

INTRODUCTION

Hematopoiesis

Hematopoiesis is the process of producing blood cells. There are two types of hematopoietic cells: myeloid cells and lymphoid cells (Figure 1). Myeloid cells include granulocytes, monocytes, erythrocytes and megakaryocytes, whereas lymphoid cells include B lymphocytes, T lymphocytes and natural killer (NK) cells. All these cells are derived in the bone marrow from hematopoietic stem cells [1].

Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cells (HSCs) must have two key defining characteristics of stem cells: multi-potency and self-renewal. Multi-potency, in the case of HSCs, is defined as the ability to differentiate to all blood cell types. Self-renewal is defined by maintenance of stem cell number and function throughout life. In experiments, the cut-off to be considered self-renewing is 20 weeks [2]. HSCs must be able to regenerate blood following a bone marrow transplantation.

There are two types of HSCs: long-term HSCs (LT-HSCs) and short-term (ST-HSCs). ST-HSCs are derived from LT-HSCs and maintain the same level of multi-potency with a more limited capability of self-renewal, a timeframe of about 4 weeks compared to the 20 weeks of LT-HSCs. HSCs can be found in three sources: the bone marrow, peripheral blood, and umbilical cord blood [1]. Although the administration of cytokines to mobilize HSCs into the peripheral blood (PB) is becoming more common, the accepted clinical practice still remains to be bone marrow transplantation.

BACKGROUND: CURRENT FATE-TRACKING APPROACHES

Transplantation Assays

The transplantation assay is the most commonly used technique to analyze cell lineages [3]. The recipient mice are lethally irradiated, and the donor cells are then transplanted into them. There are three types of transplantation assays: competitive transplantation, limited dilution assay and xenotransplantation [4]. Competitive transplantation is where the stem cell functions in two donor samples are directly compared. The limited dilutions assay is used to estimate the frequency of stem cells within the donor cell sample, done by decreasing the number of donor cells that are injected into groups of irradiated mice recipients. Xenotransplantation is used to study the functions of human stem cells in a mouse model of human hematopoiesis and immune systems.

Inducible Knock-Out Mice

The inducible knock-out mice technique makes use of the Cre-LoxP system. This technique allows for the selective and inducible knock out of a gene of interest when investigating a cell lineage. The Cre recombinase gene is often combined with an estrogen receptor (ER) to form CreER. The ER in CreER is insensitive to endogenous estrogen, only induced following the injection of Tamoxifen. The caveat of this technique is that it requires a promoter specifically expressed in the cells of interest to drive Cre expression, which hematopoietic stem cells do not have. A singular molecular marker expressed exclusively by HSCs has yet to be discovered [1].

Lineage Tracing Studies

The lineage tracing study works similarly as it also uses the CreLoxP system. In this mouse model, a reporter transgene and CreER transgene is used. In this case, a stop codon upstream of the reporter gene is floxed. Upon expression of CreER, the stop codon is excised and the reporter gene is then expressed. Cell lineage is then traced via the reporter protein, where all progeny from those cells of interest will inherit the transgene with the excised stop codon, continuing to express the reporter protein later on. However, the same caveat is present in this method, where a cell-specific promoter is essential [3].

Lineage Ablation Studies

The lineage ablation study works by ablating all cells of interest to study cell lineage. A common method used is diphtheria toxin (DT) mediated cell depletion. In this method, DT receptor cDNA is combined with a cell specific promoter and then microinjected to fertilized eggs. Upon injection of DT into the transgenic mice that express DT receptors on only the cells of interest, those cells of interest are damaged or die, ablating those cells and any of its progeny down the cell lineage [4]. This method, like others previously mentioned, require the use of a HSC-specific promoter which is still undiscovered at this point in time.

OBJECTIVE

Due to the lack of HSC- or progenitor-specific drivers, fate tracking approaches used in solid tissue stem cells cannot be applied to study native hematopoiesis. Because of this gap in knowledge, the mechanisms behind non-transplant hematopoiesis have been scarcely studied, resulting in a deeper hole in our understanding. Despite holding a crucial role in blood production, stem or progenitor cells characteristics, such as number, lifespan and lineage potential, have yet to be analyzed. To fill this gap in the stem cell biology field, Sun et al. present a new experimental system where they perform in situ labelling and fate tracking of HSCs which can then be used to investigate lineage and origins in their 2014 paper, “Clonal dynamics of native hematopoiesis” [5].

