No output is represented in black. Shown are values from several animals (n=5 for both groups (collected over two experiments)). For all bar graphs mean and S.D. between mice are depicted.

3.5.9 Detection of individual HSC over time

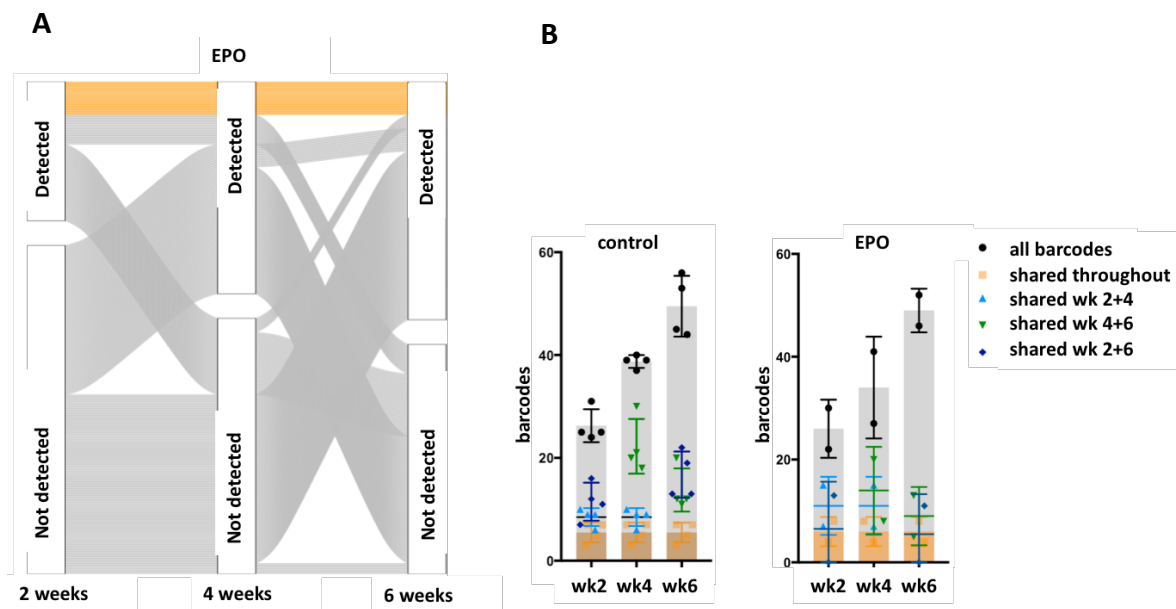


Figure S8: Detection of individual HSC over time. (A-B) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen 6 in the same individual mice. (A) Sankey diagram showing detection of barcodes (irrespective of lineage) over time for all mice combined in EPO-group as indicated. Orange color is used for barcodes detected at all three timepoints. (B) Bar graph showing variability between mice in detection of barcodes over time in control and EPO-group. Mean and S.D. between mice are depicted. Shown are values from the same animals throughout (n=3 from one experiment for both groups).

3.5.10 Changes in lineage restrictions of transplanted EPO-exposed HSC I

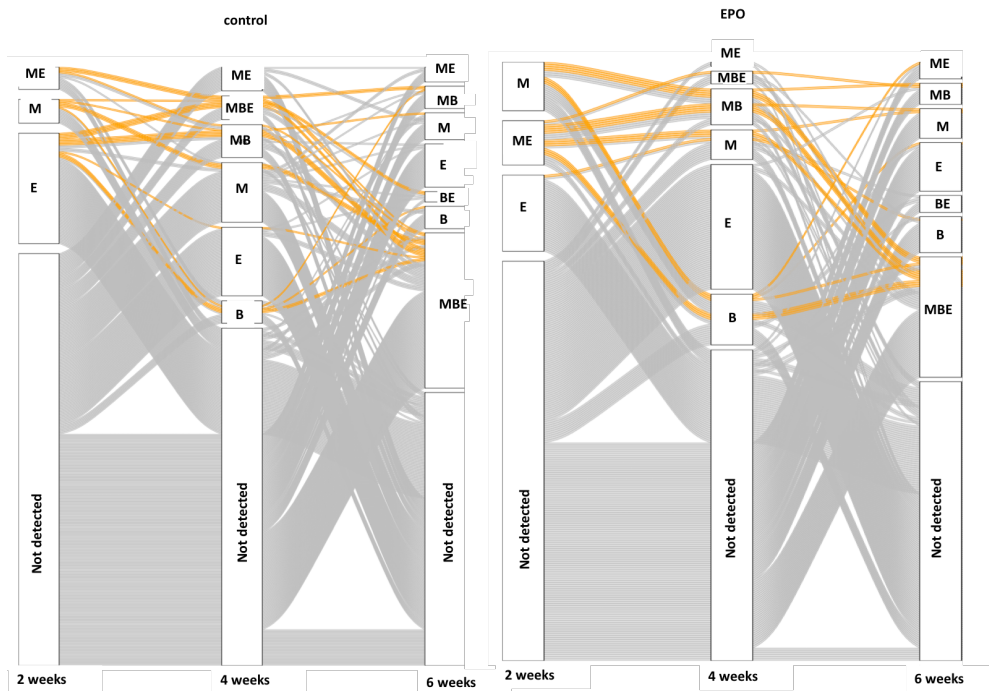


Figure S9: Changes in lineage restriction of individual HSC over time in control and EPO-group. HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/or without 1000 ng/ml EPO for 16h, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen 6 in the same individual mice. Sankey diagram showing detection of barcodes in different lineages over time for all mice combined in control and EPO-group as indicated. Orange color is used for barcodes detected at all three timepoints. Shown are values from the same animals throughout (n=3 from one experiment for both groups).