EXPERIMENTS

Transposon Tagging

The method developed by Sun et al. for clonal marking uses transposon tagging. The premise of this method is based on the transient expression of a hyperactive Sleeping Beauty (HSB) transposase, an enzyme that mobilizes cognate DNA transposon (Tn). This approach is performed using a doxycycline (Dox)-inducible HSB cassette and a single-copy non-mutagenic Tn. When Dox is administered, it activates the Dox-dependent transcriptional activator (M2), which is driven from the ubiquitous *Rosa26* locus. In these mice (known as M2/HSB/Tn), M2 and Dox bind to TetO, which allows for the transcription of HSB to occur. Once the HSB transcript is translated, the HSB transposase mobilizes Tn to a random chromosomal locus. Simultaneous to the mobilization of Tn, a STOP signal is removed, allowing for the transcription of DsRed reporter, shown in Figure 1a of the original paper. The presence of DsRed can now be used as a marker for successful Tn mobilization. Due to the quasi-random integration of Tn after mobilization, each cell with successful transposition (DsRed+) will carry a single and distinct insertion site, which, even after Dox withdrawal, will continue to be a stable genetic tag for that cell and its progeny [5].

To verify this technique, further analysis was performed. Further analysis of this technique showed that Tn mobilization could be induced in approximately 30% of long-term (LT)-HSCs, short-term (ST)-HSCs, multipotent progenitors (MPPs) and myeloid progenitors (MyP). In addition, no labelling (DsRed⁺) or Tn mobilization was found in un-induced (Dox⁻) mice. Upon transplantation, DsRed⁺ HSC/progenitors produced myeloid and lymphoid lineages for 10 months, signifying that those DsRed⁺ cells were LT-HSCs. Along the same vein, DsRed⁻ HSC/progenitors produced only DsRed⁻ progeny, verifying insignificantly low levels of Tn mobilization under Dox⁻ conditions. It was also found that there were minimal levels of Tn mobilization in peripheral blood granulocytes, B cells and T cells of Dox⁻ un-induced mice, levels that were orders of magnitude lower than that of Dox⁺ cells. This further analysis performed demonstrated that the M2/HSB/Tn model created by Sun et al. is in fact strictly Dox-dependent Tn mobilization in hematopoietic cells, and can be effectively used to study the clonal dynamics of native hematopoiesis [5].

Clonal Dynamics of Granulopoiesis

Given the rapid turnover of granulocytes, the clonal dynamics of granulopoiesis need to be studied to fully comprehend the clonal dynamics of native hematopoiesis. After each Dox injection, 3-4 months of 'chase' was provided to ensure that mature PB populations would most likely be derived from longer-lived HSCs. During this time frame, 90-98% of granulocyte tags were only identified at one time point. The remaining 2-10% were found at adjacent multiple time points, indicating short-lived, unstable granulocytic clones. On the other hand, only seven Tn tags were found in Dox⁻ PB granulocytes. This data confirms the inverse correlation between the number of PB clones found in the BM and time elapsed. This instance continued for up to 12 months of 'chase', signifying that this clonal instability is not a transient occurrence. In a further experiment, out of 290 single granulocytes, 270 unique Tn tags were detected. Of those 270 Tn tags, 254 tags were found once, 14 found twice and 2 found thrice. Finally, this phenomenon was observed in induction periods of merely one day, so this pattern cannot be attributed to the resulting clonal complexity associated with a longer induction period. This allows for the definitive conclusion that long term steady-state granulopoiesis is polyclonal in nature and mostly driven by the recruitment of unique, non-overlapping progeny [5].

Myeloid and Lymphoid Lineages of Hematopoietic Clones

Next, Sun et al. investigated the lineage output of hematopoietic clones by comparing the Tn tags of granulocytes, B lymphocytes and T lymphocytes, the terminally differentiated cells of HSCs. The lineage of HSCs is of hierarchal organization, as demonstrated in Figure 1. Granulocytes are derived from the myeloid progenitor, whereas lymphocytes are derived from the lymphoid progenitor. Interestingly enough, very few granulocytes tags were shared with B or T cells, around 7% found in pro/pre-B-cells, despite their progenitors both originating from HSCs. This indicates that most of granulocyte-producing clones are inclined to the myeloid lineage as opposed to the lymphoid one, for up to 45 weeks [5].