3.5.11 Changes in lineage restrictions of transplanted EPO-exposed HSC II

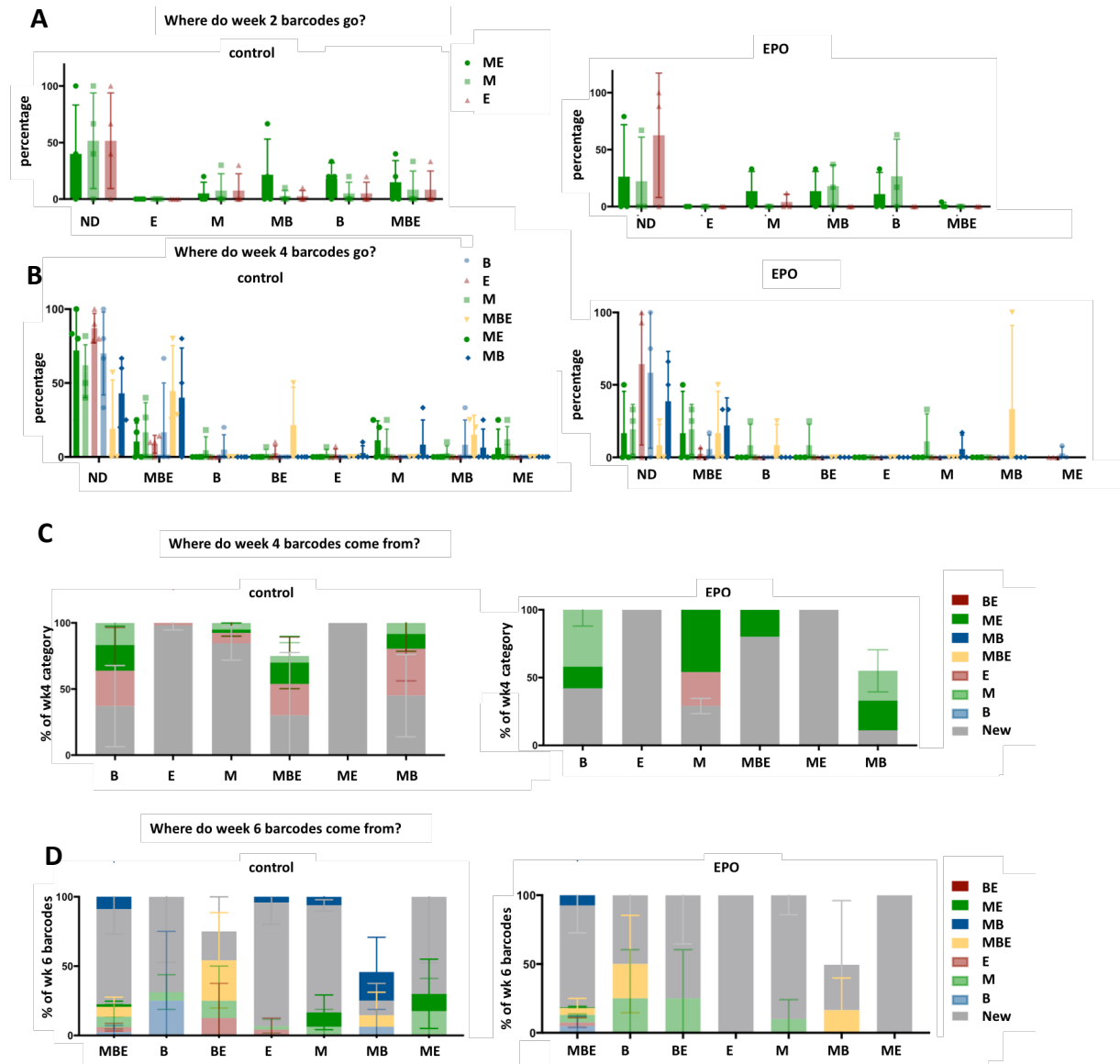


Figure S10: Changes in lineage restriction of individual HSC after transplantation of EPO-exposed HSC. (A-D) HSC were sorted from CD45.1 mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ul EPO for 16h, and transplanted into sublethal-irradiated wt mice. Analysis of barcodes in the E, M, and B lineage from blood at week 2, 4, and spleen 6 in the same individual mice. (A-D) Bar-graph showing the fate (A, B) and origin (C, D) of barcodes detected with the indicated lineage restriction at the indicated timepoint in the indicated condition. Fate and origin are defined based on the detection and lineage restriction at the respectively previous (origin) and next (fate) analysis timepoint. Shown are values from the same animals throughout (n=3 for both groups from one experiment). For all bar graphs mean and S.D. between mice are depicted.

3.5.12 HSC self-renewal vs differentiation 4 months after transplantation

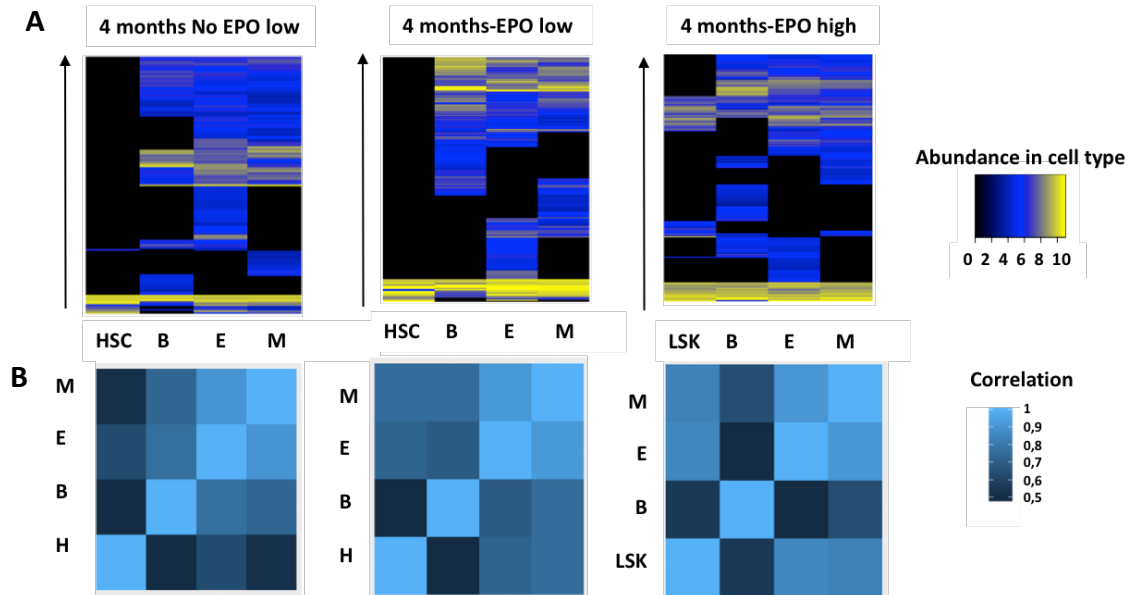


Figure S11: HSC self-renewal vs differentiation 4 months after transplantation of EPO-exposed HSCs. (A-B) HSC were sorted from Rosa26CreER^{T2}; mTdTtomato/mGFP mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with/without 1000 ng/ml (EPO high) or 160 ng/ml EPO (EPO low) for 16h, and transplanted into sublethal-irradiated mice. The barcode identity in the E, M, and B lineage was assessed at month 4 in spleen and compared to the barcode identity in HSC (or LSK EPO high) in bone. (A) Heatmaps showing the output of individual barcodes (rows) in different samples as indicated. Data is log transformed and clustered by complete linkage using Euclidean distance. No output is represented in black. (B) Pearson correlation between the indicated cell types at the indicated timepoints (H=HSC). Shown are values from several animals (n=4 EPO and n=3 control (collected over 2 different experiments)).