However, when examining lymphoid clones, only 10% of the pro/pre-B cell tags were found in granulocytes at 9 and 26 weeks, whereas when examined again at 40-45 weeks, 47% of pro/pre-B cell tags were detected to be in granulocytes. This shift from 10% to 47% over the course of 14-19 weeks supports the notion that B-cell production shifts from the lymphoid progenitor to a multipotent progenitor, with the potential to differentiate down the myeloid lineage. To support this analysis, monocyte tags were also examined. Monocytes and granulocytes both differentiate from a common progenitor, the granulocyte, monocyte progenitor (GMP), so they are expected to share a significant number of tags. As expected, 60% of monocyte tags were shared with granulocytes, consistently at

three time points. This suggests that myeloid-producing clones are at least bi-potent, capable of both lineages [5].

Differences with Transplant Hematopoiesis

This technique proposed by Sun et al. is unique in that it is a method of lineage tracing in native hematopoiesis, instead of transplant hematopoiesis. In this sense, some results presented differed from those of transplant hematopoiesis. In those transplants, a few dominant LT-HSC clones were able to stably produce multiple blood lineages, in contrast to what Sun et al. found. However, this population of LT-HSC clones could be attributed to some stable and multipotent clones observed in retrovirus-infected DsRed⁺ Lin⁻ c-Kit⁺ Sca1⁺ cells. Similar results of clonal diversity were also seen when DsRed⁺ Lin⁻ c-Kit⁺ cells due to the higher regenerative potential of cells that have been less manipulated through the transplantation process [5].

Further analysis in native hematopoiesis even revealed that less than 5% of LT-HSCs shared tags with MPPs and MyPs, progenitors thought to be their immediate downstream progeny (Fig 1). In contrast, in transplantation hematopoiesis, almost all PB clones are derived from LT-HSCs, a stark difference from the native 5%. This suggests that under undisturbed conditions of native hematopoiesis, progenitors play a larger role than LT-HSCs, which play a larger role in transplantation hematopoiesis. These discrepancies indicate that there are intrinsic core differences between the clonal dynamics of post-transplant hematopoiesis versus steady-state hematopoiesis, and that conclusions drawn from one cannot be directly applied to the other [5].

DISCUSSION

Major Conclusions

In Sun et al.'s paper, the authors present a novel approach for *in situ* fate-tracking of HSCs. By using this approach in their experiments, the authors can conclude various points. Regarding granulopoiesis, it can be determined that steady-state granulopoiesis is polyclonal and driven by the recruitment of unique, non-overlapping clones. Regarding the myeloid and lymphoid lineages, the authors discovered that granulocyte-producing clones are of the myeloid lineage for up to 45 weeks, while lymphoid progenitors have the potential to become MPPs and shift lineages. Finally, the writers concluded, most importantly, that in an unperturbed system of native hematopoiesis, LT-HSCs have limited contribution whereas MPPs hold a central role, the antithesis of what is found in transplantation [5].

Significance on the Research Field

Before Sun et al.'s discovery of this new technique, there was no method to track HSCs *in situ*, simulating a native hematopoiesis environment, as opposed to a perturbed, transplantation environment. As demonstrated by this paper, the contribution of LT-HSCs and MPPs in native and transplantation hematopoiesis differ greatly, a discovery that would have never been made if this new technique was not presented. With the publishing of this paper, more research questions of native hematopoiesis can be addressed. As Sun et al. stated in their paper, it is now possible to investigate the effects of clonal diversity or lifespan of progenitors on severely aged mice, or how the clonal dynamics of native hematopoiesis are influenced by stress. Further studies can now also be conducted *in situ* on LT-HSCs and their role in the production of blood. A caveat to most studies like this is that extrapolated information cannot be directly applied otherwise, such that *in vitro* results cannot be

assumed to be the same *in vivo*, where in this research field, transplantation results cannot be applied to native circumstances. As seen in this paper, they most certainly differ from one another. This novel M2/HSB/Tn mouse model allows for the studying of hematopoiesis lineages under normal physiological conditions, with minimal disturbances to their natural niche [5].

Limitations and Criticisms

As with all studies involving transgenic mice, a lot of time and effort is put into generating them, even though end results may not be what was desired. Since Sun et al. worked with transposons, there would appear to be unpredictability in the Tn mobilization, where some insertion sites would cause disruption of coding regions. The additional screening process to select for non-mutagenic Tn insertions would mean that there were mice generated that could be lethally mutant, a constant ethical issue in the scientific community. Furthermore, in many of the data generated, it was stated that the data was extracted from a cohort of merely three mice, a surprisingly small sample size for a study of such magnitude of significance.