3.5.13 HSC self-renewal vs differentiation after transplantation of EPO-exposed HSC

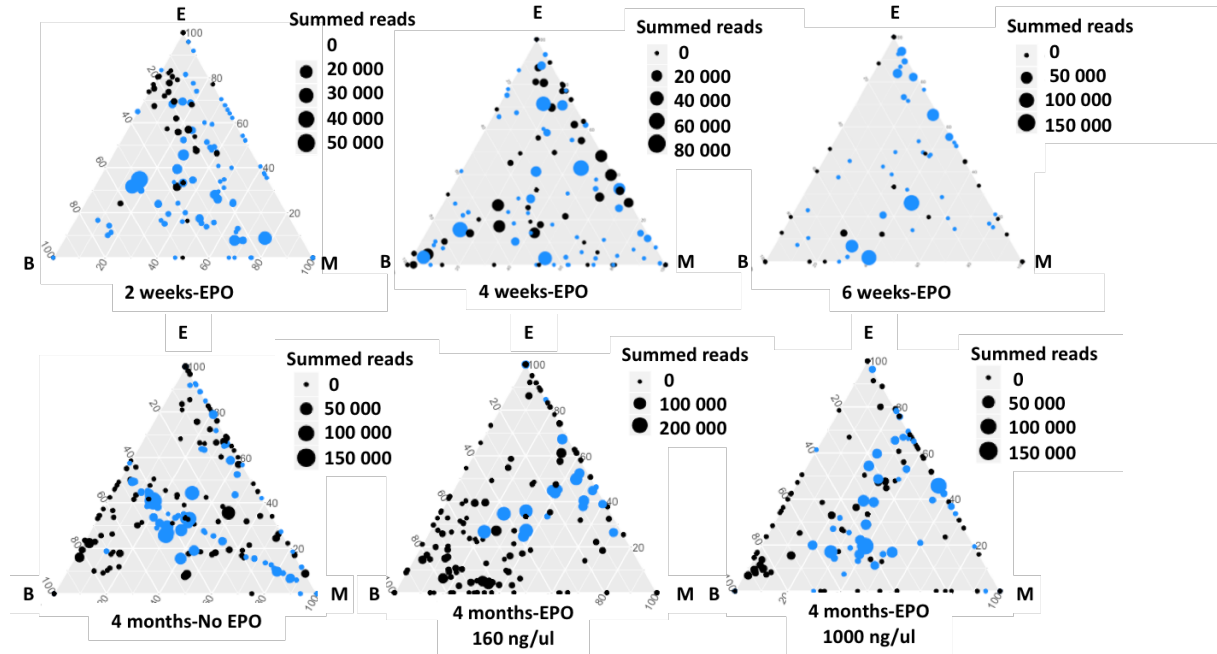


Figure S12: HSC self-renewal vs differentiation in the different lineages after transplantation of EPO-exposed HSC. HSC were sorted from *Rosa26CreER^{T2}*; *mTdtTomato/mGFP* mice, barcoded using the LG2.2 cellular barcoding library, cultured in vitro with 1000 ng/ml (EPO high) or 160 ng/ml EPO (EPO low) for 16h, and transplanted into sublethal-irradiated mice. The barcode identity in the E, M, and B lineage was assessed at week 2, week 4, week 6, or month 4 in spleen and compared to the barcode identity in HSC (or LSK EPO high) in bone (A) Triangle plots showing the relative abundance of barcodes (dots) in the E, M, and B lineage with respect to the summed output in the 3 lineages at the indicated timepoints after transplantation for barcodes found back in bone HSC (or LSK EPO high) (blue) or not (black). Shown are values from several animals (n=1 week 2, n=5 week 4, n=3 week 6, month 4 n=4 (collected over 6 different experiments)).

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Chapter 4: What next? Outlook on cellular barcoding

In this thesis, I have performed experiments to better understand the kinetics of differentiation of single HSC over the first six-weeks after bulk transplantation. By detecting cellular barcodes in the erythroid lineage from RNA, we could do so in myeloid, B-cells but also erythroid cells. By monitoring clonality in mature cells and progenitor populations, we could establish that early high erythroid cell production after transplantation is maintained by distinct short-lived HSC clones. In contrast, high myeloid cell production after transplantation is maintained by long-lived multi-outcome. We determined the effect of EPO on these kinetics. While clonal succession and stability persisted, high output ME- and MB-biased HSC dominated the early hematopoietic reconstitution. In this chapter, I will discuss some outstanding general issues related to these two studies with respect to the future applications of cellular barcoding.

4.1 The promises of available HSC barcoding libraries

Single HSCs behave differently in bulk

In the 2000s single cell transplantation became possible as the prime assay to follow HSC on a clonal level. Till today this assay gives important insights on the lineage restrictions and engraftment durations of single cells immunophenotypically defined as HSC (Carrelha et al., 2018) (Yamamoto et al., 2013a). Drawbacks of the technique are however not alone the large number of mice required for high throughput. The particular stress a cell can encounter when transplanted alone could also influence its behavior and does not readily reflect its behavior during bulk transplantation. We could observe these differences in Chapter II and III of this thesis when applying cellular barcoding to trace the differentiation of hundreds of single HSC after bulk transplantation. When monitoring the reconstitution kinetics after bulk transplantation on a single cell level, we could observe that HSC lineage restriction types and biases were similar to findings from single cell transplantation studies. However, the differentiation kinetics and importance of each HSC subset seemed different in the bulk than in the single cell transplant setting. Also in Chapter III, where we monitored the effect of EPO on HSC differentiation, the interactions of HSCs during bulk transplantation were prominent. Although EPO-exposure did not lead to changes in bulk lineage output, clonality of single HSC was changed. A combination of high output ME- and MB-biased HSC was apparent, suggesting the functional compensation between HSC clones, as previously reported for lymphoid-deficient HSCs (L. Nguyen et al., 2018). Likely, as for the cytokine EPO, other alterations of HSC differentiation have been missed to date because they were masked on the bulk level by feedback mechanisms. Already available cellular barcoding libraries will be an important tool to assess such hematopoietic compensation and feedback mechanisms.

Combining cellular barcoding libraries

The number of cellular barcoding libraries available is increasing and the detailed assessment of methodological challenges over the recent years, makes barcoding results more and more reliable. To assess compensation and feedback mechanisms of single cells it will be especially useful to transplant several differently barcoded HSC populations together. Most of the cellular barcoding libraries available to date already have the possibility for multiplexing. The libraries of Lu et al. and Wu et al. can be multiplexed through library IDs (Lu et al., 2011) (Wu et al., 2014). The libraries by Cornils et al. and Thielecke et al. can be multiplexed by different reporter proteins encoded in the fixed triplet of the barcode sequence (Cornils et al., 2014) (Thielecke et al., 2017) and a first proof of principle has been performed (Aranyosy et al., 2017). The library used in this thesis is not only available in conjunction with a GFP reporter, but also with a Thy1.1 reporter (unpublished). Alternatively, one same cellular

barcoding library can be used with congenically marked cell populations (L. Nguyen et al., 2018), and high-diversity libraries can be sub-sampled in smaller libraries which's constitution is controlled and optimized for simultaneous use.

4.2 HSC barcoding entering the 'omics' era

HSC transcriptome and lineage differentiation

Recently, an increasing number of HSC scRNAseq data sets has emerged (Kowalczyk et al., 2015) (Haas et al., 2015) (Nestorowa et al., 2016) (J. Yang et al., 2017) (Velten et al., 2017) (Giladi et al., 2018) (Tusi et al., 2018). Based on this data the binary decisions of the "classical" model of hematopoietic differentiation (hematopoietic tree) have been more and more doubted and a picture of HSC differentiation as a "flow" or "continuum" has emerged. Gene expression signatures for different mature hematopoietic cells and progenitors become more and more detailed. Often the expression of lineage specific genes in HSC is equated to an actual change in lineage differentiation. In how much HSC transcriptome indeed reflects lineage differentiation is however unclear and would require a combination of scRNAseq and cellular barcoding. In zebrafish three such systems have already been developed: LINNAEUS (LINEage tracing by Nuclease-Activated Editing of Ubiquitous Sequences) (Spanjaard et al., 2018), scGESTALT (Raj et al., 2018), and ScarTrace (Alemany et al., 2018). In mouse some low throughput solutions exist as the combination of scRNAseq and lineage tracing by fluorescent proteins (Montoro et al., 2018) (V. W. C. Yu et al., 2016). The detection of cellular barcodes by scRNAseq in HSC could link HSC transcriptome to lineage differentiation. By detecting cellular barcodes from RNA of erythroid cells in Chapter II and III, and the comparison of RNA and DNA barcode profiles in Chapter II we made a first step in this direction. Not all current cellular barcoding libraries will be suitable for such application. For several libraries the barcode is not expressed (also to avoid biological effects) (Lu et al., 2011) (Wu et al., 2014) (Ludwig et al., 2019) (H. E. C. Bhang et al., 2015). For a number of other libraries detection of barcodes by scRNAseq should and will certainly be possible soon (Cornils et al., 2014) (Thielecke et al., 2017) (Gerrits et al., 2010), (L. V. Nguyen et al., 2014a) (Naik et al., 2013).

HSC epigenome, proteome and barcoding

Besides the transcriptome of hematopoietic progenitors also epigenome and proteome are starting to be analyzed on the single cell level (Buenrostro et al., 2018) (Corces et al., 2016) (Palii et al., 2019). A study by Yu et al. using the HUE mouse reported that the lymphoid and myeloid bias of two analyzed HSC clones could not clearly be matched to transcriptomic changes, but rather to epigenomic profiles (X. Yu et al., 2017). The epigenetic hard-wiring remains however still controversial. Also for

the effect of cytokines on HSCs it remains to be determined if the epigenome of HSCs could be influenced. In Chapter III we saw that the effect of EPO on HSC is transient. This is in line with the effect of other cytokines on HSCs (Pietras et al., 2016) (Mossadegh-Keller et al., 2013) (Wilson et al., 2008). The question remains however if there is a memory for cytokine exposure to ease subsequent changes. Besides, scATAC-seq, scCHIP-seq should soon be spreading (Rotem et al., 2015). Like for scRNAseq and transcriptome, the detection of cellular barcodes in scCHIP-seq data, could enable a high-throughput matching of epigenome to lineage information. Single-cell proteome analyzes of hematopoietic progenitors are to date restricted. Mass cytometry has been employed (Palii et al., 2019). Besides a microfluidics single cell proteomics platform has been used to assess cytokine production of individual HSCs (J. L. Zhao et al., 2014). Technical developments will be needed to combine single cell proteome to barcode lineage information in the next decade.

4.3 HSCs barcoding and other dimensions

In situ lineage tracing of HSCs

HSC in the bone marrow are thought to reside in special microenvironments, termed “niches”. Many different cell types have been identified as niche components, including MSC, fibroblasts, endothelial cells, osteoblast, adipocytes, nerve cells, and mature hematopoietic cells as T-cells, macrophages, or megakaryocytes (Morrison & Scadden, 2014) (Birbrair & Frenette, 2016). The signaling between niche and HSC is considered to be crucial for HSC survival, proliferation, and migration. More recently, the influence of the niche on lineage differentiation has been focused too. It has for example been started to be elucidated if HSC and differently committed early progenitors reside in distinct niches (Ding & Morrison, 2013) (Greenbaum et al., 2013) (Cordeiro Gomes et al., 2016). Very recently it was suggested that even differently biased HSC could already reside in different niches (Pinho et al., 2018). Pinho et al. showed that megakaryocyte and myeloid-biased HSC are highly enriched in proximity of megakaryocytes (Pinho et al., 2018). A combination of barcoding information with spatial information would enable to study the influence of niche cells on HSCs in even more detail. Different approaches to start such “spatial barcoding” exist. The MEMOIR system (memory by engineered mutagenesis with optical in situ readout) allows readout of “barcodes” in situ through single molecule RNA fluorescence hybridization in mouse embryonic stem cells (Frieda et al., 2017). The application of such system to HSC, e.g. the detection of cellular barcodes in HSC through FISH, could make it possible to assess the influence of HSC niches on lineage differentiation. Complementary to the barcode readout in situ, also some first steps towards barcode induction in situ have been developed. The NICHE-seq system, combines for example photoactivatable fluorescent reporters, two-photon microscopy, and scRNAseq to infer the cellular and molecular composition of specific niches (Medaglia

et al., 2017). Combining such system with cellular barcoding of HSC, the fluorescent labeling of barcoded HSC or their progeny in specific niches, and readout by NGS or scRNAseq would be a great step towards the characterization of the influence of niches on HSC differentiation. Other approaches for “spatial barcoding” could be especially interesting for in situ HSC lineage tracing and barcoding systems. These include for example the photoactivation of CreERT2 recombinase to induce reporter expression or to generate barcodes in HSC in specific locations. Recombinations in single cells have been possible using such system in zebrafish (Sinha et al., 2010). An application to the polylox barcoding or barcode mouse could help to follow the fate of HSC in specific locations (Pei et al., 2017) (Gerlach et al., 2013) (Thesis Jeroen W.J. van Heijst, 2010).