Preceding Studies

Many studies preceded the publishing of this paper that used various lineage tracing techniques, such as dyes and radioactive markers, genetic markers by transfection, transplantation, genetic recombination, etc. However, these methods did not allow each and every cell of interest to possess a unique and stable genetic tag as they do with the use of Tn mobilization, especially without the use of a cell-specific promoter. Although a HSC-specific promoter is unknown, studies could be performed on differentiated cells for lineage tracing. For example, in 2012, Calaminus et al. was able to trace megakaryote lineages using Pf4-Cre driver [6]. This demonstrates that there are cell-specific drivers that can be used but no HSC markers expressed solely by HSCs. This is also the case in the 2013 review paper by Joseph et al., where various driver used for lineage tracing were listed, but those markers were not exclusive to HSCs [7]. As exhibited by a review article in *Cell*, 2012, lineage tracing *in situ* was relatively easily done for solid tissue cells, but fairly difficult with hematopoietic cells until this novel M2/HSB/Tn model was established [3].

Following Studies

Despite the novel M2/HSB/Tn model discovered by Sun et al. in 2014, there were no major studies that conducted further experiments based on this mouse model. However, this paper did set the precedent for investigating fundamental properties of unperturbed hematopoiesis *in vivo* [8]. In this study, a Tie2-CreER driver along with a YFP reporter was used, although Tie2 is not exclusively expressed in HSCs. Additionally, another study in *Cell Stem Cell* conducted a human study using gene-corrected cells in four WAS patients treated with LV HSPC, where patients showed reconstitution of engineered cells [9]. Finally, in a 2017 review article, the authors discuss how lineage tracing allows for the diagnosis of the origin of disorders which can then be treated via hematopoietic stem cell-directed gene therapy [10]. Most importantly though, these papers written after the publishing of Sun et al.'s paper address the issue classical LT-HSCs do not seem to play as large of a role as MPPs in native hematopoiesis, the most significant finding from that paper. The lack of use of the M2/HSB/Tn model is in fact, not concerning. Sun et al.'s paper was published in 2014, merely three years ago. There could be studies using their mouse model that have yet to be published in this three-year time frame, as research takes time, as does the editing and publishing process. It is expected that there will be studies to come that will make excellent use of this novel M2/HSB/Tn mouse model for the *in situ* studying of native hematopoietic clonal dynamics.