Barcoding, kinetics, and HSC biases

The kinetics of HSC differentiation have started to be studied. Well known are bulk lineage differentiation kinetics after HSC transplantation (Forsberg et al., 2006) (Boyer et al., 2019). The development of inducible HSC reporter mice, allowed to assess the bulk lineage production kinetics of HSCs (Säwen et al., 2018) (Gazit et al., 2014) (Busch et al., 2015) (Sawai et al., 2016) (Upadhaya et al., 2018) (Chapple et al., 2018b). In Chapter II we assessed these kinetics at the single cell level for the first six weeks after transplantation.

The very low number of barcodes we could record in same lineage combinations over time made it clear how important kinetic phenomena are for the consideration of HSC lineage biases (Figure 4 Chapter I). We sampled blood over time, to allow kinetic analysis. Other groups employed partial splenectomies and bone marrow aspirations (Upadhaya et al., 2018) (Naik et al., 2013). Each of these approaches induces also some alterations. An alternative could be “temporal barcoding” approaches, for example through induction of different reporter proteins in barcoded cells over time. Also, the induction of different barcode types in single cells at different timepoints could be imagined.

Closely linked to influence of kinetics on HSC differentiation biases are also division numbers. In Chapter II we could see a high correlation between barcode abundance in HSC and mature erythroid and myeloid, but not B-cells. One explanation for this observation could be a higher number of divisions in the generation of erythroid and myeloid cells from HSC, than for B-cells. The comparison of barcode abundance in HSC, MPP, P and mature cells implied this could already occur at the P stage. Different studies have previously studied the number and rate of divisions in different hematopoietic progenitor subsets including the HSC. These often made use of the dilution of a pulsed histone 2B-green fluorescent protein (H2BGFP) label (Bernitz, Kim, MacArthur, Sieburg, & Moore, 2016) (Qiu, Papatsenko, Niu, Schaniel, & Moore, 2014) (Oguro et al., 2013) (Wilson et al., 2008) (Foudi et al., 2009). Also different labelled nucleoside analogues as BrdU and ErdU (Oguro et al., 2013) (Wilson et al., 2008) (Akinduro et al., 2018) and the dilution of a Tomato protein expression (Upadhaya et al., 2018) have been employed

to estimate proliferation rates. A combination of cellular barcoding and proliferation tracking would be a great tool to assess the influence of kinetics on HSC differentiation and HSC biases.

4.4 Beyond the single-cell level, intra-clonal heterogeneity

The heterogeneity of HSC in terms of differentiation and proliferation at the single cell level is being established. The current applications of cellular barcoding are an important tool in this development. New applications of cellular barcoding, as a combination with scRNA-seq, scCHIP-seq, or with spatial and/or temporal information will further our understanding of hematopoiesis at the single cell level. Besides inter-clonal HSC heterogeneity also intra-clonal variability, less studied to date, will become an important aspect to consider. Not all HSC cells within a clone might behave in the same way. A barcoded HSC, might through self-renewal give rise to HSC with a same barcode, but different behavior in terms of proliferation and differentiation. Any link of HSC barcode (and scRNA-seq, scCHIP-seq profile) to a barcode in mature cells, or as in Chapter II, already the link of an HSC barcode to a barcode in mature cells, is subject to these biases. Several studies implied high intra-clonal. For example proliferation of HSC families seems synchronous *in vitro* (Tak, 2019). Also upon transplantation a high homogeneity of HSC clones was suggested (V. W. C. Yu et al., 2016). To study the intra-clonal heterogeneity in different physiological and pathological settings will be an important challenge for the future.

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Appendix

Summary

Hematopoietic cells are the most numerous cells in our body, and their overall short half-life requires their steady production. At the apex of the hematopoietic system resides a small number of hematopoietic stem cells (HSC) which give rise to all mature hematopoietic cell types through self-renewal and differentiation divisions. For long it was assumed that these HSC are a homogeneous population of multipotent cells. Technical advancements, which allowed to trace HSC at the single cell level, revealed however that single HSC behave differently with respect to the number of lineages and the relative amounts of different lineages they produce and are heterogeneous with respect to their long-term engraftment capacity. Notably different lineage restricted and biased cells, as well as cells with long-term and short-term engraftment potential have been identified in the HSC gate. One technique allowing the lineage tracing of single HSC is cellular barcoding, which relies on the introduction of an artificially created DNA fragment, the barcode, in the genome of HSCs through viral transfection. As these barcodes are transmitted to all daughter cells, the analysis of the barcode identity of progeny cells reveals which lineages and how much of each is produced by each individual HSCs.

In this thesis we used a new cellular barcoding library to analyze how the different HSC subsets previously described interact and behave in the first six weeks after bulk transplantation, as well as the influence of erythropoietin (EPO) on this process. More in detail, we described the reconstitution kinetics of HSC in the myeloid (M; macrophage, monocytes, neutrophils, eosinophils), lymphoid (B-cells), dendritic cells, megakaryocyte and erythroid lineage (E; erythroblasts) at the single cell level. For the analysis of HSC differentiation towards the erythroid lineage we established the detection of cellular barcodes from RNA, unpublished to date. We discovered that HSC clonal succession and clonal stability co-occur in the first weeks after transplantation, but are not evenly distributed over the different hematopoietic lineages. Notably the production of erythroid cells 2-weeks after transplantation was maintained by distinct short-lived HSC clones, while high myeloid cell production after transplantation was guaranteed by long-lived multi-outcome HSCs. *In vitro* EPO exposure of HSC before transplantation, did not change the overall lineage output of transplanted HSC, or HSC differentiation kinetics, lineage restrictions, and biases at the single cell level in the first six weeks after transplantation. However, after transplantation of EPO-exposed HSC long-lived unbiased multi-outcome HSCs lost preponderance with respect to cellular output. Rather, changing clones of two types of highly biased HSCs, myeloid-erythroid (ME)-biased and myeloid B-cell (MB)-biased HSC, produced now the majority (>60%) of erythroid, myeloid and B-cells. This effect was transient but stable over different EPO concentrations and after *in vivo* EPO treatment during transplantation. It suggests a functional compensation mechanism at work.

We hope the detailed description of the engraftment kinetics of single control and EPO-exposed HSC after bulk transplantation will have relevance both for fundamental research and the clinics. The

cytokine EPO is one of the most prescribed biopharmaceuticals worldwide and routinely used in the clinics to counteract anemia in many different settings. It is important to understand the effect of EPO on HSC to guarantee the long-term safety of such application. Besides, the description of the effect of EPO on HSC can help to understand general mechanisms by which cytokines act on HSCs. The transplantation of HSC is today widely used in the clinics as treatment for a large number of different pathologies. A phase which remains particularly critical for success are the first weeks after transplantation in which the different blood lineages did not normalize yet. To understand the differentiation of HSC in this phase, might lead to improved transplantation protocols enabling faster erythroid reconstitution, and causing less burden to the patient. The detection of cellular barcodes from RNA we described paves the way for new applications of cellular barcoding in the future, especially its combination with scRNAseq profiling.

Résumé complet

La majorité des cellules de notre corps sont des cellules hématopoïétiques. Un petit nombre de cellules souches hématopoïétiques produit toutes ces cellules par divisions de maintenance and différenciation. Il a longtemps été considéré que ces cellules souches étaient une population homogène et que chaque cellule produit le même nombre et type de cellule hématopoïétique mature. Cette idée a été réfutée quand il est devenu possible de suivre la différenciation de cellules uniques. Ainsi les cellules hématopoïétiques souches individuelles produisent différents types et quantités relatives de cellules hématopoïétiques matures, amenant la reconstitution après transplantation des cellules souches peut être hétérogène. Quelques cellules qui expriment les marqueurs de cellule souche hématopoïétique survivent uniquement pour une courte durée après transplantation, d'autres pour des années.

Dans cette thèse, nous avons utilisé la technique de traçage de lignée des codes-barres cellulaires pour comprendre comment les différents types de cellules souches hématopoïétiques se comportent dans les six premières semaines après transplantation et l'influence de la cytokine érythropoïétine sur ce processus. Les codes-barres cellulaires sont des fragments artificiels d'ADN qui sont introduit dans les cellules souches hématopoïétiques murines par transfection virale. Après transplantation de cellules souche hématopoïétique barcodées de cette manière, chaque division cellulaire va transmettre le code-barre aux cellules filles. Par l'analyse des codes-barres dans les cellules hématopoïétiques matures on peut ainsi comprendre quels types et quelles quantités relatives de cellules matures les cellules souches hématopoïétiques ont produites. Nous avons observé que certaines cellules souches contribuaient de manière stable dans les premières semaines après transplantation, ainsi qu'une succession clonale. Des différences au niveau de la production de différents types de cellules hématopoïétiques matures a aussi été observées. Les globules rouges sont produits par des clones de vie courte et les cellules myéloïdes par des clones stables sur 6 semaines. Le traitement des cellules souches hématopoïétiques avec

l'érythropoïétine avant leur transplantation, ne change pas ces phénomènes généraux. Cependant en réponse à l'érythropoïétine, deux types de clones deviennent prépondérants : des clones produisant beaucoup plus de globules rouges et myéloïdes que lymphoïdes, et des clones produisant beaucoup de cellules lymphoïdes et myéloïdes et très peu de globules rouges, suggérant une compensation clonale entre les cellules souches hématopoïétiques. Cet effet était transitoire et disparaît 4 mois après transplantation. Cet effet est dose dépendant en fonction de la concentration d'érythropoïétine.

Nous espérons que l'étude détaillée de la différenciation de cellules souches hématopoïétiques uniques wt ou traitées avec l'érythropoïétine après transplantation ait un impact pour notre compréhension pour l'hématopoïèse fondamentale, mais aussi pour des applications cliniques. L'érythropoïétine est utilisée couramment comme médicament. La description détaillée de son effet sur les cellules souches hématopoïétiques est importante pour garantir la sécurité de telles utilisations. De plus, elle peut aider à comprendre les principes généraux de l'actions de cytokines sur les cellules souches hématopoïétiques. La transplantation de cellules souches est utilisée comme traitement dans de nombreuses pathologies. Une phase importante pour le succès d'une transplantation sont les premières semaines quand les niveaux de cellules sanguines ne sont pas encore normalisés. Comprendre quelles cellules produisent quels types de cellules dans cette phase peut servir de base pour le développement de nouvelles stratégies pour raccourcir cette phase critique pour le patient

Résumé chapitre 1

La majorité des cellules de notre corps sont des cellules hématopoïétiques. Un petit nombre de cellules souches hématopoïétiques produit toutes ces cellules par divisions de maintenance et différenciation. Le contrôle précis de cette différenciation est ainsi crucial pour une vie en bonne santé. Les hormones et cytokines, comme l'érythropoïétine, sont des facteurs importants pour ce contrôle et déjà utilisé en tant que traitement pour normaliser la composition sanguine dans des situation critiques. Aussi pendant la transplantation de cellules souches hématopoïétiques pour améliorer la greffe et la reconstitution des différents types de cellules hématopoïétiques matures. Il est très important de comprendre mieux la différenciation de cellules souches hématopoïétiques après transplantation et l'action de cytokines pour améliorer ces traitements.

Dans cette thèse nous décrivons la cinétique de la différenciation de cellules souches hématopoïétiques uniques et l'action de la cytokine érythropoïétine sur cette différenciation. Dans ce chapitre je récapitule la recherche actuelle sur les cellules souches hématopoïétiques et l'impact de nouvelles techniques de traçage de lignée sur cette recherche. Je décris le savoir actuel sur l'hétérogénéité des cellules souches hématopoïétiques, et l'action de cytokines, et en particulier l'érythropoïétine, sur les cellules souches hématopoïétiques.