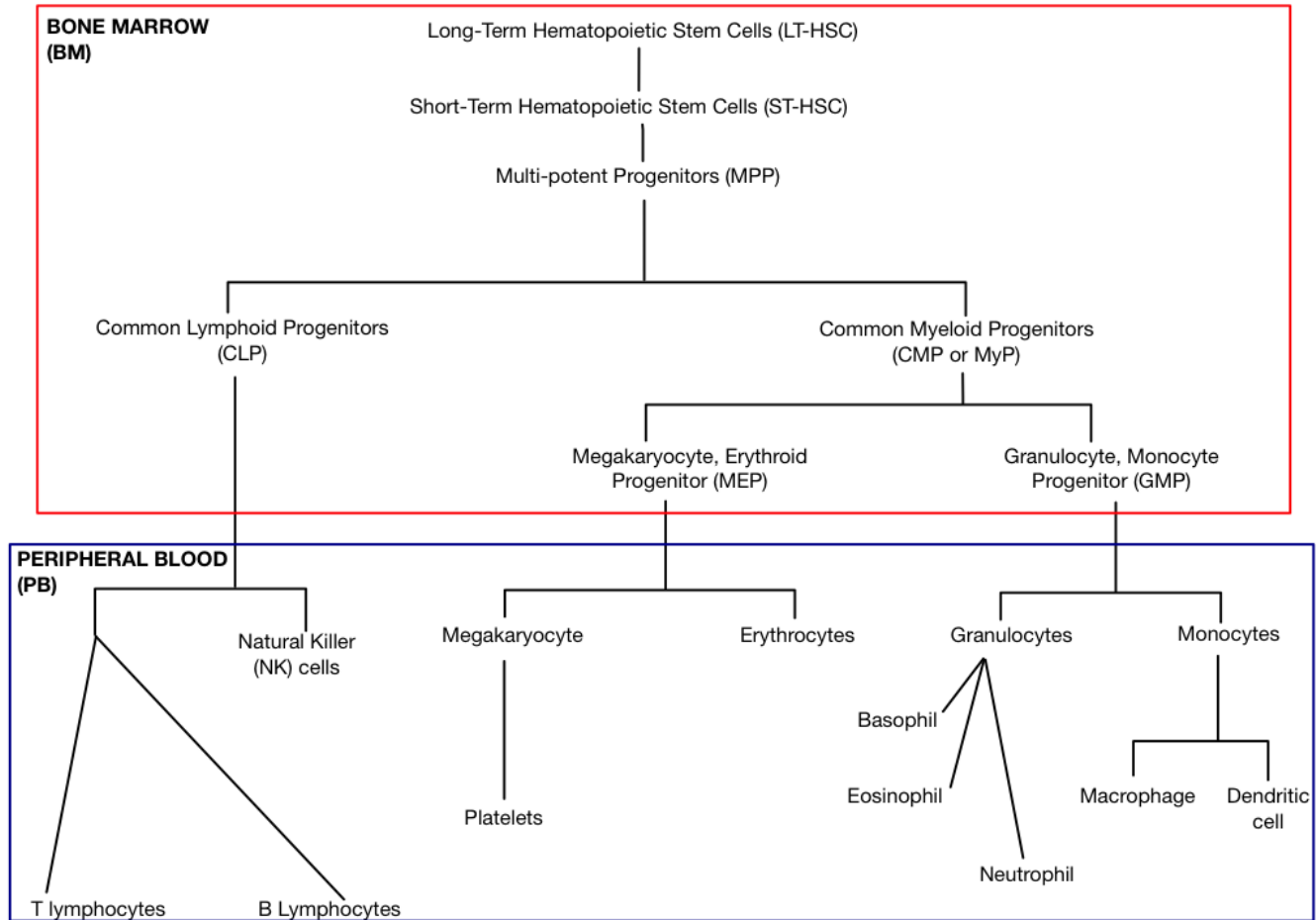
FIGURES

Figure 1. *The hierarchal organization of hematopoiesis [2].*

REFERENCES

1. Mosaad YM: **Hematopoietic stem cells: An overview.** *Transfusion and Apheresis Science* 2014, **51**(3):68-82.
2. Nijnik A: **Introduction to Hematopoietic Stem Cells.** In: *PHGY 488: Stem Cell Biology.* Montreal, QC: McGill University; 2017.
3. Kretzschmar K, Watt Fiona M: **Lineage Tracing.** *Cell*, **148**(1):33-45.
4. Nijnik A: **Methodologies for the Study of Tissue Stem Cells.** In: *PHGY 488: Stem Cell Biology.* Montreal, QC: McGill University; 2017.
5. Sun J, Ramos A, Chapman B, Johnnidis JB, Le L, Ho Y-J, Klein A, Hofmann O, Camargo FD: **Clonal dynamics of native haematopoiesis.** *Nature* 2014, **514**:322.
6. Calaminus SD, Guitart AV, Sinclair A, Schachtner H, Watson SP, Holyoake TL, Kranc KR, Machesky LM: **Lineage tracing of Pf4-Cre marks hematopoietic stem cells and their progeny.** *PloS one* 2012, **7**(12).
7. Joseph C, Quach Julie M, Walkley Carl R, Lane Steven W, Lo Celso C, Purton Louise E: **Deciphering Hematopoietic Stem Cells in Their Niches: A Critical Appraisal of Genetic Models, Lineage Tracing, and Imaging Strategies.** *Cell Stem Cell* 2013, **13**(5):520-533.
8. Busch K, Klapproth K, Barile M, Flossdorf M, Holland-Letz T, Schlenner SM, Reth M, Höfer T, Rodewald H-R: **Fundamental properties of unperturbed haematopoiesis from stem cells in vivo.** *Nature* 2015, **518**:542.
9. Biasco L, Pellin D, Scala S, Dionisio F, Basso-Ricci L, Leonardelli L, Scaramuzza S, Baricordi C, Ferrua F, Cicalese Maria P *et al*: **In Vivo Tracking of Human Hematopoiesis Reveals Patterns of Clonal Dynamics during Early and Steady-State Reconstitution Phases.** *Cell Stem Cell* 2016, **19**(1):107-119.
10. Kohlscheen S, Bonig H, Modlich U: **Promises and Challenges in Hematopoietic Stem Cell Gene Therapy.** *Human gene therapy* 2017, **28**(10):782-799.