Résumé chapitre 2

La reconstitution des différents types de cellules hématopoïétiques matures après transplantation de cellules souches hématopoïétiques dure plusieurs semaines. Dans cette première phase, les patients sont particulièrement prônes de subir des infections et peuvent avoir besoin de transplantation de globules rouges ou de plaquette sanguines. La différenciation de cellules souches hématopoïétiques uniques en globules rouges après transplantation n'a pas encore été étudié en détail par manque de technique de traçage de lignée pour les globules rouges matures sans noyau. Nous avons introduit ici, la détection de codes-barres cellulaires à partir d'ARN, et avons suivi la différenciation de centaines de cellules souches hématopoïétiques uniques pendant les six premières semaines après transplantation en souris irradiées. Nous avons observé que certaines cellules souches contribuaient de manière stable dans les premières semaines après transplantation, ainsi qu'une succession clonale. Des différences au niveau de la production de différents types de cellules hématopoïétiques matures a aussi été observées. Les globules rouges sont produits au début par des clones de vie courte puis par des clones plus stables et les cellules myéloïdes par des clones stables sur 6 semaines. Nous espérons que l'étude détaillée de la différenciation de cellules souches hématopoïétiques uniques après transplantation ait un impact pour notre compréhension pour l'hématopoïèse fondamentale, mais aussi pour des applications cliniques, en particulier le développement de protocoles de transplantation qui facilitent la reconstitution rapide de globules rouges matures.

Résumé chapitre 3

La cytokine érythropoïétine (EPO) est puissante à induire le développement de globules rouges et est utilisée couramment comme traitement. L'action de l'érythropoïétine a été attribuée à des progéniteurs spécifiques de globules rouges qui expriment fortement le récepteur pour l'érythropoïétine, mais un effet sur les cellules souches hématopoïétiques a aussi été suggéré. Des effets de l'érythropoïétine sur les cellules souches hématopoïétiques pourrait induire des effets secondaires pendant l'utilisation en clinique. Nous avons étudié ici, l'effet d'un traitement in vitro avec de l'EPO sur la différenciation de cellules souches hématopoïétiques uniques après transplantation en utilisant un système de traçage de lignée de codes-barres cellulaires. Pendant les six premières semaines après transplantation nous avons analysé la production de globules rouges, de cellules myéloïdes, cellules dendritiques, de mégacaryocytes, et de cellules lymphoïdes produit par différents clones de cellules souche hématopoïétiques. Nous avons observé qu'en réponse à l'érythropoïétine, deux types de clones deviennent prépondérants : des clones produisant beaucoup plus de globules rouges et myéloïdes que lymphoïdes, et des clones produisant beaucoup de cellules lymphoïdes et myéloïdes et très peu de globules rouges, suggérant une compensation clonale entre les cellules souches hématopoïétiques. Cet

effet était transitoire et disparaissait 4 mois après transplantation. De plus l'effet était dose dépendant en fonction de la concentration d'érythropoïétine. Nous espérons que la description détaillée de l'effet de l'érythropoïétine sur les cellules souches hématopoïétiques contribuera à garantir la sécurité de son utilisation en cliniques et aidera à comprendre les mécanismes généraux de l'action de cytokines sur les cellules souches hématopoïétiques.

Résumé chapitre 4

Dans cette thèse, nous avons décrits les cinétiques de différenciation de cellules souches hématopoïétiques uniques pendant les six premières semaines après transplantation. La détection de code-barres cellulaires à partir d'ARN, à la détection non-seulement dans cellules myéloïdes, lymphoïdes, mais aussi globules rouges matures sans noyau. Dans ce chapitre, je vais décrire les applications futures possibles de la technique de traçage de lignée de code-barres cellulaires par détection à partir d'ARN, et d'autre développement récent.

PhD portfolio

PhD candidate

Almut Eisele performed her PhD work from October 2016 till November 2019 in the group of Dr. Leïla Perié on Quantitative Immuno-hematology at UMR168 Laboratoire Physico-Chimie Curie of Institut Curie Paris within the international IC-3i PhD program funded by the European Commission's MSCA COFUND scheme. She was affiliated to the doctoral school N° 474 « Frontière du vivants » of the Centre for Interdisciplinary research (CRI) Paris and had the specialty cellular and developmental biology. During her PhD she was enrolled at Paris Sciences et Lettres (PSL) research university.

Supervision

Almut Eisele was supervised by Dr. Leïla Perié (2016-2019) head of the Quantitative Immuno-Hematology group, and Dr. Maxime Dahan † (2016-2017) head of UMR168 of Institut Curie. She was monitored through a yearly Thesis Advisory Committee meeting which members were Dr. Philippe Bousso head of Dynamics of Immune Responses group at Institut Pasteur and Dr. Thierry Jaffredo head of Migration and Differentiation of Hematopoietic Stem Cells at Institut de Biologie Paris Seine.

Courses and “mission doctorale”

As part of the training by her PhD program and doctoral school, Almut Eisele attended courses and performed a “mission doctorale”. The courses and trainings included: “Animal Experimentation-Designer” Institut Pasteur Paris. (2017) “ZIDAS 2017 Zurich image and data analysis school 2017” ETH Zurich (2018) Training animal surgery “Formation réglementaire de base en chirurgie chez le rongeur de laboratoire” Institut Curie Paris (2018) “Sensibilisation à l’Histoire, l’Epistémologie et l’Ethique des Sciences” ENS Paris (2016-2017) “Scientific integrity” Institut Curie Paris (2016) “Next-generation sequencing and data analysis training” Institut Curie Paris (2016) “You think you have an invention, now what?” Institut Curie Paris (2017) “sécurité laser” Institut Curie Paris (2017) “Brevet d’invention appliqué aux produits et aux procédés du domaine de l’immunothérapie” Institut Curie Paris (2017-2018) “Scientific writing” CRI Paris (2017) “Critical analysis of research articles” CRI Paris (2017) “Creating interdisciplinary research projects” CRI Paris (2017) “Public speaking” CRI Paris (2016-2017) Seminar series “Interdisciplinary Fridays” CRI Paris (2017-2018) Workshop organization “Thematic workshops” (2018) “Well-being and integrity in research” CRI Paris (2018). Besides, Almut Eisele was performing a “mission doctorale” and organized the monthly “TechWatch” seminar series at Institut Curie for the CurieCoreTech (CCT) network of central technology platforms, under guidance of the CCT head Dr. Andrea Hutterer (2016-2018) and Dr. Lavanya Premvardhan (2018-2019). Finally, Almut Eisele received technical trainings in the lab of Dr. Cristina Lo Celso at Imperial College London

by Dr. Delfim Duarte and in the group of Dr. Ludovic Jullien at ENS Paris by Dr. Isabelle Aujard, and Dr. Thomas Lesaux.

Scientific meetings

During her PhD, Almut Eisele could attend several scientific meetings. These include: EMBL Conference: Hematopoietic Stem Cells: From the Embryo to the Aging Organism, Heidelberg, June 2018. Second International Symposium on Red Blood Cells: Genesis and Pathophysiology, Paris, April 2018. Institute Curie “Single-cell symposium”, Paris December 2018. 47th Annual Scientific Meeting International Society for Experimental Hematology, Los Angeles, August 2018. Poster presentation “Erythropoietin changes the clonality of HSC after transplantation”. GDR Stem Cell meeting, Paris, November 2017 and November 2018. Talk “Erythropoietin changes the clonality of HSC after transplantation”.

CV PhD candidate

Almut Eisele performed her studies in Utrecht, The Netherlands and Berlin, Germany. She graduated “cum laude” from the Bachelor program in Biomedical Sciences at Utrecht University in 2013. Subsequently she completed the International Master program Molecular Medicine at Charité Berlin “excellent” (2014-2016) before enrolling in the IC-3i PhD program at Institut Curie Paris. During the first year of her PhD she graduated from the Master program Infection and Immunity at Utrecht university (2013-2017).

Publications:

- van Balkom BW, **Eisele AS**, Pegtel DM, Bervoets S, Verhaar MC, “Quantitative and qualitative analysis of small RNAs in human endothelial cells and exosomes provides insights into localized RNA processing, degradation and sorting”, *Journal of Extracellular Vesicles*. 2015 May 29.
- Serra M, Mai TD, Serra AL, Nguyen M-C, **Eisele A**, Perié L, Viovy J-L, Ferraro D, Descroix S. “Droplet microfluidic device for DNA size selection in NGS libraries preparation based on magnetic particles handling” submitted to *Nature Communications* May, 2019.
- Babic M, Hammer Q, Heinrich F, Sarsenbayeva A, **Eisele A**, Romagnani C. “NK cell receptor NKG2D enforces pro-inflammatory features and pathogenicity of Th1 and Th17 cells” submitted to *JEM* on January 21, 2019.
- **Eisele AS**, Magniez A, Tubeuf E, Tenreiro Bento S, Tak T, Cosgrove J, Lyne AM, Urbanus J, Schumacher T, Perié L. “Early hematopoietic stem cell engraftment kinetics at the single cell level”, to be submitted.
- **Eisele AS**, Magniez A, Tubeuf E, Tenreiro Bento S, Tak T, Cosgrove J, Lyne AM, Urbanus J, Schumacher T, Perié L. “Erythropoietin alters hematopoietic stem cell clonality after transplantation”, to be submitted

Acknowledgments

The PhD work would not have been possible without my supervisor Leïla Perié, her team Perié and associated members, Aurelie Magniez, Emilie Tubeuf, Alessandro Donada, Jacques Vargaftig, Lucie Laplane, Tamar Tak, Anne-Marie Lyne, Sabrina Tenreiro Bento, Jason Cosgrove, Lucie Hustin, Alessandro Donado, Pierre Bost, Giulio Prevedello, Idan Milo and interns. Thank you all. I experienced a special time of the lab--the start-up and expansion. Many changes took place in this time, including the renovation of the building, the death of Maxime Dahan, new group organizations. I learnt many, many things, also about French culture, and French working culture, or the pros and cons of interdisciplinarity. I am sure there are many exciting projects to come for team Perié. I would consider the openness to do things in a new or non-standard way as one major characteristic of the team, which can be fruitful ground for discoveries. I would like to especially thank Leïla for allowing me to defend my second master degree during the PhD. I will graduate as first student of the IC-3i PhD program and within three years. Thank you.

I received scientific and technical help from a multitude of people for which I am very grateful. These include the members of many Institut Curie platforms: the flow cytometry platform (Sophie, Annick, Zosia, Coralie, Lea) the microscopy platform (Francois, Marie, Lucie, Olivier) the animal facility (Cedrick, Mickael, Isabelle, Virginie, Casper, Anne, Colin, Nicolas), the NGS facility (Sylvain, Sonia, Virgine, Benoit) the radiation facility (Sophie), the UMR168 intern mechanics (Eric, Remy) and cell biology facilities (Fanny, Aude). Besides, I received help from the Daniela Vignjevic (Daniela, Fatima) and Sebastian Amigorena teams at Institut Curie, the team of Cristina Lo Celso at Imperial College London (Cristina, Delfim), the team of Ludovic Jullien at ENS Paris (Ludovic, Isabelle, Thomas), and the team of Philippe Bousso at Institut Pasteur (Philippe, Idan, Ruby).

I would like to thank all UMR168 members (you are too many to name you all) for scientific and non-scientific support and exchange during meetings, retreat, student seminars, Wednesday night outings, visits of the cherry blossom festival, raclette evenings, barbecue on the IPGG terrace.. I hope we will stay in contact and I wish you all the best for the futures you strive for.

Throughout my PhD I received guidance besides Leïla Perié by Philippe Bousso, Thierry Jaffredo, Lavanya Premvardhan, Andrea Hutterer, Leanne De Koning, and Hervé Isambert. I was supported by the PhD program (Zeina, Melanie, Graca) and doctoral school (David, Eugenia, Elodie), and PSL (Odile) administration. Thank you all. Thanks to the jury members Ana Cumano, Michael Rieger, Leonid Bystrykh, Catherine Sawai, and Charles Durand. Thank you, Joanna Podkalicka, for the cover

drawing. Thank you David Gentien and Thiago Trovati Maciel. Thanks to the many great people I met at the conferences I could attend (Lena, Anik and Xun). Thanks to my family and close friends.

Résumé

Un petit nombre de cellules souches hématopoïétiques produit toutes les cellules sanguines et immunitaires de notre corps. Dans cette thèse nous avons utilisé la technique de traçage de lignée des codes-barres cellulaires pour comprendre comment différents types de cellules souches hématopoïétiques se comportent dans les six premières semaines après transplantation et l'influence de la cytokine érythropoïétine sur ce processus.

Abstract

Hematopoietic stem cells reside at the apex of one of the most complex and dynamic cellular systems of our body. In this thesis we used a new cellular barcoding library for lineage tracing to analyze how different hematopoietic stem cell subsets interact and behave in the first six weeks after transplantation, as well as the influence of erythropoietin on this process.

Mots Clés

Cellules souches hématopoïétiques, traçage de lignée, codes-barres cellulaires, érythropoïétine, cellules uniques

Keywords

Hematopoietic stem cells, lineage tracing, cellular barcoding, erythropoietin, single cell, stem cell heterogeneity, clonality, stem cell fate, stem cell differentiation, transplantation